

Preclinical research on novel therapies and neurochemistry in intractable epilepsy

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Statement of originality and author attribution

This thesis is submitted to the University of Sydney in fulfilment of the requirements for a degree of Doctor of Philosophy. This is to certify that to the best of my knowledge; the content of this thesis is my own work, and that all assistance received in preparing this thesis has been acknowledged. This thesis has not been submitted for any degree or other purposes. The research in this thesis was carried out at the Brain and Mind Centre and animal facility at the University of Sydney, unless otherwise specified. Investigations were performed as part of the Discipline of Pharmacology, Sydney Pharmacy School, and Faculty of Medicine and Health. Specific contributions will be outlined below.

For the publication in Chapter 2, I am the primary author and conducted experiments for all animal studies under the supervision of Prof Jonathon Arnold. Dr Micheal Udoh performed cell experiments on T-type calcium channels and cannabinoid CB1 and CB2 receptors. I performed statistical analyses, interpretations of the data of animal studies and manuscript writing. Ms Laura Sharman assisted in analyzing atypical absence seizures. Mr. Thomas Harman assisted with the maximal electroshock (MES) test. Chapter 2 has been published upon the submission of this thesis. Prof Arnold is the corresponding author and oversaw the progress of the publication.

For the work in Chapter 3 was designed by me, together with Prof Arnold. I conducted most experimental work. Chemical compounds of GAT591 and GAT1102 was developed by our collaborator Ganesh Thakur in Northeastern University, USA. Ms Sharman helped hyperthermia-induced seizure experiments and analyzing atypical absence seizures. Mr. Harman assisted with the MES test. I conducted all sample collection and data analyses.

For the work in Chapter 4, experiments were designed by me, Prof Arnold and Dr Lyndsey Anderson. For the analysis of specialized pro-resolving mediators, samples were collected by

Dr Nicole Hawkins from Northwestern University, Illinois, USA. Liquid Chromatography with tandem mass spectrometry (LC/MS/MS) experiments with these samples were performed by Ambiotis SAS, France. I conducted all *Scn1a*^{+/-} mouse experiments, and sample collection for RT-qPCR with guidance from Dr. Cilla Zhou. I performed all sample preparation, LC/MS/MS analyses and data analyses.

I wrote all chapters of these with guidance and feedback from Prof Arnold.

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Additional publications

I also made a substantial contribution to the following publications which are complimentary to the present thesis but do not form a part of its:

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Abbreviations

ACSF	Artificial cerebrospinal fluid
ALOX	Lipoxygenase genes
ASMs	Anti-seizure medications
BAM	Biased allosteric modulators
CB1	Cannabinoid 1 receptors
CB2	Cannabinoid 2 receptors
CBC	Cannabichromene
CBCA	Cannabichromene acid
CBCV	Cannabichromevarin
CBCVA	Cannabichromevarinic acid
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBDV	Cannabidivarin
CBDVA	Cannabidivarinic acid
CBE	Cannabielsoin
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBGV	Cannabigerivarin
CBGVA	Cannabigerovarinic acid
CBL	Cannabicyclol
CBN	Cannabinols
CBND	Cannabinodiol
CBT	Cannabitriol
CNS	The central nervous system
DEE	Developmental and epileptic encephalopathy
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EEG	Electroencephalogram
EPA	Eicosapentaenoic acid
FDA	U.S. Food and Drug Administration

GABA _A	GABA receptor type-A
GAERS	Genetic Absence Epilepsy Rat from Strasbourg
GPP	Prenylation of geranyl diphosphate
GTCS	Generalized tonic-clonic seizures
HIV	Human immunodeficiency virus
I.P.	Intraperitoneal
IL-1 β	Interleukin 1 β
ILAE	International League Against Epilepsy
LGI1	Leucine-rich glioma-inactivated protein 1
MES	Maximal electroshock seizure
MRI	Magnetic resonance imaging
NAM	Negative allosteric modulators
Nav1.1	Voltage-gated sodium channel
NMDA	N-methyl-D-aspartate
PAM	Positive allosteric modulators
PPAR γ	Peroxisome proliferator-activated receptor
PTZ	Pentylenetetrazol
RVD	D-series resolvins
S.C.	subcutaneous
SAR	Structure-activity relationships
<i>Scn1a</i> ^{-/-}	Homozygous <i>Scn1a</i> knockout
<i>Scn1a</i> ^{+/-}	Heterozygous <i>Scn1a</i> knockout
SPECT	Single-photon emission computed tomography
SUDEP	Sudden unexpected death in epilepsy
TANGO	Targeted Augmentation of Nuclear Gene Output
TGA	Therapeutic Goods Administration
TNF- α	Tumor necrosis factor alpha
Δ^8 -THC	Δ^8 -trans-tetrahydrocannabinol
Δ^9 -THC	Δ^9 -tetrahydrocannabinol
Δ^9 -THCA	Δ^9 -tetrahydrocannabinolic acid

Abstract

Epilepsy is a condition involving underlying hyperexcitability of neurons in the brain leading to various seizure-related phenotypes. However, there remains unmet clinical need in epilepsy for around 30% of patients that remain resistant to treatment. This requires new therapeutic targets to be explored, through the characterization of novel neurochemical pathways that respond to seizures and testing novel candidate molecules for anti-seizure effects in state-of-the-art preclinical models of seizures.

Cannabidiol (CBD), a non-psychoactive cannabinoid derived from *Cannabis sativa*, displays promising anti-seizure effects in patients of Dravet syndrome and Lennox-Gastaut syndrome, two types of epilepsy that are resistant to current medications. The chemical structure of CBD provides a scaffold for developing novel anti-seizure agents. Additionally, this has led to further investigations into the role of the endogenous cannabinoid system and other lipid-related signaling pathways in epilepsy and whether these systems could be leveraged to develop new anti-seizure drugs. The present thesis then aimed to further advance this field of research by identifying novel cannabinoid-related anti-seizure agents and investigating the impact of seizures on the novel lipid signaling mediators called the specialized pro-resolving mediators which assist in brain repair.

This thesis begins in Chapter 1 with an introduction to epilepsy, covering recent seizure classification updates and definitions of epilepsy, and then narrows the focus to two epilepsy syndromes, Dravet syndrome and Lennox-Gastaut syndrome. It then explores the basis of *Cannabis sativa* and its role in epilepsy treatment, and the potential of cannabinoid-related molecules in the treatment of epilepsy by reviewing the anti-seizure effects of phytocannabinoids and synthetic cannabinoids in preclinical models. Finally, the introduction

also reviews not only the endocannabinoid system, but the related emerging lipid signaling pathways of the specialized pro-resolving mediators.

This thesis comprises three original studies in Chapters 2, 3 and 4 which focused on developing novel treatments for Dravet syndrome and Lennox-Gastaut syndrome. All studies used animal models of these syndromes to evaluate the therapeutic effects of novel compounds and identify potential treatment targets. The first two studies, presented in Chapters 2 and 3, employed these models to investigate the anti-seizure potential of cannabinoid-related compounds with different therapeutic rationales. For evaluating the anti-seizure activities, we employed the maximal electroshock seizure (MES) test to induce symptomatic generalized tonic-clonic seizures (GTCS) and used two drug-resistant mouse models to examine anti-seizure effects on disease-specific seizures in Dravet syndrome and Lennox-Gastaut syndrome. In the Dravet syndrome model, we utilized heterozygous knockout *Scn1a* mice (*Scn1a*^{+/-} mice) to assess the temperature threshold for hyperthermia-induced GTCS. If anti-seizure effects were confirmed in both GTCS models, we extended to evaluate the anti-seizure efficacy in suppressing atypical absence seizures in the Lennox-Gastaut syndrome model. A heterozygous knock-in mutation in the *Gabrb3* gene in mice (*Gabrb3*^{+/*D120N*} mice) mimics seizure phenotypes observed in patients with Lennox-Gastaut syndrome. The final study, described in Chapter 4, examined whether seizure burden impacts brain concentrations of the specialized pro-resolving mediators in *Scn1a* mice.

Chapter 2 evaluated the anti-seizure effect of non-cannabis-derived phytocannabinoid-like compounds - amorfrutin 2, magnolol and honokiol. Amorfrutin 2 has structural similarity to the phytocannabinoid cannabigerolic acid (CBGA) which has anti-seizure activity. Magnolol and honokiol resemble biaryl cannabinoids found in nature (e.g. CP 55,940). In addition, we characterized their molecular pharmacology at CB1 and CB2 receptors, as well as at T-type calcium channels, which are established targets of cannabinoids. Amorfrutin 2 and magnolol,

but not honokiol, exhibited anti-seizure effects against both hyperthermia-induced seizures in *Scn1a*^{+/-} mice and GTCS severity in the MES test. In *Scn1a*^{+/-} mice, amorfrutin 2 was more potent than magnolol. However, magnolol showed stronger anti-seizure effects than amorfrutin 2 in the MES-model. In addition, magnolol reduced atypical absence seizures in *Gabrb3*^{+/*D120N*} mice. All compounds inhibited T-type calcium channels subtype Cav3.1, Cav3.2 and Cav3.1. Interestingly, while honokiol displayed the greatest potency in inhibiting Cav3.1, it was ineffective in preventing seizures. None of the compounds displayed any specific effects on cannabinoid receptors.

The endogenous cannabinoid system is comprised of endocannabinoids and cannabinoid receptors. Research has shown that targeting this system may reduce seizures. For example orthosteric agonists of the cannabinoid CB1 receptor reduce seizures in animal models like the main psychoactive constituent of cannabis, Δ^9 -tetrahydrocannabinol (Δ^9 -THC). However, orthosteric agonists have sedative and intoxicating side-effects. Allosteric modulators of the CB1 receptor have emerged that do not have such side-effects by targeting a topographically distinct site on the receptor. Chapter 3 investigated the anti-seizure effects of GAT591 and GAT1102, two mixed positive allosteric modulators (PAMs) and allosteric agonists (ago-PAMs) targeting CB1 receptors. Our findings demonstrated that GAT591 and GAT1102 exhibit anti-seizure effects in hyperthermia-induced GTCS in the *Scn1a*^{+/-} mouse model, with GAT1102 showing greater potency at lower doses. GAT1102 also protected against MES-induced GTCS, while GAT591 showed no effect in MES test. However, despite its strong anti-seizure effects in GTCS, GAT1102 increased atypical absence seizures in *Gabrb3*^{+/*D120N*} mice, suggesting proconvulsant effects in this model of Lennox-Gastaut syndrome. Moreover, GAT1102 had hypothermic effects typical of orthosteric agonists.

Chapter 4 investigated how seizure burden impacts the expression of specialized pro-resolving mediators, which are key to understanding the resolution mechanism in epilepsy syndromes.

Resolution emerges as an endogenous defensive response to resolve excessive inflammation. In epilepsy, persisting neuroinflammation is recognized as one of the contributing factors to worsening neuronal hyperexcitability. Pro-resolving mediators derived from polyunsaturated fatty acid precursors such as arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) initiate anti-inflammation and promote tissue repair. Our previous study found that a high seizure burden in *Scn1a*^{+/-} mice increased prostaglandin markers in the hippocampus, so Chapter 4 extends on this work by examining whether the high-seizure burden increases specialized pro-resolving mediators. We analyzed the expression changes of pro-resolving mediators in the hippocampus, a key region for seizure propagation. *Scn1a*^{+/-} mice with high seizure burden displayed increased concentrations of pro-inflammatory mediators, leukotriene B4 and E4, suggesting an ongoing neuroinflammation in the hippocampus. Further, we also observed that the high seizure burden in *Scn1a*^{+/-} mice increased hippocampal concentrations of different special pro-resolving mediator families, that is, the maresins (maresin1), D-series resolvins (RVD1 and RVD4), and protectins (PCTR1). Despite these changes, the mRNA expression of lipoxygenase genes (Alox5, Alox12, and Alox15), which synthesize these mediators, remained unaffected.

Finally, Chapter 5 provides a general discussion to summarize the findings from all research chapters and discuss their significance in the context of seizure management for Dravet syndrome and Lennox-Gastaut syndrome. The discussion also addresses limitations in experimental design and proposes suggestions for future research directions.

Chapter 1

Introduction

1.1 Seizure, epileptogenesis and epilepsy

Neuronal activity governs circuit formation in controlling a wide range of brain functions, including emotion, memory and motor movement.¹ An imbalance in neuronal activity can become pathological where excessive excitatory and insufficient inhibitory neuronal activities lead to hypersynchronous discharges. A seizure refers to the transient occurrence of these abnormal discharges in the brain, which can be either provoked or unprovoked.² However, the onset of a seizure does not necessarily imply a diagnosis of epilepsy.³

Epileptogenesis describes the series of pathological processes that convert a healthy brain into one with epilepsy.⁴ Generally, epileptogenesis progresses through three phases: the initial phase, the silent phase, and the chronic phase. During the initial phase, an epileptogenic insult triggers structural and physiological changes in the brain that increase its susceptibility to seizures. This is followed by the silent phase, a latent period during which ongoing modifications to the brain lead to the chronic phase, characterized by spontaneous recurrent seizures.⁴ These pathological changes often result in irreversible brain damage, creating an enduring predisposition to generate seizures, thereby leading to epilepsy.⁴

Epilepsy is a chronic neurological disease, clinically characterized by (1) the occurrence of two or more unprovoked seizures at least 24 hours apart or (2) the occurrence of a single unprovoked seizure with more than 60% of seizure recurrence risk over the next ten years or (3) the diagnosis of epilepsy syndrome.³ Examples of epilepsy syndromes include Dravet syndrome and Lennox-Gastaut syndrome, which are characterized by different seizure types, findings in electroencephalogram (EEG), imaging features and potential underlying genetic causes.³ However, in some cases the etiology remains unknown, although pathogenic mutations are identified in the majority of Dravet syndrome diagnoses and in Lennox-Gastaut syndrome.

Depending on which regions of the brain are affected, epileptic seizures can result in brief episodes of impaired awareness, cognitive symptoms, and motor manifestations.

1.2 Epidemiology

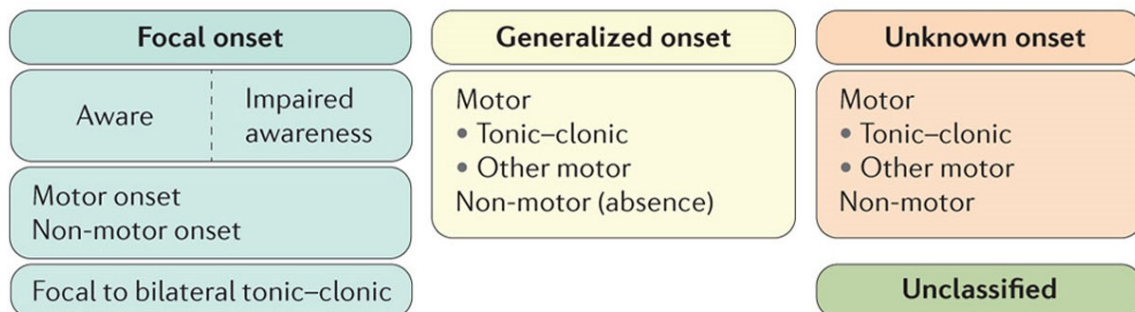
1.2.1 Global statistics

Epilepsy remains the most prevalent neurological disease, affecting over 70 million people in the world.^{5, 6} Pooled data from a meta-analysis reported that the incidence rate of epilepsy was 61.4 per 100,000 person-years.⁷ The disease onset has a bimodal distribution with peaks in the childhood and elderly populations. The overall lifetime prevalence of epilepsy was 7.60 per 1,000 population, while 6.38 per 1,000 population received regular treatment for active epilepsy.⁷ Incidence and prevalence were higher in low- or middle-income countries than in high-income countries. This disparity is partly attributed to higher rates of birth injuries, infectious disorder and limited healthcare resources, which often result in inadequate care and progression to epilepsy.⁸ Nearly 75% of these patients live in low-income countries and do not have access to necessary treatments.⁹

1.2.2 Australian statistics

Australia comprises urban and rural areas with a population of over 25 million people of different ethnic origins, including Aborigines and Torres Strait Islanders, Caucasians and worldwide immigrants.¹⁰ The Australian government estimated in 2017 – 2018 that around 151,000 people were diagnosed with epilepsy, and 9,000 of them were Indigenous Australians.¹¹ Epilepsy expenditure accounted for around 134 billion Australian dollars in the health system with an average of 31,400 hospitalizations yearly.¹¹

The most recent classification framework was introduced by the International League Against Epilepsy (ILAE) in 2017.² It applies to both adults and children seizure but not neonatal seizure. This classification is organized into three key layers that describe the critical features of a seizure: (1) the onset location in the brain; (2) the level of awareness during the seizure and (3) other characteristics of the seizure (Figure 1).²



The classification begins by determining whether the seizure originates as a focal or generalized onset of the brain.² A focal seizure refers to an episode that starts in one hemisphere of the brain. It may remain localized within a specific neuronal network or spread more widely within the same hemisphere. In contrast, a generalized seizure involves both hemispheres. If there is insufficient information to determine the origin of the seizure, it is classified as having an unknown onset.²

4

is made by assessing whether the patient can recall their surroundings during the seizure, although it is not necessary for them to be aware of the onset of seizures. The final stage of classifying focal seizures involves identifying motor or non-motor symptoms as part of the seizure's semiology.

Generalized-onset seizures can also be categorized based on motor or non-motor symptoms.² Seizures with a non-motor generalized onset are typically considered as absence seizures. This classification system also facilitates the identification of seizure evolution, either through propagation within a single hemisphere or through involvement of both hemispheres from the onset. Similarly, seizures with unknown onset can be categorized based on motor or non-motor features. The expanded classification framework provides a detailed description of seizure semiology according to the different possible onset locations (Figure 2).

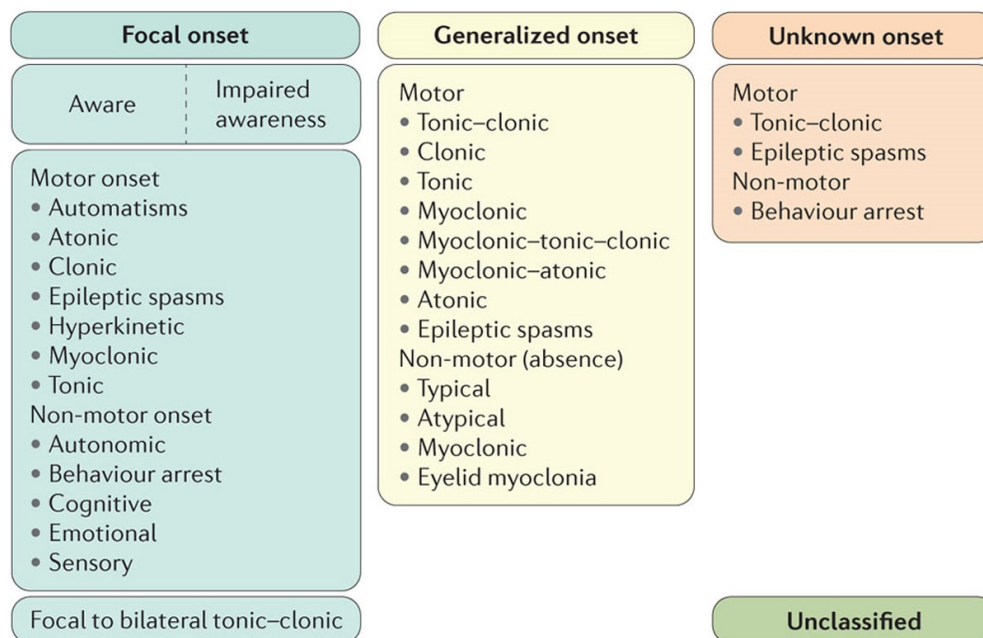


Figure 2. The expanded the International League Against Epilepsy 2017 operational classification of seizure types. The following clarifications should guide the choice of seizure type. Fisher RS, Cross JH, French JA, Higurashi N, Hirsch E, Jansen FE, et al. Operational classification of seizure types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology Epilepsia. 2017 Apr;58:522-530.

1.4 Classification of epilepsy

In 2017, the ILAE also revised its classification of epilepsy, which serves as the principal clinical tool for evaluating epilepsy types, along with associated comorbidities and etiologies.¹² This classification functions as a three-tier diagnostic guideline (Figure 3), emphasizing treatment considerations at each level. It begins by categorizing seizure types, followed by the classification of epilepsy into one of four categories: (1) focal epilepsy, (2) generalized epilepsy, (3) combined generalized and focal epilepsy, and (4) unknown epilepsy. Once seizure or epilepsy types are identified, the etiology and any comorbidities should be incorporated into the treatment plan.¹²

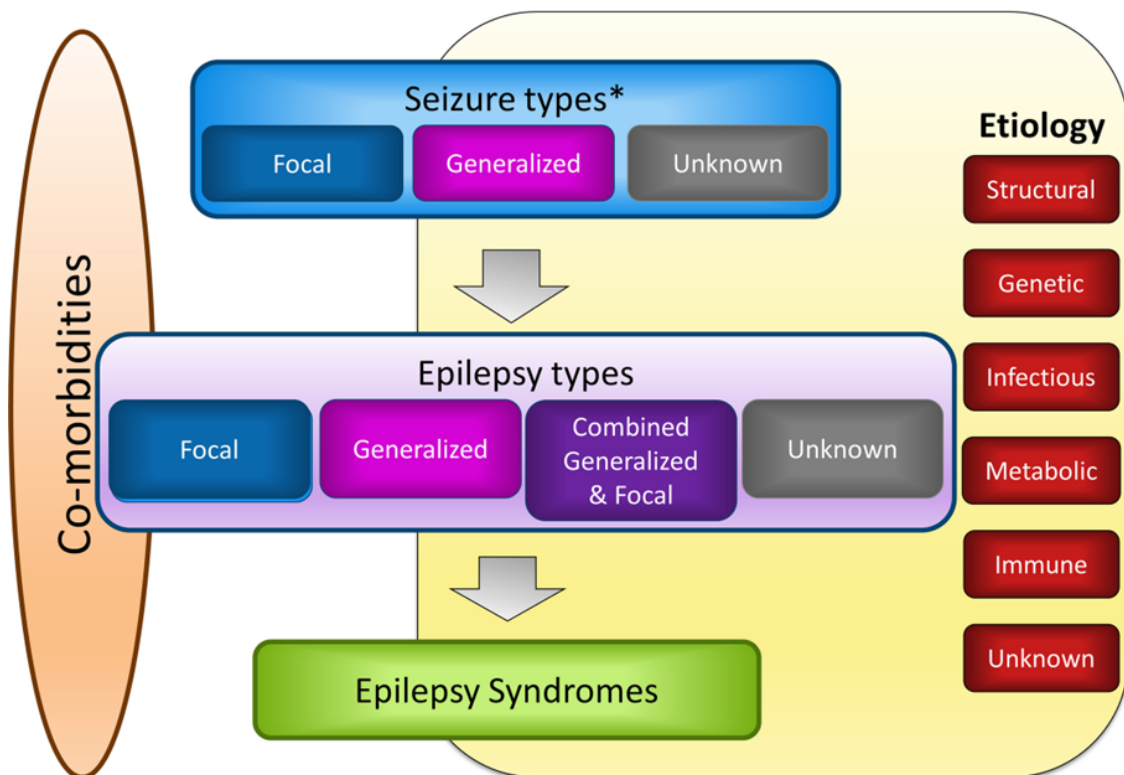


Figure 3. Framework for classification of epilepsies. Epilepsies are classified into four types on the origin of seizure activity. Scheffer IE, Berkovic S, Capovilla G, Connolly MB, French J, Guilhoto L, et al. ILAE classification of the epilepsies: Position paper of the ILAE Commission for Classification and Terminology Epilepsia. 2017 Apr;58:512-521.

Patients with focal epilepsy may exhibit more than one seizure type and can be further classified as having unifocal or multifocal epilepsy, based on the presence of interictal focal discharges.¹² In contrast, patients with generalized epilepsy typically show generalized spike-wave activity on their EEG. Combined generalized and focal epilepsy describes patients who experience both generalized and focal seizures, as evidenced by clinical and EEG findings, such as in Dravet syndrome and Lennox-Gastaut syndrome. Unknown epilepsy is diagnosed when clinical information is insufficient to determine the epilepsy type, often due to an unclear seizure onset.

The third diagnostic level focuses on epilepsy syndromes, which are defined as clusters of features, including seizure types, EEG patterns, and imaging findings that tend to occur together.¹² Syndrome-specific features, such as age of onset, diurnal variation, and prognosis, are crucial factors to consider in diagnosis and treatment. In 2022, the ILAE further refined syndrome classification by subdividing syndromes according to specific age of onset.¹³ Additionally, they introduced the category of developmental and epileptic encephalopathy (DEE), which refers to epilepsy syndromes associated with developmental impairment.¹³

1.5 Etiology

1.5.1 Structural etiology

The ILAE identifies six epilepsy etiologies, each carrying significant implications for treatment and prognosis.¹² An etiological diagnosis should be considered at seizure onset, considering the progression of epileptogenesis. Given the dynamic nature of epilepsy, multiple etiologies may be relevant to a single patient. As a result, continuous re-evaluation is necessary to adapt the treatment plan and ensure the most effective management as the condition evolves.

A structural etiology refers to a visible abnormality detected through neuroimaging, such as magnetic resonance imaging (MRI), with corresponding localization in the EEG. Structural abnormalities may result from trauma, genetic lesions, or tumors.¹⁴ The diagnosis of a structural etiology relies on the alignment of findings from both neuroimaging and EEG.¹² If discrepancies exist, a structural etiology should not be assumed.

1.5.2 Genetic etiology

A genetic etiology is characterized as a known genetic mutation where seizures occur as the primary symptom, accounting for approximately one-third of epilepsy cases in children and adults.^{12, 15, 16} Genetic mutants in epilepsy are often heterogeneous, and these mutated alleles can be inherited. Therefore, genetic testing and the assessment of family history are critical components in diagnosing this etiology.

Based on the advances in sequencing techniques, the role of genetics in epilepsy is increasingly recognized. The ILAE Consortium on Complex Epilepsies conducted the largest genome wide association study (GWAS) on epilepsy, and recruited over 15,000 patients with epilepsy and 29,000 control participants.¹⁷ This study revealed 11 novel genome-wide loci associated with epilepsy and identified eight genes (*BCL11A*, *GJAI*, *ATXN1*, *GABRA2*, *KCNAB1*, *SCN3A*, *PCDH7*, *STAT4*) that contributes to this association.¹⁷ Mapping of genetic architecture of epilepsy has help characterized the biological functions of these mutations, particularly in ion-channels (*SCN1A*, *SCN2A*, *SCN3A*, *GABRA2*, *KCNN2*, *KCNAB1*, and *GRIK1*) and transcription factors (*ZEB2*, *STAT4* and *BCL11A*). The discovery of epilepsy-related genes highly has greatly enhanced the understanding of some forms of epilepsy, such as Dravet syndrome and Lennox-Gastaut syndrome. Notably, over 900 gene have been identified as risk

factors to epilepsy with around 90% of these genes associated with developmental and epileptic encephalopathies.¹⁸

1.5.3 Infectious etiology

An infectious etiology refers to cases where infectious pathogens directly induce epilepsy, with seizures being the primary symptom.¹² In some cases, epilepsy may arise as a secondary condition following a prior infection, which is still considered an infectious etiology. This classification indicates that the patient has developed epilepsy, rather than experiencing seizures as a symptom of an acute infection.¹⁹ Structural abnormalities, often identified in the post-infection stage, may contribute to the development of epilepsy. Consequently, an infectious etiology has specific treatment implications.¹² Common causes within this category include neurocysticercosis,²⁰ human immunodeficiency virus (HIV),²¹ cerebral malaria, cytomegalovirus, and cerebral toxoplasmosis.²²

1.5.4 Metabolic etiology

Secondary epilepsy caused by a known metabolic disorder falls under the category of metabolic etiology.¹² Such metabolic disorders can lead to biochemical changes throughout the body, resulting in conditions like porphyria,²³ uremia, or pyridoxine-dependent seizures.^{24, 25} A genetic defect may overlap with these metabolic disorders, suggesting that metabolic epilepsy could have a genetic basis.^{12, 26} Early identification of a metabolic etiology is crucial, as patients in this group require specific therapies and preventive measures to manage the disease effectively.

1.5.5 Immune etiology

Immune-related epilepsy arises as a result of immune disorders that are frequently associated with brain inflammation.¹² The incidence of immune epilepsies increases as our understanding of immune disorders improves, particularly with the discovery of various antibodies in autoimmune encephalitis. Accessing antibody expression aids diagnosis of immune-related epilepsy, such as anti-N-methyl-D-aspartate (NMDA) encephalitis²⁷ and anti-Leucine-rich glioma-inactivated protein 1 (LGI1) encephalitis.²⁸ With the emergence of antibody entities, patients with this etiology may receive special immunotherapies for seizure control.

1.5.6 Unknown etiology

Up to one-third of epilepsy patients have an unclear cause of epilepsy and should be classified under unknown etiology.^{15, 16} In clinical practice, treatment for patients with unknown etiology should be tailored based on their medical history, age, and seizure characteristics.¹² In developing countries, limited access to advanced brain imaging techniques and genetic testing may further hinder the accurate identification of epilepsy etiologies.

1.5.7 Etiology distribution by age

The distribution of seizures and epilepsies is strongly dependent on the age of onset.²⁹ A study implemented this new etiological classification retrospectively to population-based child population aged 3 – 13-years-old¹⁵ reported compatible results with another study reassessed the etiology in adults aged at least 18-years-old.¹⁶ Over one-third of the patients had unknown etiology in both age groups.^{15, 16} Child populations had a higher proportion in genetic etiology

than adults while infection-caused epilepsy was more common in adults. The proportion of patients with structural and metabolic etiology were similar in both populations.^{15, 16}

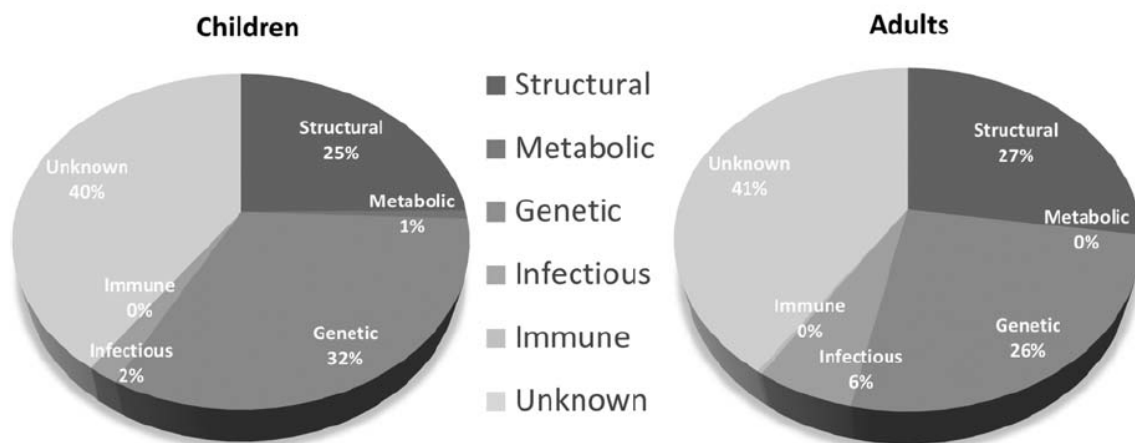


Figure 4. Etiologies of epilepsy by age. Data are obtained from two different studies that evaluated etiologies of epilepsy in children and adults using the new the International League Against Epilepsy classification of the epilepsies. Falco-Walter J. Epilepsy-Definition, Classification, Pathophysiology, and Epidemiology Semin Neurol. 2020 Dec;40:617-623.

1.6 Neonatal seizure

The classification of neonatal seizures differs from the seizure types outlined above. The ILAE neonatal task force established a classification framework for neonatal seizures.³⁰ In line with the 2017 seizure classification, a three-tier guideline was adopted for seizures in neonates with modifications.³⁰

In newborns, seizures occur exclusively with focal onset.^{30, 31} Thus, the division of generalized onset is unnecessary. Rapid propagation may spread bilaterally in rare conditions, such as spasms or myoclonic seizures.³¹ Instead, clinical presentations on whether the neonate is critically ill or remains suspicious are the first step for seizure diagnosis. Additionally, accessing their level of awareness is not applicable. Therefore, a differential diagnosis is

introduced using video-EEG recording or amplitude-integrated EEG to determine whether a seizure is electroclinical or electrographic-only (without clinical signs). Finally, a seizure can be subdivided according to the predominant clinical manifestation, as this is more likely to have clinical implications for etiology than determining the seizure-onset zone.³⁰

1.7 Epilepsy syndrome

Epilepsy syndromes present as age-dependent onset with distinctive EEG patterns and seizure phenotypes. Patients with epilepsy syndrome display a range of comorbidities and atypical features that require different therapeutic and prognostic implications. The ILAE Nosology and Definitions Task Force published guiding principles to assist with clinical diagnosis.¹³ These syndromes are grouped according to age at onset: (1) neonatal and infantile, (2) childhood, (3) variable age and (4) idiopathic generalized epilepsies.¹³ The syndromes are then subdivided into each age group based on the seizure types in line with the 2017 epilepsy classification.¹³

Developmental and epileptic encephalopathy is considered when patients exhibit cognitive and developmental impairment with a specific age of onset (Figure 5), such as Dravet syndrome^{32, 33} and Lennox–Gastaut syndrome.^{13, 34} In the following parts of this thesis, we will focus on studying and discussing Dravet syndrome and Lennox–Gastaut syndrome.

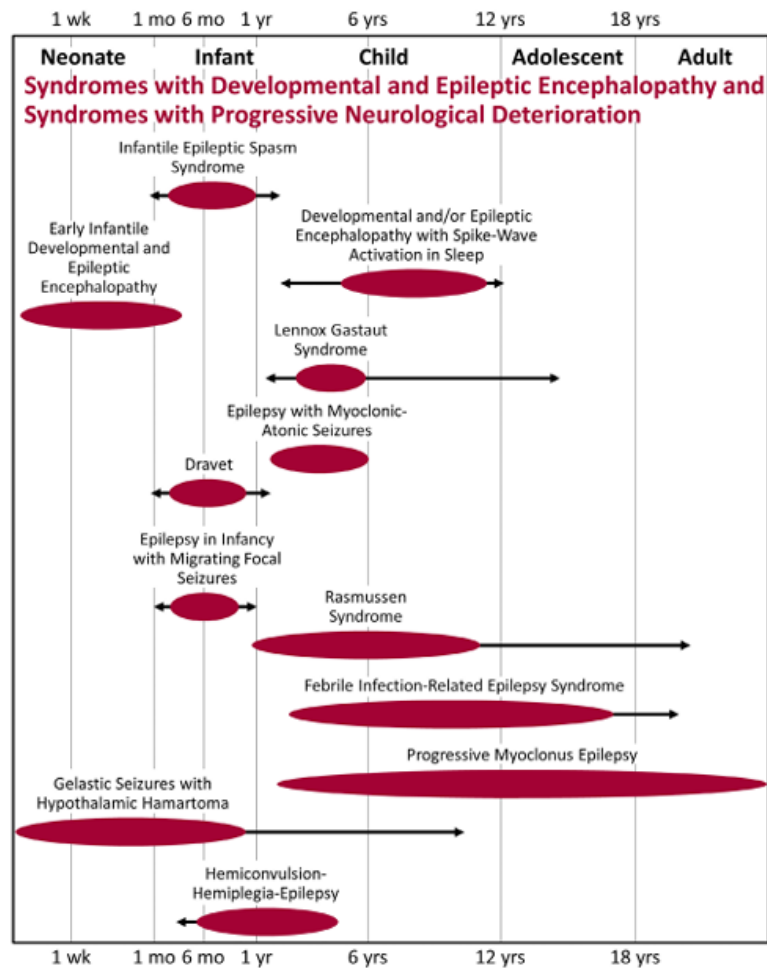


Figure 5. Classification of syndromes with developmental and/or epileptic encephalopathy or with progressive neurological deterioration based on age at presentation. Wirrell EC, Nabbout R, Scheffer IE, Alsaadi T, Bogacz A, French JA, et al. Methodology for classification and definition of epilepsy syndromes with list of syndromes: Report of the ILAE Task Force on Nosology and Definitions *Epilepsia*. 2022 Jun;63:1333-1348.

1.8 Dravet Syndrome

1.8.1 Overview

Dravet syndrome is an infantile-onset epilepsy syndrome with an average onset age of 6 months (Figure 5). It is a lifelong condition classified as a developmental and epileptic encephalopathy, affecting 6.5 infants in every 100,000 births.³⁵⁻³⁷ Despite available treatments, seizures are often intractable and evolve across the lifespan. Typical seizure phenotypes include febrile and

afebrile, focal clonic and generalized tonic-clonic seizures (GTCS).³⁵ Cognitive impairments and developmental delays emerge slowly in the second year of life, for example, speech delays and gait instability.^{38, 39} Although these patients experience lower health-related quality of life than patients with other chronic illnesses, improvements can be seen by reducing seizure frequency.⁴⁰

1.8.2 Diagnosis

Patients suspected of having Dravet syndrome typically experience prolonged seizures triggered by fever or photosensitivity as their first seizure.^{35, 41} Diagnosing Dravet syndrome can be challenging, as EEG investigations are sometimes not feasible in infants. Slow-wave patterns with interictal discharges are typically found in the EEG profiles of Dravet syndrome.⁴² However, no EEG abnormalities does not exclude the possibility of Dravet syndrome. MRI imaging may provide additional diagnostic support when brain atrophy is present, although an unremarkable MRI cannot rule out the condition.⁴³ Genetic testing is considered a critical diagnostic tool, as *SCN1A* pathogenic variants are the most common cause of Dravet syndrome. Identifying *SCN1A* variants as genetic biomarkers has significantly improved the accuracy of diagnosis.

1.8.3 Disease mechanisms

In 2001, genetic screening captured a *de novo* heterozygous mutation of the *SCN1A* gene in patients with Dravet syndrome and confirmed that this disease has a genetic basis. (Figure 6).⁴⁴

⁴⁵ Notably, over 80% of these patients carrying *SCN1A* mutations exhibit disruption in the transmembrane region with approximately half of these cases involving missense mutations.⁴⁶⁻

⁴⁸ Familial inheritance accounts for around 10% of other cases.⁴⁹ The *SCN1A* gene is located on chromosome 2q24 and encodes the alpha subunit of the voltage-gated sodium channel (Nav1.1). Nav1.1 is predominantly expressed in cortical and hippocampal GABAergic inhibitory interneurons. A reduced sodium current in inhibitory GABA interneurons is the proposed mechanism of Dravet syndrome, which causes neuronal hyperexcitability via disinhibition.⁴⁴ For patients carrying a loss-of-function mutation in Nav1.1, sodium channel blockers are not ideal drug to treat seizure, and drug such as lamotrigine and carbamazepine are contraindicated for Dravet syndrome patients.

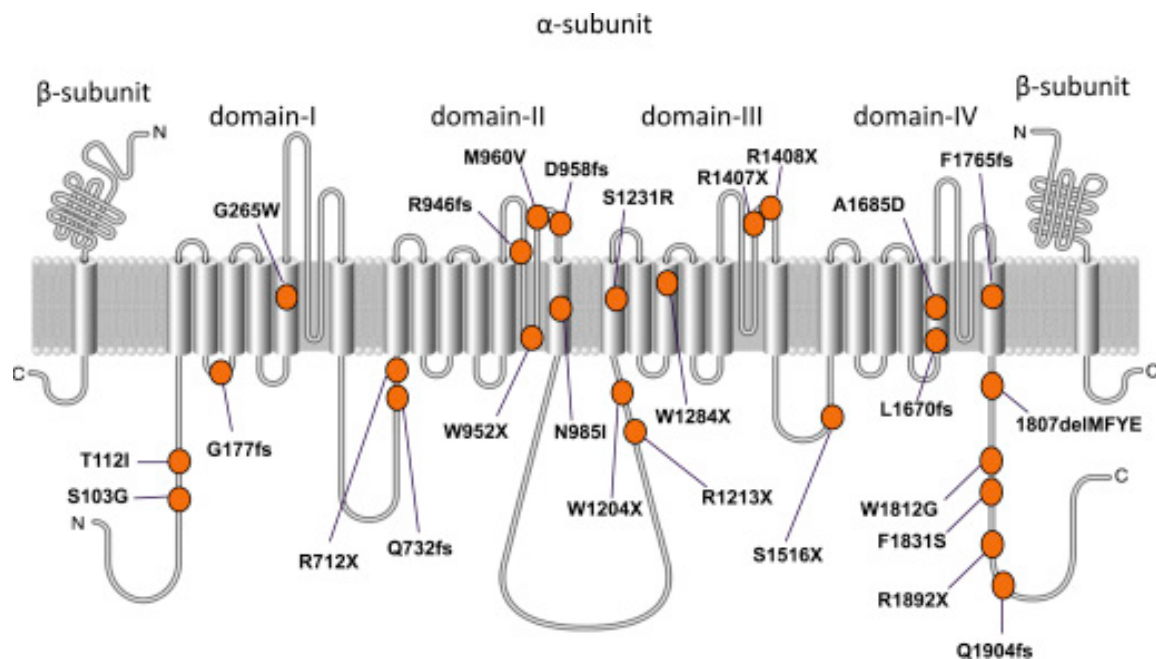


Figure 6. De novo *SCN1A* mutations across the *SCN1A* protein. Four homologous domains are in the alpha subunit and each form of six transmembrane segments. Fujiwara T, Sugawara T, Mazaki-Miyazaki E, Takahashi Y, Fukushima K, Watanabe M, et al. Mutations of sodium channel alpha subunit type 1 (*SCN1A*) in intractable childhood epilepsies with frequent generalized tonic-clonic seizures *Brain*. 2003 Mar;126:531-546.

Studies have recently discovered gain-of-function *SCN1A* variants in early-infantile onset epilepsy, expanding the disease spectrum of *SCN1A*-related Dravet syndrome (Figure 7).⁵⁰

Functional analysis revealed that single point variants in sodium channel (such as p.I1498M⁵⁰ and p.R1636Q⁵¹) increased current amplitude in action potential-clamp and display an increase of persistent current, suggesting gain-of-function features.⁵¹ Patients carrying gain-of-function mutants exhibit three distinct clinical phenotypes: more severe seizures, congenital arthrogryposis and movement disorder.⁵⁰ Unlike patients with loss-of-function variants, most of these patients respond to sodium channel blocking therapies.^{50, 52}

Besides the *SCN1A* gene, other genetic variants in *PCDH19*, *GABRA1*, *CHD2*, *STXBP1* and *SCN1B* are associated with Dravet syndrome.⁵³⁻⁵⁷ Potential genotype-phenotype correlations may exist, for example a missense *SCN1A* mutation, p.Thr226Met lead to infantile *SCN1A* encephalopathy but not Dravet syndrome. This conversely suggests that not all *SCN1A* mutations cause Dravet syndrome.⁵⁸ However, the physiological mechanism by which these changes alter neuron networks to produce excessive firing remains unclear. A biophysical mechanism suggests a homomeric interactions between a functional dominant mutation and wild type. This interaction not just alters membrane potential but also further reduces the availability of the wild-type channel, thereby resulting in more profound effects than simple haploinsufficiency.⁵⁹ This mechanism may explain the severity of clinical phenotypes in patients with gain-of-function *SCN1A* mutation, such as p.Thr226Met in *SCN1A* gene.

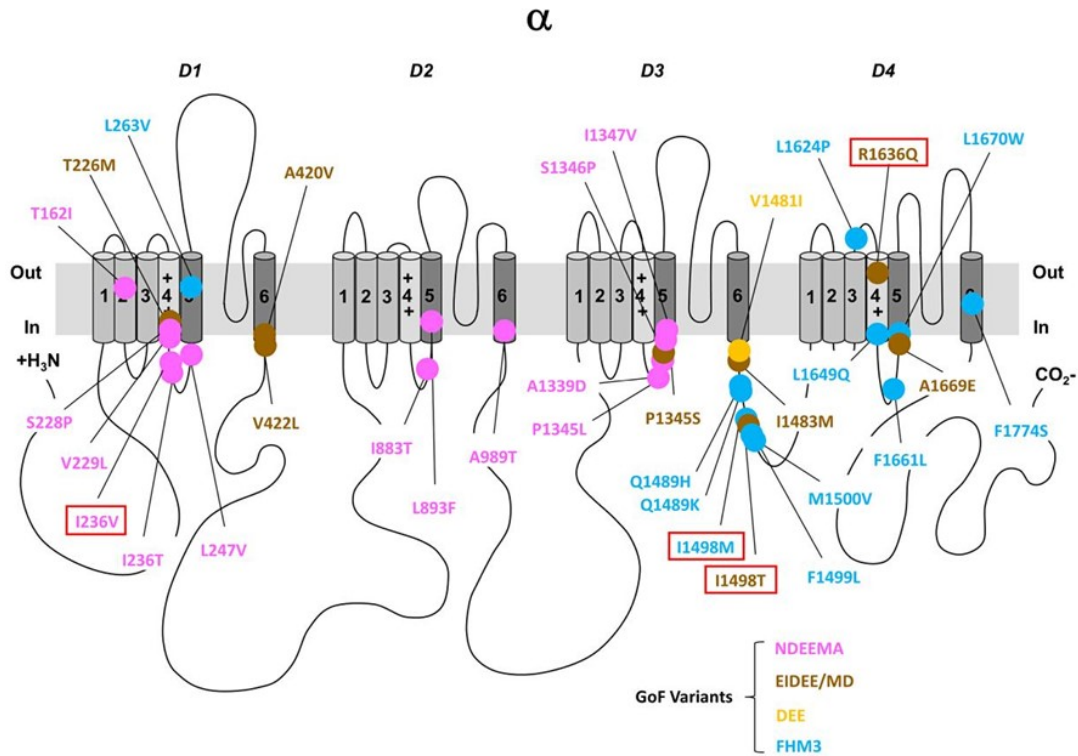


Figure 7. Gain-of-function variant phenotypes across the *SCN1A* protein. Four homologous domains (D1–4) are in the alpha subunit and each form of six transmembrane segments (S1–S6). Segment 4 represents the voltage sensor and segments S5–6 the pore region. Individual missense variants are displayed as different coloured circles according to phenotype: Pink denotes neonatal developmental and epileptic encephalopathy with movement disorder (NDEEMA), brown denotes early infantile developmental and epileptic encephalopathy (EIDEE) / movement disorder, amber denotes developmental and epileptic encephalopathy (DEE), and blue denotes familial hemiplegic migraine 3 (FHM3). Functionally studied variants are framed in red. Brunklaus A, Brunker T, Feng T, Fons C, Lehikoinen A, Panagiotakaki E, et al. The gain of function *SCN1A* disorder spectrum: novel epilepsy phenotypes and therapeutic implications *Brain*. 2022 Nov 21;145:3816-3831.

1.8.4 Mortality

Children with Dravet syndrome show a higher risk of premature death and sudden unexpected death in epilepsy (SUDEP) is the leading cause of death. Mortality rates in Dravet syndrome have been reported to range from 3.7% to 17%, compared with only 3.5% in other newly diagnosed childhood epilepsy.⁶⁰⁻⁶² SUDEP is a fatal complication of epilepsy without a death cause. The mechanism of this complication is not yet fully understood but current data implies

that heart arrest and respiratory dysfunction may play a role in causing this emergency condition.^{63, 64} A recent study suggests that the *SCN1A* gene is correlated to cardiac arrhythmia, leading to premature mortality during seizure onset.⁶³ Additionally, high frequency of GTCS and status epilepticus are other risk factors that cause death.

1.8.5 Surgery

Surgical management in Dravet syndrome is generally limited to the implantation of a vagus nerve stimulation device. The device is programmed to deliver an intermittent electrical stimulation to the vagus nerve to modulate the neural circuit in cortex and the limbic system.⁶⁵⁻⁶⁷ At present, only a few studies have reported the efficacy of vagus nerve stimulation.^{68, 69} Of note, approximately half of the patients showed a 50% reduction in seizures, but the adverse events were inconsistent across different studies.⁶⁹ Early complications include hoarseness, throat pain and facial nerve paralysis, which often improve over time.^{70, 71} The North American Consensus Panel recommends that vagus nerve stimulation devices should only be considered after first- and second-line anti-seizure medications (ASMs). Other epilepsy surgery, such as corpus callosotomy and resective surgery are, in general, less common in Dravet syndrome.⁴¹ Among 16 patients with Dravet syndrome who underwent either temporal lobectomy or corpus callosotomy, only 19% of them experienced 50% seizure reduction.⁴¹

1.8.6 Ketogenic Diet

A ketogenic diet is an alternative dietary treatment consisting of a high-fat, low-protein and low-carbohydrate diet for managing refractory seizures.⁷² This diet shifts the brain's energy composition from glucose to the ketone body. Although the exact mechanisms by which these

changes suppress seizures are unclear, beta-hydroxybutyrate (a key metabolite) produced by the high-fat diet decreases neuronal excitability.⁷³ A prospective study following patients on a ketogenic diet for two years showed that 75% of patients reached a 50% seizure reduction rate in reduction, with two patients becoming seizure-free.⁷⁴ Similarly, the most recent meta-analysis study also reported that 47% of patients experienced a 50% reduction in seizure frequency.⁷⁵ While short-term side effects, such as gastrointestinal discomfort and urinary ketosis, are common, no long-term toxic side effects have been documented.

1.8.7 Genetic therapeutic

Given the genetic basis of Dravet syndrome, genetic therapy is gaining attention to restore the functional expression of mutated gene targets. These therapies can be delivered via viral vectors and non-viral genetic therapeutic technologies.

Several genetically engineered viral vectors have been developed, and one recently translated to a phase I/II clinical trial. ETX-101 specifically targets GABAergic interneurons to enhance endogenous expression of *SCN1A*.⁷⁶ This treatment is designed as a one-time gene editing therapy administered by intracerebroventricular delivery. In a mouse model of Dravet syndrome, a single dose of ETX-101 reduced spontaneous and hyperthermia-induced seizures and prolonged survival.⁷⁶

Another approach involves antisense oligonucleotides to increase mRNA transcript levels and, subsequently, the NaV1.1 protein in the brain. STK-001 employed the proprietary Targeted Augmentation of Nuclear Gene Output (TANGO) technology⁷⁷ that binds to the functional *SCN1A* pre-mRNA, preventing the inclusion of the nonproductive exon and thereby increasing the wild-type *SCN1A* transcript.⁷⁸ An ongoing clinical trial of STK-001 showed multiple doses

of STK-001 promoted seizure reduction that was well-tolerated by patients.⁷⁹ It is worth noting that these gene therapies cannot only reduce seizure frequency but also diminish ASM requirements.⁷⁹ However, these approaches are still under development, where the treatment is highly invasive due to the delivery by intrathecal administration. This procedure carries potential risks, such as the central nervous system (CNS) infection and neurological complications and not all individuals are suitable for genetic therapeutics.⁷⁹

1.8.8 Drug treatment

The scientific understanding of Dravet syndrome has increased since its original description in 1978, but the treatment options are still limited. Seizures in Dravet syndrome are a refractory in nature, providing substantial challenges in management. For most patients, ASMs are the primary treatment option (Figure 8).⁸⁰

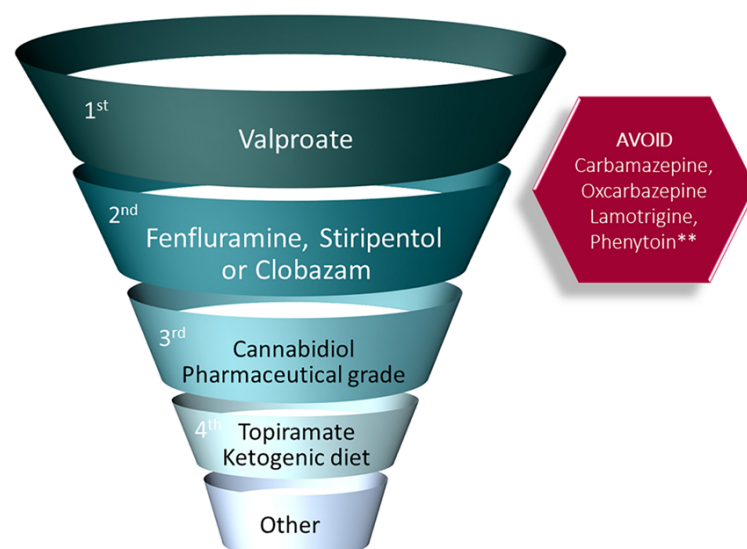


Figure 8. Therapeutic algorithm for maintenance therapies for management of seizures in Dravet syndrome. Wirrell EC, Hood V, Knupp KG, Meskis MA, Nabbout R, Scheffer IE, et al. International consensus on diagnosis and management of Dravet syndrome *Epilepsia*. 2022 Jul;63:1761-1777.

Valproic acid is an appropriate first-line drug, as it is a broad-spectrum ASM that targets multiple ion channels and receptors.⁸⁰ However, many patients rely on polytherapy for effective seizure control. In such cases, clobazam can be considered either as an initial monotherapy or a second ASM with valproic acid. Stiripentol and fenfluramine are other widely used second-line drugs as an adjunctive treatment. Cannabidiol (CBD) has recently approved ASM for Dravet syndrome due to its high efficacy and well-tolerated profile. The anti-seizure effects of CBD and its mode of action will be further discussed in the following sections.

Given the underlying disease mechanism, Guerrini et al. first proposed avoiding sodium channel blockers (e.g., carbamazepine and lamotrigine).⁸¹ They suggested that these agents aggravate seizure outcomes and negatively impact cognitive outcomes.⁸¹ Although, the current management goal often aims at seizure reduction, both clinicians and caregivers agree that cognitive and behavioral comorbidities must also be addressed.^{41, 80 82} These comorbidities affect the overall quality of life beyond seizures.

1.8.9 Rodent model

An effective screening platform for drug development relies on an *in vivo* model which encompasses multifactorial disease mechanisms in the brain, but also the *in vivo* bioavailability of the test molecules. Replicating a genetic mutation in rodent models becomes a strategy for developing a reliable disease model. Several rodent models resemble *SCN1A* gene deficit,⁸³⁻⁸⁶ which leads to a loss-of-function deficiency in the sodium channel subunit Na_v1.1 that is an underlying pathophysiological mechanism.⁸³ These mouse models of Dravet syndrome carry either *Scn1a* knock-out or a mutation, such as a heterogenous deletion,⁸³ A1783A mutation⁸⁴,⁸⁵, E1099X mutation⁸⁶ and R1648H mutation.⁸⁷ The first Dravet syndrome-specific mouse

model was established in 2006 with a homozygous *Scn1a* knockout (*Scn1a*^{-/-}).^{83, 88} This model exhibited spontaneous seizures and behavioral comorbidities. However, the knockout mice only lived on average to postnatal 15 (P15), and so the mice did not live to adulthood.⁸³ Therefore, mouse models involving heterozygous deletion of *Scn1a* (*Scn1a*^{+/-}) became more prominent allowing experimentation into adulthood, although there are still sporadic deaths after P21 in these lines.⁸⁹ *Scn1a*^{+/-} mice resemble key clinical features, such as hyperthermia-induced and spontaneous seizures and behavioral deficits.⁸⁹ Additionally, mouse strain is correlated to their phenotypes, in which a more severe phenotype was induced by crossing with C57BL/6J genetic background compared to a 129 background strain.⁹⁰ These validated mouse models offer a functional assessment to identify novel ASMs and allow further study of the pathological mechanism of the disease formation.

1.9 Lennox-Gastaut syndrome

1.9.1 Overview

Lennox-Gastaut syndrome belongs to an epilepsy syndrome with an onset typically 18 months to 8 years of age, with a peak onset between 3 and 5 years of age (Figure 5).⁹¹ It is classified as an epileptic encephalopathy that can evolve from other infantile epilepsy syndromes, such as epileptic spasms. The incidence of Lennox-Gastaut syndrome ranged from 14.49 to 28 per 100,000 individuals.^{92, 93} The prevalence was reported as 6.5 to 39.2 per 100,000 individuals, accounting for 1 - 2 % of the epilepsy population diagnosed with Lennox-Gastaut syndrome.^{94, 95} Patients with Lennox-Gastaut syndrome experience intractable seizures that are resistant to drug treatment. Multiple seizure types usually co-occur, for example, tonic, atonic and atypical absence seizures.^{34, 96} Tonic and atypical absence seizures persist in adulthood whereas atonic

seizure can be managed in most cases.⁹⁷ While one-third of patients display normal development before seizure onset, intellectual deficits become apparent over time.⁹⁸ The long-term prognosis for these patients is unsatisfactory due to the delayed diagnosis in some cases.

1.9.2 Diagnosis

Current clinical diagnosis of Lennox-Gastaut syndrome follows three hallmark criteria: (1) multiple drug-resistant seizures before the age of 18 (must include tonic seizure), (2) cognitive and behavioural impairments, which may not be present at seizure onset, and (3) diffuse slow spike-and-wave and generalized paroxysmal fast activity on EEG.⁹⁷ Since this syndrome can overlap with other childhood epilepsy syndromes, EEG patterns are crucial for differential diagnoses. For instance, the onset of atypical absence seizures is accompanied by a slow spike-and-wave pattern or consists of bursts of diffuse paroxysmal fast activity during sleep.⁹⁹ Other diagnostic strategies include clinical follow-up on family history, genetic investigations and cognitive assessments. In addition, brain MRI can help detect subtle abnormalities in Lennox-Gastaut syndrome. Structural malformations are common etiology, evolving from cortical malformations and acquired brain injury. Unlike Dravet syndrome, there are no predominant genetic variants linked to Lennox-Gastaut syndrome, although some genetic mutations have been identified from different studies (Table 1).¹⁰⁰

Table 1. Number of studies screening genetic mutations implicated in Dravet syndrome or Lennox-Gastaut syndrome. Sullivan J, Benitez A, Roth J, Andrews JS, Shah D, Butcher E, et al. A systematic literature review on the global epidemiology of Dravet syndrome and Lennox-Gastaut syndrome: Prevalence, incidence, diagnosis, and mortality *Epilepsia*. 2024 May;65:1240-1263.

Gene	Number of articles	
	Dravet syndrome	Lennox-Gastaut syndrome
<i>SCN1A</i>	62	1
<i>PCDH19</i>	9	0
<i>ALDH7A1</i>	1	1
<i>CHD2</i>	2	1
<i>STXBP1</i>	0	1
<i>SCN1B</i>	3	0
<i>STX1B</i>	1	0
<i>SCN8A</i>	4	1
<i>GABRA1</i>	1	0

1.9.3 Disease mechanism

Most cases of Lennox-Gastaut syndrome have an identifiable cause with brain malformations and brain damage, accounting for approximately 75% of the suspected etiology.¹⁰¹ However, with the advancement of whole-genome sequencing, genetic factors are believed to play an important role in causing Lennox-Gastaut syndrome.¹⁰² A study that sequenced the exomes of patients with Lennox-Gastaut syndrome highlighted several genes associated with the condition.¹⁰² Notably, de novo mutations in the *GABRB3* gene have been increasingly recognized. The *GABRB3* gene encodes the $\beta 3$ subunit in GABA receptor type-A (GABA_A) receptors, which are highly expressed in the developing brain, underscoring the importance of this gene in childhood epilepsy.¹⁰³ One possible molecular pathology suggests that *GABRB3*

mutations decrease the expression of the $\beta 3$ subunit on the synaptic cell surface, thereby inactivating synaptic clustering.¹⁰⁴

1.9.4 Mortality

Data from a 10-year follow-up indicated that mortality rate in Lennox-Gastaut syndrome was 2.88%, significantly higher than the age- and gender-matched general population (0.01%).⁹⁵ A longer follow-up period tends to suggest an even higher risk of death in this syndrome. Another retrospective study that followed patients with Lennox-Gastaut syndrome for over 20 years reported a mortality rate of 14.8%.¹⁰⁵ The causes of death in Lennox-Gastaut syndrome are attributed to statue epilepticus and SUDEP. Structural brain damage, refractory seizures and gene mutations are common features among Lennox-Gastaut syndrome patients which are also recognized as risk factors for premature death.^{106, 107}

1.9.5 Surgery

Resective surgery offers favorable long-term seizure outcome for patients who are diagnosed with malformations of cortical development or brain lesion. The effectiveness of surgical outcomes depends on comprehensive examinations to accurately define the epileptogenic regions. A full assessment that includes EEG, MRI and ictal single-photon emission computed tomography (SEPCT) is required prior surgical procedures. A retrospective study reviewed surgical outcome from 90 patients over ten years and found that half of them achieved seizure-freedom.¹⁰⁸ In this report, patients who became seizure-free also shown improvements in behavior and social competence.

Seizures in Lennox-Gastaut syndrome involve highly synchronized discharges across bilateral brain networks. Palliative surgery disconnects two hemispheres across the corpus callosum to prevent propagation of epileptic discharges. Corpus callosotomy can involve either partial resection in the anterior corpus callosum or complete resection. In 23 patients who underwent anterior corpus callosotomy, 8.7% maintained seizure-free for 5 years.¹⁰⁹ For patients with refractory generalized, complete corpus callosotomy was found to be effective in controlling generalized tonic-clonic, atypical absence, atonic seizures.¹¹⁰ Overall, the surgical interventions improved the quality of life for the patients and their caregivers.¹⁰⁹

1.9.6 Ketogenic diet

Similar to the management of Dravet syndrome, the ketogenic diet is an alternative treatment option for Lennox-Gastaut syndrome. A meta-analysis reported that 72% of patients with Lennox-Gastaut syndrome experienced a 50% reduction in seizure frequency across seven retrospective studies.¹¹¹ Among these patients, 11.7% achieved seizure freedom when the ketogenic diet was combined with their ASMs.¹¹¹ EEG data further support these findings, demonstrating a reduction in interictal epileptic discharges.¹¹² Additionally, a separate retrospective study showed that 75% of patients remained on the ketogenic diet after 18 months of treatment, indicating good tolerance.¹¹³

1.9.7 Drug treatment

Complete seizure-freedom is usually not achievable in Lennox-Gastaut syndrome because of the intractable and highly resistant nature of the seizures to available ASMs. Therefore, effective seizure control remains the primary goal in managing Lennox-Gastaut syndrome.

Patients often require multiple ASMs, as a single effective drug with a strong evidence base for Lennox-Gastaut syndrome is limited. Consequently, treatment decisions should be individualized based on seizure semiology and underlying etiology.⁹⁷

Currently, the U.S. Food and Drug Administration (FDA) has approved eight ASMs for treating Lennox-Gastaut syndrome (Figure 9).¹¹⁴ Valproate remains the first-line treatment among many clinicians despite not having specific approval for Lennox-Gastaut syndrome.¹¹⁵ This is because valproate effectively reduces multiple seizure types, including atonic and atypical absence seizures.¹¹⁶ When valproate alone does not provide adequate seizure control, lamotrigine and rufinamide are often considered adjunctive treatments. Lamotrigine is also recommended as a first-line option. Further, clobazam may be used as an add-on treatment for atonic seizures, although it has been associated with tolerance and cognitive side effects.¹¹⁶

Other approved adjunctive therapies include topiramate and CBD, both of which demonstrate a well-tolerated profile. CBD, a non-psychoactive phytocannabinoid, is approved for use in patients aged two years and older and has been shown to reduce overall seizure frequency, particularly for atonic seizures.¹¹⁷ Felbamate is less frequently used due to its associated safety concerns (serious cardiovascular conditions and hepatic failure),¹¹⁸ while fenfluramine is the most recent FDA-approved drug for Lennox-Gastaut syndrome. A recent study reported that fenfluramine also reduced the risk of SUDEP.¹¹⁹ In addition to the approved drug for Lennox-Gastaut syndrome, other ASMs may be considered precision medicine for individual cases. For example, ethosuximide can be used for absence seizures.¹¹⁶

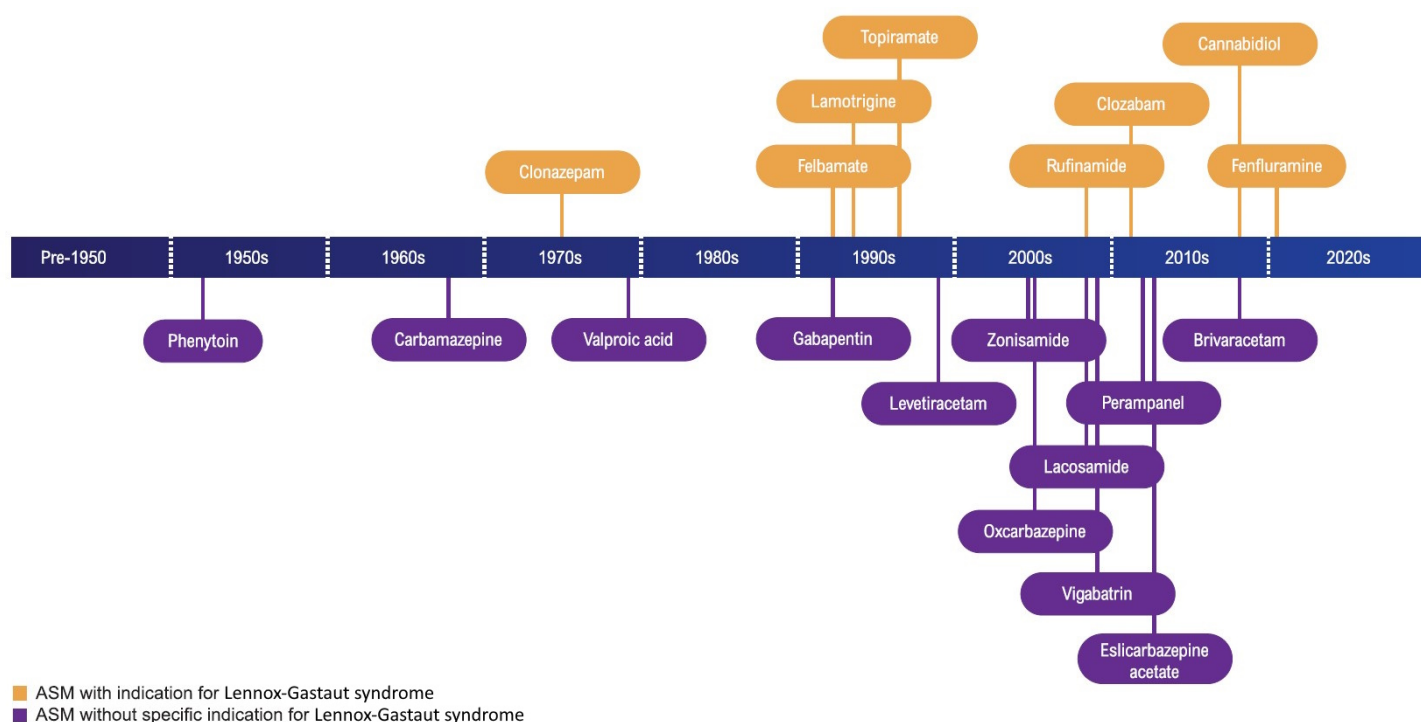


Figure 9. Timeline of U.S. Food and drug administration (FDA) approval of anti-seizure medicines (ASMs) for Lennox-Gastaut syndrome. Sankar R, Chez M, Pina-Garza JE, Dixon-Salazar T, Flamini JR, Hyslop A, et al. Proposed anti-seizure medication combinations with rufinamide in the treatment of Lennox-Gastaut syndrome: Narrative review and expert opinion Seizure. 2023 Aug;110:42-57.

1.9.8 Rodent model

The leading cause of Lennox-Gastaut syndrome has not been established, and multiple etiologies are thought to contribute to the disease. Next-generation sequencing identified several rare *de novo* mutations in the *GABRB3* gene in patients diagnosed with Lennox-Gastaut syndrome.¹²⁰ These mutations impair the inhibitory function of GABA_A receptors, resulting in insufficient inhibitory neurotransmission and triggering seizures.¹²¹ Mutations in the *GABRB3* gene affect the assembly and trafficking of the $\beta 3$ subunit of the receptors. Notably, phosphorylation of the $\beta 3$ subunit regulates the accumulation of GABA_A receptors at synapses, influencing long-term potentiation inhibition.¹²²

One particular mutation, c.G358A, results in a structural change in GABA_A receptors where an aspartic acid residue at position 120 is substituted with an asparagine (p.D120N). Genetic knock-in of the p.D120N in mice (*Gabrb3*^{+/*D120N*}) recapitulates clinical features of Lennox-Gastaut syndrome.¹²³ *Gabrb3*^{+/*D120N*} mice exhibited delayed development, characterized by lower body weight and a limited reproductive window. Atypical absence seizures and myoclonic seizures were observed most frequently between P14 to P17 and persisted into adulthood.¹²³ Interestingly, more atypical absence seizures occurred in the dark hours and peaked at light-to-dark transition. As the mice reach adulthood, they gradually develop memory deficits, social and behavioral impairments, and hyperactivity.¹²³ Therefore, the *Gabrb3*^{+/*D120N*} mice model shares most of the clinical features in Lennox-Gastaut syndrome. These models enable wide drug screening and allow the study of pathological mechanisms.

1.10 Development of anti-seizure medicine

1.10.1 Novel anti-seizure agents

Drug resistance remains a major unsolved problem in Dravet syndrome and Lennox-Gastaut syndrome, increasing the risk of premature death. Thus, the unmet clinical needs of these patients underscore the necessity for more therapeutic options, including new ASMs and potential disease-modifying therapies. The development of ASMs aims to explore novel agents that modulate selective targets to inhibit excessive neuronal discharges. At present, only around 30 ASMs are approved and available on the market.¹²⁴ These medications are classified into four broad classes based on their mechanism of action, although some drugs target multiple molecular pathways. The four classes include (1) voltage-gated ion channel modulators, (2) γ -aminobutyric acid (GABA) enhancers, (3) inhibitors of synaptic excitation mediated by

ionotropic glutamate receptors and (4) synaptic neurotransmitter release modulators (Figure 10).¹²⁵

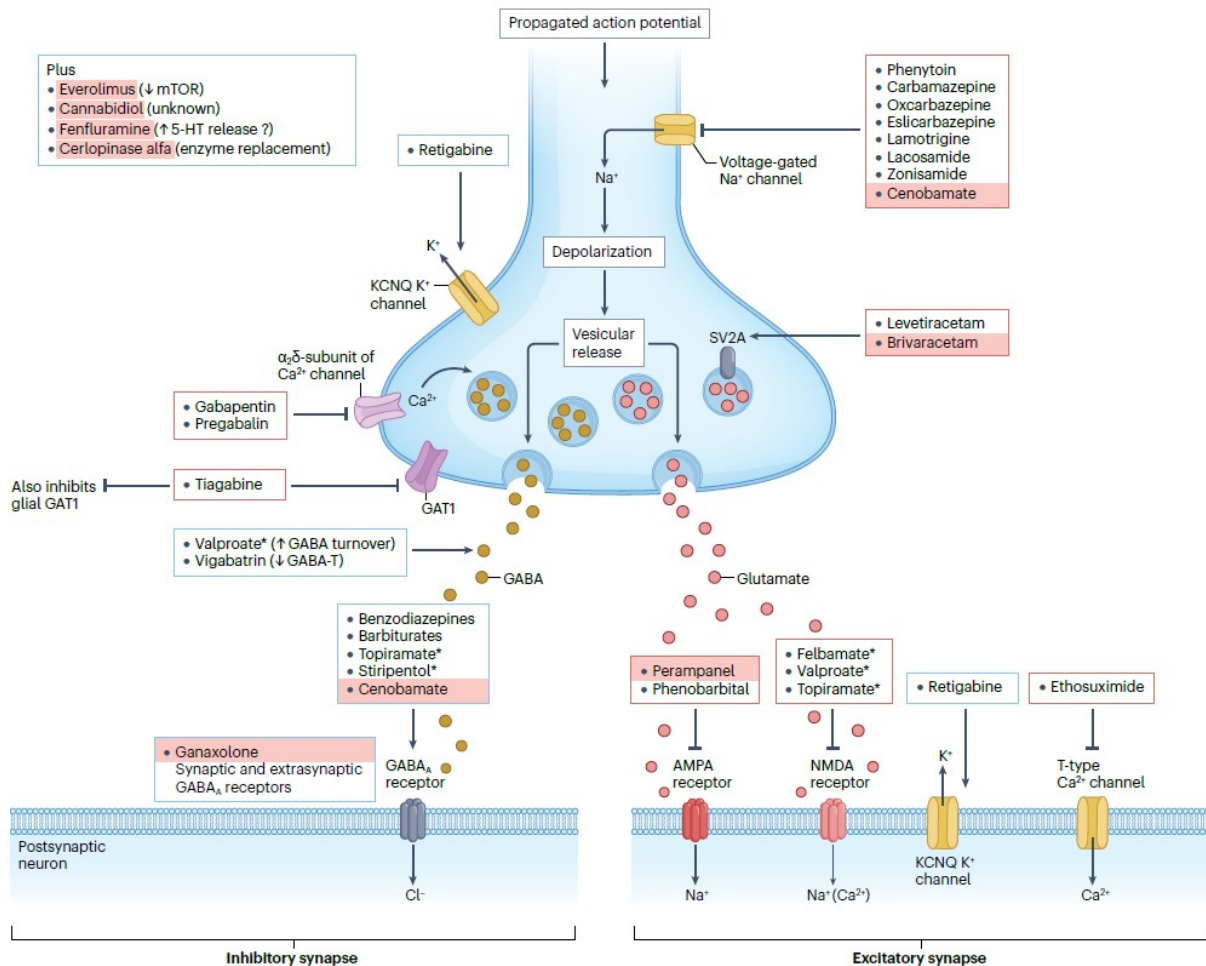


Figure 10. Molecular targets of clinically approved anti-seizure medications (ASMs). ASMs that act more broadly via several targets are marked by asterisks (for example, valproate, topiramate and felbamate). ASMs that have been approved in the past 10 years are highlighted in red. Klein P, Kaminski RM, Koepp M, Loscher W. New epilepsy therapies in development Nat Rev Drug Discov. 2024 Sep;23:682-708.

In the last decades, epilepsy therapy screening programs including that at the University of Utah have implemented a systemic scheme using various seizure and epilepsy models to identify novel ASMs.¹²⁶ The process begins with higher-throughput models, such as the MES test for GTCS and the 6-Hz seizure test for focal seizure.^{126, 127} Anticonvulsant compounds that

demonstrate promising efficacy in these initial models are then subjected to drug-resistant models to further characterize their effects and spectrum of activity against recurrent seizures.^{126, 127} In my experimental design, I employed the MES test to screen anti-seizure effects in GTCS and mouse models of Dravet syndrome and Lennox-Gastaut syndrome to explore anti-seizure effects against phenotypic-specific seizures. These disease-specific models enable a more comprehensive evaluation of potential ASMs across different seizure types and resistance profiles.

In addition to drug development, discovering pharmacological targets is equally important. Recent attention has focused on cannabinoid 1 (CB1) receptors and cannabinoid 2 (CB2) receptors due to their role in epilepsy. Initially, a CB1 and CB2 receptor agonist (WIN 55,212-2) produced anticonvulsant effects, reducing both epileptiform discharges in hippocampal culture and status epilepticus in pilocarpine-treated rats.¹²⁸ Subsequently, a hallmark study genetically knocked out CB1 or CB2 receptors in mice, leading to convulsive behavior with EEG recordings capturing epileptic discharges concurrent with the seizures, suggesting the onset of epilepsy.¹²⁹ Taken together, these findings implicate CB1 and CB2 receptors in neuronal modulation and brain excitability.

To date, the field of novel agent development targeting CB1 and CB2 receptors against seizures is expanding. This target-focused drug discovery approach has been adopted to develop agents with selection to prevent off-target events. Such a strategy is particularly beneficial for patients with Dravet syndrome and Lennox-Gastaut syndrome, who often require a polytherapy regime. In the following session, the role of the endocannabinoid system, phytocannabinoids and synthetic cannabinoids in epilepsy will be presented.

1.10.2 Cannabis

The term “cannabis” is derived from the genus *Cannabis* (Family Cannabaceae).¹³⁰ According to the current taxonomy classification, cannabis includes up to three species: *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis*.^{131, 132} Generally, *Cannabis sativa* was predominantly cultivated in Europe (Figure 11),¹³³ *Cannabis indica* in Asia, and *Cannabis ruderalis* in southern Russia.^{130, 132, 134}



Figure 11. Flowering structures of male (left) and female (right) of Cannabis sativa. Bonini SA, Premoli M, Tambaro S, Kumar A, Maccarinelli G, Memo M, et al. Cannabis sativa: A comprehensive ethnopharmacological review of a medicinal plant with a long history J Ethnopharmacol. 2018 Dec 5;227:300-315.

The medical use of cannabis dates back to 2800 B.C., when Chinese Pharmacopeia documented its use for fatigue, improving appetite and alleviating gastrointestinal problems.¹³⁵ In ancient Egypt, the Ebers Papyrus (an Egyptian medical document written in 1500 B.C.) also noted people in their time who used cannabis for pain relief and mood enhancement.¹³⁶ This historical

evidence suggests an early medicinal use of cannabis worldwide. In 2016 the World Health Organization (WHO) reported that cannabis is the most common illicit recreational drug.¹³⁷ 181.8 million people used cannabis for medical and recreational purposes globally with Western Central Africa, North America and Oceania displaying the highest rates of use.¹³⁷

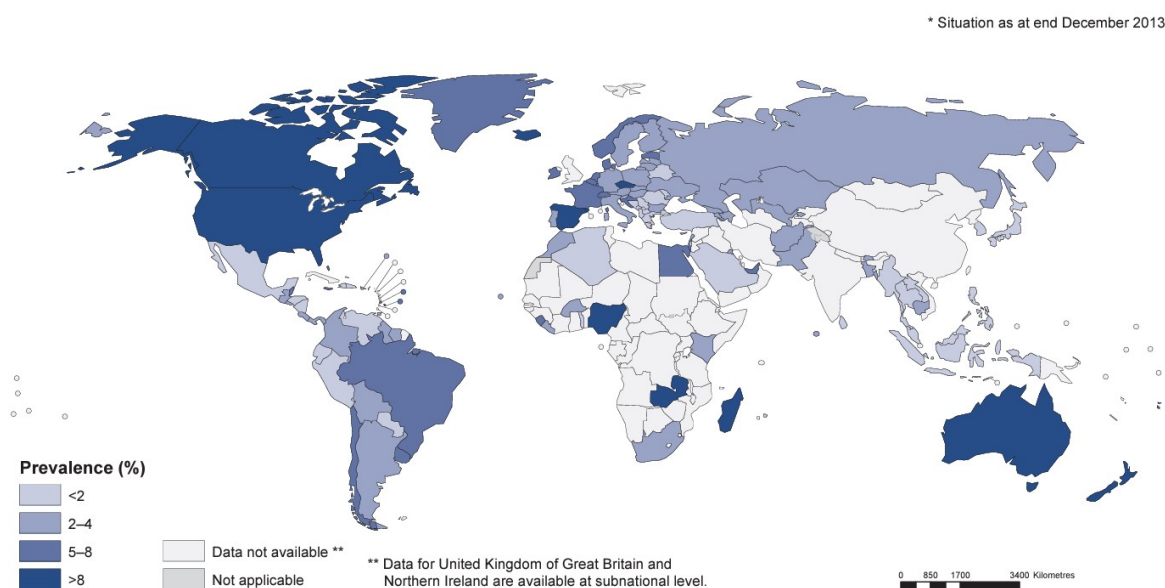


Figure 12. Global prevalence of cannabis used for population ages 15 -65 years in 2015. World Health Organization. The health and social effects of nonmedical cannabis use. Geneva 2016 11 November 2016.

Cannabis has a complex chemical composition of 750 potentially bioactive molecules, including cannabinoids, terpenoids, flavonoids, and lignanamides.^{138, 139} The two major active compounds, CBD and Δ^9 -tetrahydrocannabinol (Δ^9 -THC) have been the most studied for their potential medicinal properties. Δ^9 -THC¹⁴⁰⁻¹⁴² is the main intoxicating constituent of cannabis which is abundant in recreational cannabis, whereas CBD is not intoxicating and is abundant in hemp cannabis varieties.^{143, 144} CBD and Δ^9 -THC are plant cannabinoids (phytocannabinoids), and are structural isomers, although they have a markedly different

pharmacology. In addition to CBD and Δ^9 -THC, there are approximately 200 identified as phytocannabinoids, many of which are referred to as “minor” phytocannabinoids, despite some of them being more abundant in raw cannabis than the “major” phytocannabinoids.¹⁴⁵

Biosynthesis of phytocannabinoids occurs within pistillate glands which combine both enzymatic and non-enzymatic synthesis pathways. Phytocannabinoids are a structurally diverse class of terpenophenolic metabolites produced in cannabis, with their structural motifs determined by the chain length of starting fatty acid in independent biosynthetic pathways (Figure 13).¹⁴⁶ The biosynthesis begins with cannabigerolic acid (CBGA), a central precursor, formed through prenylation of geranyl diphosphate (GPP), and olivetolic acid. CBGA is then converted into pentyl side chain phytocannabinoids (Figure 13).^{147, 148}

Through the action three enzymes – cannabidiolic acid (CBDA) synthase¹⁴⁹, cannabichromenic acid (CBCA) synthase¹⁵⁰ and Δ^9 -tetrahydrocannabinolic acid (Δ^9 -THCA) synthase¹⁵¹ – CBGA undergoes oxidative cyclization to form CBDA, CBCA, and Δ^9 -THCA, respectively. These acid forms of phytocannabinoids are relatively unstable and then converted into CBD, cannabichromene (CBC) and Δ^9 -THC, through heat-driven decarboxylation, for example under sunlight exposure. Under varying conditions, such as oxidation, light exposure or isomerization, CBD, CBC and Δ^9 -THC are further transformed into minor phytocannabinoids. For example, Δ^9 -THC undergoes oxidation to form cannabinol (CBN)¹⁴⁸, or CBC can be transformed to cannabicyclol (CBL).

In contrast to the phytocannabinoids that contain a pentyl alkyl side chain, those with propyl alkyl side chain are derived from cannabigerovarinic acid (CBGVA), another central precursor, synthesized from geranyl diphosphate and varinolic acid (Figure 13).¹⁵² Like CBGA, CBGVA can be converted into cannabidivarinic acid (CBDVA), cannabichrovarinic acid (CBCVA), and Δ^9 -tetrahydrocannabivarinic acid (Δ^9 -THCVA) through the actions of CBDA synthase, CBCA

synthase and Δ^9 -THCA synthase, respectively.¹⁵³ Subsequent decarboxylation yields other neutral forms of the cannabinoids such as cannabidivarin (CBDV) from CBDVA, or Δ^9 -tetrahydrocannabivarin (Δ^9 -THCV) from Δ^9 -THCVA.

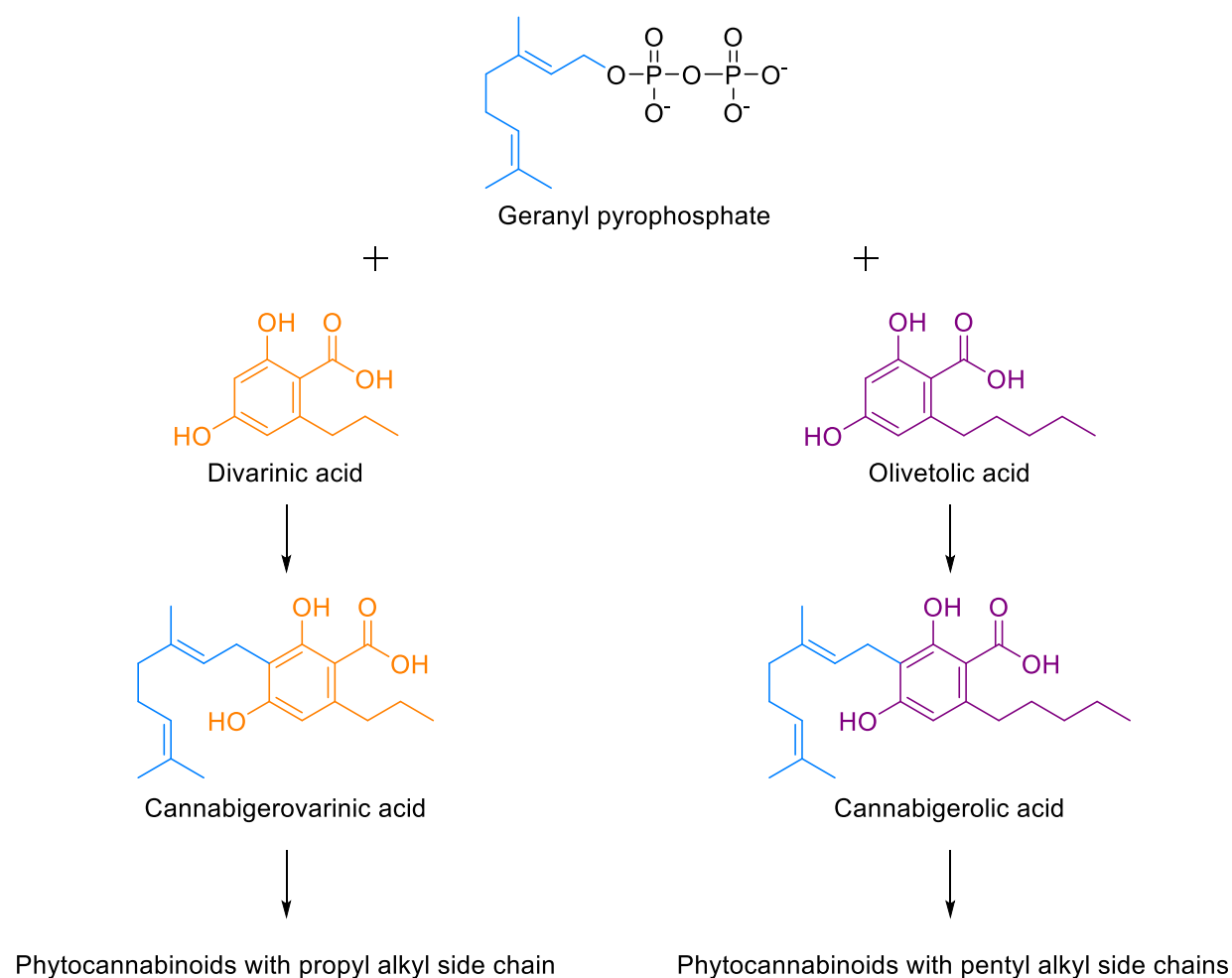
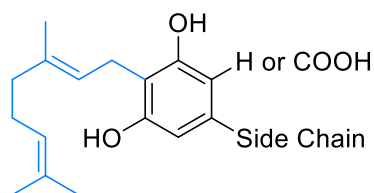


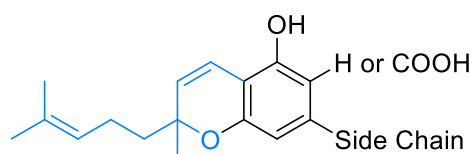
Figure 13. Two independent pathways for the biosynthesis of phytocannabinoids with propyl alkyl side chains and pentyl alkyl side chains

The glandular trichomes are the primary sites secrete phytocannabinoids in resin form, these compounds are classified into 11 different categories from the olivetol series as leads: CBG, CBC, CBD, CBL, cannabielsoin (CBE), cannabinol (CBN), cannabinodiol (CBND),

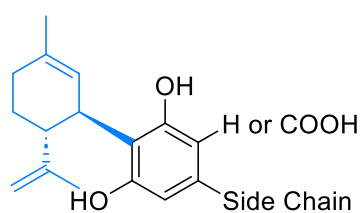
cannabitriol (CBT), Δ^8 -trans-tetrahydrocannabinol (Δ^8 -THC), Δ^9 -THC and the miscellaneous cannabinoids (Figure 14).^{145, 154}



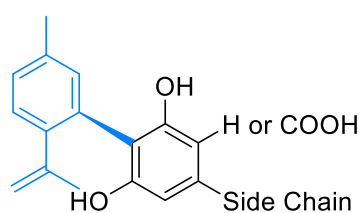
CBG-type



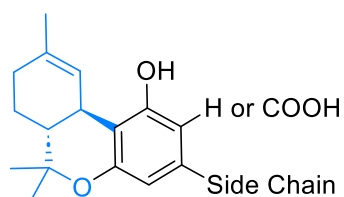
CBC-type



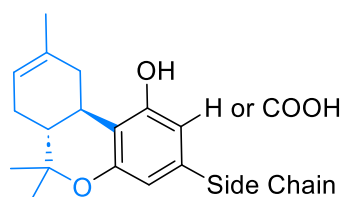
CBD-type



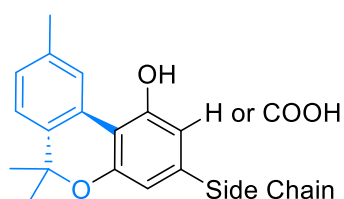
CBND-type



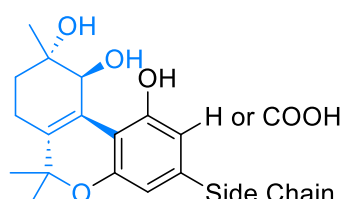
Δ^9 -THC-type



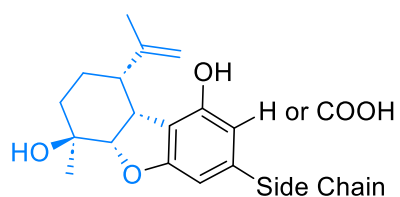
Δ^8 -THC-type



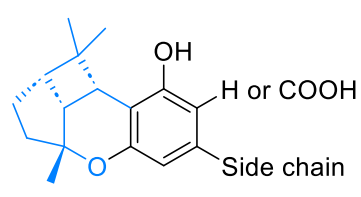
CBN-type



CBT-type



CBE-type



CBL-type

Figure 14. Structural overview of different leads in phytocannabinoids

1.10.3 Endocannabinoid system

Following the identification of psychoactive compounds in cannabis, an analogue of Δ^9 -THC (CP 55,940) was found to bind to an endogenous receptor to induce intoxication, resulting in the discovery of an endocannabinoid system.¹⁵⁵ The system consists of cannabinoid receptors,^{156, 157} endogenous cannabinoids¹⁵⁸⁻¹⁶⁰ and the enzymes responsible for endocannabinoid synthesis and degradation.¹⁶¹⁻¹⁶³ The endocannabinoid system is widely distributed throughout the CNS and plays a key role in modulating physiological responses, synaptic plasticity and the regulation of neurotransmitter release.¹⁶⁴⁻¹⁶⁶

The two cannabinoid receptors, CB1 and CB2 receptors, are G-protein coupled receptors that primarily couple with Gi or Go protein classes. The CB1 receptor is predominantly expressed in the CNS and peripheral nerve terminals,¹⁵⁶ while the CB2 receptor is mainly found in immune cells and the hematopoietic system.¹⁵⁷ Emerging evidence also suggests that CB2 receptors exist in astrocytes and microglia.^{167, 168} These breakthroughs led to the discovery of endogenous CB1 and CB2 receptors ligands. They are endogenous lipids that act on CB1 or CB2 receptors that activate these receptors, collectively known as endocannabinoids.¹⁵⁸⁻¹⁶⁰

Anandamide (AEA) was the first endocannabinoid identified that acts as an agonist of CB1 receptors, while 2-arachidonoylglycerol (2-AG) was subsequently characterized as having agonist activity on both CB1 and CB2 receptors.^{158, 159} The release of endogenous ligands occurs in an on-demand fashion triggered by physiological stimuli, and in the brain at the level of the synapse in a retrograde manner. Briefly, non-cannabinoid post-synaptic receptors (e.g. glutamatergic receptors) initiate the synthesis of 2-AG, which is liberated from the lipid post-synaptic membrane in response to excessive neurotransmitters.^{169, 170} 2-AG then traverses the

synaptic cleft to activate CB1 receptors that are located on the presynaptic terminal. Activation of CB1 receptors reduces the presynaptic calcium influx and then suppresses the release of neurotransmitters.^{164, 169, 170} The retrograde signaling pathway serves as a self-protective mechanism against overstimulation by neurotransmitters. These highlight the therapeutic potential of endocannabinoids and cannabinoid receptors in regulating neuronal circuit excitability.

The endocannabinoid system may be involved in epilepsy development and seizure severity based on several studies in humans. A study evaluated the disruption of endocannabinoid in patients with refractory temporal lobe epilepsy and found that the concentrations of AEA was decreased in their cerebrospinal fluid compared to drug naïve control subjects who were diagnosed for suspected CNS disorders (multiple sclerosis, subarachnoid hemorrhage and loss of consciousness) other than epilepsy.¹⁷¹ Another study showed that hippocampal brain concentrations of 2-AG and cannabinoid CB1 receptors expression was reduced in biopsies from refractory temporal lobe epilepsy compared to autopsies from control subjects who died from unrelated to CNS disorder.¹⁷² In contrast, during acute seizures, AEA concentrations were found to be elevated following kainic acid (KA)-induced seizures in animal models.¹⁷³ These findings highlight the dynamic regulation of endocannabinoid signaling and suggest targeting the endocannabinoid system might aid in seizure control and epilepsy management.

Several lines of evidence also support the role of the endocannabinoid system in regulating brain hyperexcitability and seizures using preclinical models. For example, AEA treatment decreased the amplitude of excitatory postsynaptic potentials in hippocampal slices in rats.¹⁷⁴ AEA treatment also protected mice against MES-induced seizures in mice that was mediated by CB1 receptors because the effects were blocked by the cannabinoid receptor antagonist SR141716.¹⁷⁵ Moreover, systemic intracerebroventricular administration of AEA shortened seizure duration and recovery time in a electrical kindling rat model of epilepsy.¹⁷⁶ Similarly,

exogenous treatment with 2-AG prevented the prolonged high-frequency epileptiform bursts and this effect was reversed by a CB1 receptor antagonist (AM-251) in a hippocampal neuronal culture model.¹⁷⁷ Further, pilocarpine-induced seizures in rats were associated with increased hippocampal concentrations of 2-AG, suggesting for this endogenous cannabinoids is released on demand to curb neuronal hyperexcitability.¹⁷⁸ 2-AG treatment also suppressed seizure generation in a kainic acid-induced status epilepticus mouse model.¹⁷⁹

Beyond the exogenous delivery of endocannabinoids, enzyme inhibitors that prevent the degradation of endocannabinoids are potential anti-seizure agents. FAAH inhibitors, which block the degradation of AEA, and MAGL inhibitors, which inhibit the metabolism of 2-AG, enhance the endogenous concentrations of AEA and 2-AG receptively.¹⁸⁰ Administration of URB597, a FAAH inhibitor, delayed the onset of maximal dentate discharges following perforant path stimulation in a rat model of limbic seizures.¹⁸¹ Treatment with AM374, another FAAH inhibitor, reduced seizure severity in a kainic acid-induced seizure model in rats.¹⁸² Similarly, the MAGL inhibitors, such as JZL184 and ABX-1431, exhibited anticonvulsant seizures.^{183, 184} Acute treatment with JZL184 delayed the development of GTCS in an electrical kindling model.¹⁸³ In a *Scn1l*^{+/-} mouse model of Dravet syndrome, acute administration of ABX-1431 increased the temperature threshold for hyperthermia-induced GTCS in *Scn1l*^{+/-} mice.¹⁸⁴ Further, continuous oral administration of ABX-1431 reduced the proportion of GTCS in *Scn1l*^{+/-} mice but was ineffective in improving survival.¹⁸⁴ Overall, previous evidence demonstrates that endocannabinoids display anti-seizure effects mediated by CB1 receptors, highlighting the potential of CB1 receptors as a promising pharmacological target for the development of ASMs.

1.10.4 Cannabinoid 1 (CB1) receptors as an anti-seizure target

Given the evidence above of promising anti-seizure effects of AEA and 2-AG, modulating CB1 receptors provides a potential anti-seizure drug target. One study sought to validate CB1 receptors as an anti-seizure drug target by examining whether CB1 knockout mice (*Cnr1*^{-/-}) display greater susceptibility to kainic acid-induced seizures.¹⁷³ Indeed, *Cnr1*^{-/-} mice displayed more severe seizure-related behaviors compared to wild-type control mice, highlighting that CB1 receptors protected against kainic acid-induced seizures.¹⁷³ The relationship between CB1 activation and anti-seizure effects was then further validated pharmacologically with the CB1 receptor antagonist SR141716A, where treated mice had more severe kainic acid-induced seizures than vehicle controls.¹⁷³ The same study also investigated whether GABAergic interneurons in the cortex contributed to the anti-seizure effects. To explore this, the authors used a CB1^{f/f;CaMKIIαCre} mouse model in which CB1 receptors were selectively knocked-out in cortical principle glutamatergic neurons.¹⁷³ The CB1^{f/f;CaMKIIαCre} mice displayed increased susceptibility to kainic-acid induced seizures. Further, pretreatment with the AEA reuptake inhibitor UCM707 significantly protected wild-type control mice from kainic acid-induced seizures but was ineffective in CB1^{f/f;CaMKIIαCre} mice.¹⁷³ These findings demonstrated that the anti-seizure effect of CB1 receptors were mediated by localization of CB1 receptors on cortical glutamatergic neurons.¹⁷³

Δ⁹-THC is one of the well-known CB1 receptor orthosteric agonists. The anticonvulsant property of Δ⁹-THC was first reported in the early 1970s when multiple studies showed that Δ⁹-THC prevented electroshock-induced seizures in mice.¹⁸⁵⁻¹⁸⁷ In one rat study, treatment with 10 mg/kg Δ⁹-THC protected against the dorsal hippocampal discharges from afferent stimulation (delivered via electrodes in the fornix).¹⁸⁸ Further, exogenous Δ⁹-THC abolished spontaneous seizures in seizure sensitive strains of gerbils.¹⁸⁹ More recently in our lab, low-dose of Δ⁹-THC (0.1 mg/kg) displayed an anticonvulsant effect as it increased the body

temperature threshold at which mice has a hypothermia-induced GTCS, in the *Scn1a*^{+/-} mouse model of Dravet syndrome.¹⁹⁰ Although, subchronic Δ^9 -THC dosing did not reduce spontaneous seizures in the *Scn1a*^{+/-} mice.¹⁹⁰ By contrast, some studies have found that Δ^9 -THC exerted proconvulsant effects in different rodent models. Whalley et al., reported that a high-dose of Δ^9 -THC (40.5 mg/kg) produced motor seizures in healthy rats, which were associated with epileptiform discharges in EEG recordings.¹⁹¹ These discrepant results might be attributed to the high-dose of Δ^9 -THC activating not just CB1 receptors on the glutamatergic afferents but also those on GABAergic interneurons, which might promote disinhibition of glutamatergic projection neurons.^{192, 193} The conflicting effects of the exogenous CB1 receptor agonist Δ^9 -THC on seizures highlights the problematic nature of CB1 as an anti-seizure drug target, at least when targeted by orthosteric agonists. In addition, the on-target liability of the psychotropic-related side-effects of CB1 receptor activation further limits the therapeutic potential of CB1 agonists in drug discovery. In addition to Δ^9 -THC, other CB1 receptor orthosteric agonists, such as WIN 55,212-2, CP 55,940, and HU-210, have been tested in preclinical models of epilepsy. These compounds have demonstrated varying degrees of anti-seizure efficacy. Further review on the effects of these compounds will be presented in the following section.

1.10.5 Cannabinoid 2 (CB2) receptors as an anti-seizure target

The potential of CB2 receptors as a new anti-seizure drug target has arisen more recently, with basic science highlighting that CB2 receptors are expressed in the brain on glial cells (microglia and astrocytes) and some controversial evidence for expression on neurons.^{157, 194-196} One major advantage of targeting CB2 receptors is that their activation is devoid of psychotropic effects unlike the CB1 receptor. Indeed, several studies have reported the inducible expression of CB2

receptors the brain under various neurological conditions, including epilepsy.¹⁹⁷⁻²⁰⁰ For instance, status epilepticus induced upregulation of CB2 receptors on hippocampal neurons in rats.²⁰¹ The therapeutic advantage of inducibility is because CB2 receptors exhibit low expression in the brain under normal conditions, which reduce the likelihood of off-target effects and the interaction with normal physiological activities. Upregulation of CB2 receptors in response to seizures makes CB2 receptors a disease-associated target and allows drugs targeting CB2 receptors to act specifically in disease-affected cells, for example in glial cells.^{201, 202} This distinct feature of CB2 receptors offers new opportunities for safely targeting the endocannabinoid system in epilepsy.

Few studies have examined the role of CB2 receptors in seizure susceptibility. Interestingly, mice with double-knockout of CB1 and CB2 receptors exhibited more severe spontaneous seizures than mice with a single knockout of either CB1 or CB2 receptors, suggesting loss of CB2 receptors may further enhance seizure susceptibility.¹²⁹ Another study confirmed that heterozygous (*Cnr2*^{+/-}) and homozygous (*Cnr2*^{-/-}) CB2 receptor knockout mice had increased susceptibility to GTCS and myoclonic jerks following the chemoconvulsant pentylenetetrazol (PTZ).²⁰³ However, deleting CB2 receptors did not affect seizure outcomes in *Scn1a*^{RH/+} mutant mice, inferring that CB2 receptors do not play a direct role in Dravet syndrome.²⁰³ Further, local expression of a *Scn1a* missense mutation (A1783V) in neurons alters the endocannabinoid system by upregulating CB2 receptors in the hippocampus.²⁰⁴ These findings suggest that the upregulation of CB2 receptors in *Scn1a*^{A1783V} mice may be driven by seizure-related neuroinflammation. It would be of interest for future studies to address whether this simply reflects an adaptive response to the disease state or a maladaptive contributor to its pathogenesis.^{200, 204}

Targeting CB2 receptors has shown promising anti-seizure effects in preclinical seizure models. For example, CB2 receptor-specific agonists AM1241 and β -caryophyllene have

displayed anticonvulsant properties.^{205, 206} AM1241 administration reduced seizure frequency and was accompanied by less neuronal loss in CA3 region of the hippocampus after pilocarpine-induced status epilepticus in mice.²⁰⁵

Similarly, β -caryophyllene treatment alleviated seizure severity in a kainic acid-induced seizure mouse model and reduced seizure frequency in a mouse model of PTZ-induced seizures.²⁰⁶ This same study proposed that the neuropharmacological activities of β -caryophyllene involve GABAergic receptors to decrease neuronal excitability.²⁰⁶ Subsequent research suggested that anti-seizure effects of CB2 receptor activation are mediated through downstream signaling pathways, as CB2 receptors inhibit cAMP production, leading to reduced calcium influx and neuronal excitability.^{207, 208}

1.10.6 Anticonvulsive effects of phytocannabinoids in clinical studies

A major development in cannabis research is the approval of a highly purified CBD formulation (Epidiolex®) by drug regulatory bodies around the world including the US, Australia and the European Union for the adjunctive treatment for seizures associated with the intractable epilepsies. In several clinical trials, CBD significantly reduced seizures in patients with associated with Dravet syndrome, Lennox-Gastaut syndrome and tuberous sclerosis complex, with a favorable tolerability.²⁰⁹⁻²¹¹ In a double-blind, placebo-controlled trial, 43% of patients with Dravet syndrome experienced a 50% seizure reduction with CBD treatment compared with 27% of the placebo group.²⁰⁹ In a phase three clinical trial recruited patients with Lennox-Gastaut syndrome, drop-seizure frequency was reduced by 42% with 20 mg/kg CBD, 37% with 10 mg/kg, and 17% in the placebo group.²¹⁰ Similarly, a placebo-controlled randomized clinical trial assessed adjunctive CBD treatment at 25 mg/kg/day and 50 mg/kg/day for tuberous

sclerosis complex-associated seizures.²¹² Both doses reduced seizure frequency by ~ 48% compared to 27% for the placebo group after 16 weeks of treatment.²¹²

Long-term open-label trials have confirmed the sustained efficacy of adjunctive CBD in Dravet syndrome, Lennox-Gastaut syndrome and seizures associated with tuberous sclerosis complex.²¹³⁻²¹⁵ Patients with Dravet syndrome achieved 45% – 74% overall seizure reduction over 156 weeks,²¹³ while those with Lennox-Gastaut syndrome experienced a 48%–71% reduction in drop seizures.²¹⁴ For patients with tuberous sclerosis complex associated seizures, CBD maintained 54% to 68% efficacy in seizure reduction over 48 weeks.²¹⁵

Most adverse effects occurred during the first 14 days of dose titration among patients, including diarrhea, vomiting, fatigue and decrease appetite. Withdrawal rates due to serious adverse effects were 9% for Dravet syndrome, 11.7% for Lennox-Gastaut syndrome, and 6% for tuberous sclerosis complex.²¹³⁻²¹⁵ Overall, the introduction of CBD into mainstream medicine for the treatment of intractable epilepsies highlights that CBD might be the first in a new cannabinoid class of anti-seizure agents. Many of the cannabinoids have structural similarities, so preclinical research has been examining whether any molecules in cannabis beyond CBD and THC have efficacy.

1.10.7 Anti-seizure effects of phytocannabinoids in preclinical models of epilepsy

Preclinical models for intractable epilepsies provide effective screening platforms to facilitate the development of treatment, particular for assessing the anti-seizure effects of phytocannabinoids. In Dravet syndrome, mouse models exhibit an inducible seizure by hyperthermia, spontaneous seizure and reduced lifespan. These features closely capture the clinical phenotypes of Dravet syndrome, making the *Scn1a* mouse model favorable for the

pharmacological assessment of potential drug candidates.⁸³⁻⁸⁶ Notably, *Scn1l*^{+/-} mouse model has been pharmacologically validated with different anticonvulsant agents, such as valproic acid, clobazam, and stiripentol, and yield a compatible response with those observed in clinical populations of Dravet syndrome.²¹⁶

CBD is effective in reducing seizures in the *Scn1l*^{+/-} mouse model of Dravet syndrome.²¹⁷ Mice treated with high doses of CBD at 100 or 200 mg/kg (intraperitoneal, i.p.) showed reductions in seizure duration and severity compared to vehicle-treated control.^{190, 217} Additionally, repeated CBD administration reduced spontaneous seizure rate by 70% compared to the vehicle control.²¹⁷ The pharmacological mechanism of CBD involves the restoration of inhibitory signaling in GABAergic interneurons, perhaps via GPR55.^{217, 218} Further, CBD also reduced excitability of excitatory neurons in the dentate gyrus of the hippocampus.²¹⁷

Our previous study showed that a combination of CBD (3500 mg/kg) with clobazam (80 mg/kg) produced a greater anticonvulsant effect than clobazam alone.²¹⁹ This finding has prompted further investigation into whether the pharmacodynamic and pharmacokinetic interactions between CBD and ASMs could improve treatment efficacy. Building on this, a recent study used *Scn1l*^{A1783V/WT} mice to evaluate the treatment efficacy of a second-line adjunctive treatment combining clobazam and sodium valproate.²²⁰ The study demonstrated comparable findings that adjunctive CBD (150 mg/kg) combined with clobazam and valproate increased temperature threshold for hyperthermia-induced seizure onset in comparison to treatment with clobazam and valproate alone in *Scn1l*^{A1783V/WT} mice.²²⁰

Looking beyond CBD, recent evidence showed that CBD analogs have anti-seizure effects in preclinical models. Preclinical studies indicated that CBDA has anti-seizure properties. Anderson et al., demonstrated that CBDA (10 and 30 mg/kg i.p.) increased the temperature threshold for hyperthermia-induced GTCS in *Scn1l*^{RX/+} mice model of Dravet syndrome.²²¹

This is at 3-10 fold lower doses than the effective CBD dose in this model. Further, in the MES model, a novel magnesium-based formulation of CBDA prevented the onset of full hindlimb extension in rats (median effective dose $ED_{50} = 115.4\text{mg/kg}$).²²² CBDV is a propyl analogue of CBD that has been found to display anticonvulsant effects in *in vivo* and *in vitro* seizure and epilepsy models. In rat hippocampal slices, CBDV (10 μM and 100 μM) attenuated epileptiform local field potentials which were triggered by 4-aminopyridine (4-AP) and Mg^{2+} free artificial cerebrospinal fluid (ACSF),²²³ as the absence of magnesium removes the inhibition on NMDA receptors, leading to a hyperexcitable state that reflects epileptic discharges.^{224,225} CBDV (100 and 200 mg/kg i.p.) treatment protected mice from MES-induced tonic hindlimb extension and audiogenic-induced audiogenic seizures.²²³ Further, various doses of CBDV (95, 100 and 200 mg/kg i.p.) decreased seizure severity and delayed the onset of first seizure in PTZ-induced acute seizures and pilocarpine-induced seizures in rats.^{223, 226} Pre-treatment of CBDV (400 mg/kg per os) suppressed PTZ-induced generalized seizures in rats.²²³ Additionally, subcutaneous (s.c.) injection of CBDV at 100 mg/kg in rats attenuated their seizure severity in PTZ-induced generalized seizures. The same study demonstrated that a 200 mg/kg dose of CBDV reduced the proportion of P20 rats exhibiting hindlimb extension in MES test but was ineffective in P10 rats, showing an age-specific profile.²²⁷ To further explore the anti-seizure effects, pre-treatment of CBDV was administered prior to pilocarpine induction, which increased the latency of status epilepticus in rats, preventing epileptogenesis.²²⁸ Since these preclinical studies, CBDV has since undergone clinical investigation in patients with focal seizures and Rett syndrome.²²⁹ A phase 2 trial randomized 162 adults with focal seizure to 400 to 800 mg CBDV twice daily or placebo treatment over an 8-week treatment period.²²⁹ Whilst CBDV was ineffective against seizures in the trial, it was well-tolerated with only mild adverse effects, for example, diarrhea and nausea.²²⁹ Our group

examined the acid form of CBDV, CBDVA, and found it displayed anticonvulsant effects at 100 mg/kg against hyperthermia-induced GTCS in *Scn1a*^{+/-} mice.²³⁰

Structural variants of Δ^9 -THC have also been examined for anti-seizure activity in preclinical models. Biphasic effects of Δ^9 -THCA were reported in preclinical studies with different seizure models.^{231, 232} In our previous study a combination of Δ^9 -THC (3%) and Δ^9 -THCA (97%) at 100 and 200 mg/kg (i.p.) exhibited anticonvulsant effects against 6 Hz induced-psychomotor seizures in mice.²³¹ However, this mixture worsened the severity of spontaneous seizures and reduced the lifespan of *Scn1a*^{+/-} mice.²³¹ Further, Δ^9 -THCA alone at 200 mg/kg (i.p.) displayed proconvulsant effects by lowering the CC₅₀ in the MES threshold test for GTCS.²³¹ Similarly, in the iron-induced cortical epilepsy model in rats, Δ^9 -THCA at 20 mg/kg (i.p.) induced polyspike bursts in EEG signals and motor toxicity, mimicking epileptic discharges and excitatory behaviors.²³²

Δ^9 -THCV is the propyl analogue of Δ^9 -THC that has been assessed for anti-seizure activity in preclinical models. Δ^9 -THCV suppressed the frequency and amplitude of epileptiform discharge bursts which were induced by Mg²⁺-free artificial cerebrospinal fluid in rat piriform cortex slices.²³³ Furthermore, in a PTZ-induced seizure model, a single pre-treatment dose of Δ^9 -THCV reduced mortality and increased the proportion of seizure-free mice.²³³ A potential mechanism suggests that Δ^9 -THCV acts as a ligand for the CB1 receptors, functioning as a potential CB1 receptor antagonist.²³⁴ In contrast, Δ^9 -THCV at 3 mg/kg (i.p.) exerted proconvulsant effects that decreased the body temperature threshold for hyperthermia-induced GTCS in *Scn1a*^{+/-} mice.²³⁰

CBC has been considered a bioactive compound that contributes to the medicinal effects of cannabis as it is the third most abundant compound following Δ^9 -THC and CBD. In a MES mouse model, CBC (25 mg/kg i.p.) shortened the duration of hindlimb extension,²³⁵ while in

another study, CBC (up to 500 mg/kg i.p.) did not alter the latency to hindlimb extension in mice.²³⁶ This highlights at best CBC only has a subtle effect on MES-induced GTCS. In the *Scn1a*^{+/-} mouse model of Dravet syndrome, CBC was evaluated in three doses (10, 30, 100mg/kg i.p.) against hyperthermia-induced seizures. Treatment with 100 mg/kg CBC increased the temperature threshold for induction of GTCS induction, consistent with an anticonvulsant effect.²³⁷ Several natural and synthetic analogs of CBC were also studied: fluorinated CBC (5-fluoro-CBC), CBCA, cannabichromevarin (CBCV), and CBCVA.²³⁷ Similar to CBC, these analogs showed anticonvulsant effects against hyperthermia-induced seizures in *Scn1a*^{+/-} mice.²³⁷ Acute administration of 5-fluoro-CBC at a dose of 100 mg/kg (i.p.) increased the body temperature for the onset of GTCS from hyperthermia seizure induction.²³⁷ The acidic CBC analogs which are found in cannabis, CBCA and CBCVA (both at 100 mg/kg i.p.) also showed the anticonvulsant effects against febrile seizures in *Scn1a*^{+/-} mice.²³⁷ However, no effect was noted for CBCV on hyperthermia-induced seizures, despite CBCV's excellent brain uptake.²³⁷ Future studies are needed to assess whether CBC and its analogs affect spontaneous seizures and mortality in the *Scn1a*^{+/-} mouse model.

In addition, our group examined CBG and its analogs, including CBGA, CBGVA and cannabigerivarin (CBGV) in *Scn1a*^{+/-} mice.²³⁰ CBGA at 30 and 100 mg/kg i.p. and CBGVA at 100 mg/kg i.p. elevated the body temperature threshold for hyperthermia-induced GTCS compared to the vehicle-treated control. By comparison CBGA worked at a lower dose than CBD, which is effective at 100 mg/kg against in this model.^{190, 230} However, neither CBG nor CBGV affected the body temperature threshold. The anticonvulsant effects of CBGA at 30 mg/kg i.p. were further supported by the MES-induced GTCS mouse model, where CBGA-treated mice required a higher critical current (CC₅₀) to reach hindlimb extension.²³⁰ Inhibition of T-type calcium channels may contribute to the anti-seizure effects of CBGA, as it had submicromolar inhibitory effects on Cav3.1, with also micromolar potency at inhibiting Cav3.2

and Cav3.3 channels.²³⁸ The inhibitory effects of CBGA were associated with hyperpolarizing shift in activation, leading to larger Ca^{2+} currents in response to modest membrane depolarization, following by a steady state of inactivation on these channels.²³⁸ Interestingly, CBGA (10 and 30 mg/kg i.p.) had proconvulsant effects by lowering the CC_{50} seizure threshold in a 6-Hz seizure model in mice.²³⁰ Chronic oral administration of CBGA at 2500 mg/kg in chow also exacerbated spontaneous seizure frequency in the *Scn1a*^{+/-} mice, demonstrating proconvulsant effects. Overall, CBGA provided a promising scaffold to explore for future anti-seizure drug discovery and development although several potential liabilities and limitations would need to be considered. Olivetolic acid is a precursor of CBGA and shares a structural similarity to CBGA but does not have the geranylated sidechain. In the *Scn1a*^{+/-} mouse model of Dravet syndrome, olivetolic acid (100 mg/kg i.p.) displayed anticonvulsant effects by increasing the body temperature threshold for hyperthermia-induced GTCS.²³⁹

1.10.8 Phytocannabinoid derived from non-cannabis plants

There exist potentially bioactive compounds from natural products that have chemical similarity with the cannabinoids that are found in non-cannabis plants.¹⁴⁵ Plants have been reported to produce compounds with similar chemical characteristics and are phytocannabinoid-like such as *Rhododendron ferrugineum*, *Helichrysum umbraculigerum* and *Radula perrottetii* (liverworts).¹⁴⁵ Notably, *Helichrysum umbraculigerum* exhibits biological effects comparable to cannabis and has long been used for treating gastrointestinal disorders and pain symptoms in South Africa.²⁴⁰

The biosynthesis of CBGA has been identified not only in cannabis but also in *Helichrysum umbraculigerum*.^{241, 242} Similar to cannabis, CBGA is produced and accumulated in the glandular trichomes of their flowers.²⁴² Interestingly, a novel type of CBGA-type analogue,

amorfrutin was recently identified and isolated (Figure 15).²⁴² Amorfrutin is synthesized from similar precursors as CBGA in *Helichrysum umbraculigerum*.²⁴³ There are five subtypes of amorfrutins (Figure 15). Few studies have examined the pharmacological properties of the amorfrutins, however one study noted that they activate peroxisome proliferator-activated receptors (PPAR γ), an emerging target for anti-seizure therapies.²⁴⁴ The amorfrutins were also found to suppress nitric oxide synthase and inflammatory responses via NF- κ B pathway.²⁴⁵ Of particular interest, amorfrutin 2 is a pentyl-type cannabinoid that shares a major structural overlap with CBGA. Given that CBGA displayed anti-seizure effects in our previous study, we were motivated to explore the effect of amorfrutin 2 in Dravet syndrome and Lennox-Gastaut syndrome, which has not been characterized before.

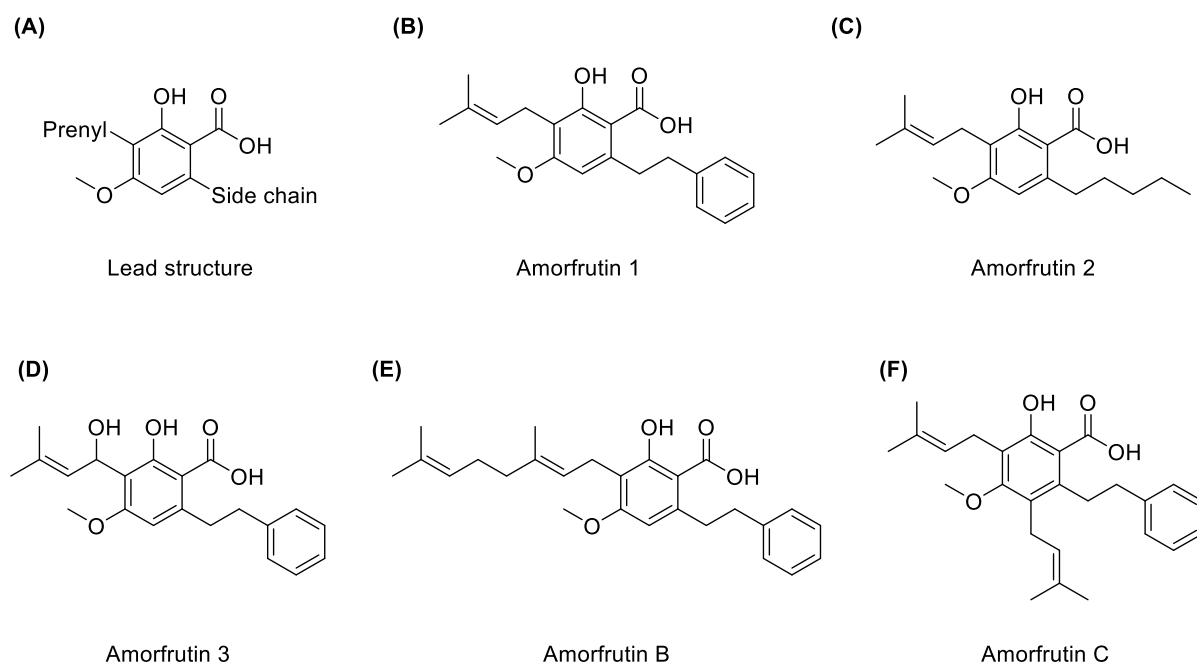


Figure 15. Structures of the lead and five derivatives of amorfrutins.

There are other non-cannabis-derived bioactive compounds that interact with cannabinoid receptors. They are abundant in numerous other plants, for example, guineensine from *Piper*

guineense (West African pepper),²⁴⁶ curcumin from *Curcuma longa* (turmeric)²⁴⁷ and magnolol and honokiol from *Magnolia officinalis*.²⁴⁸ In fact, magnolia bark has been traditionally used as medicinal material in Chinese medicine for sleep disorders, fever and anxiety. Notably, magnolol and honokiol affect cannabinoid receptors, which also share structural similarity with CP 55,940, a synthetic agonist of CB1 and CB2 receptors. In particular, magnolol acts as a partial agonist on CB2 receptors, while honokiol showed low-affinity modulation on CB1 receptors.²⁴⁸

Previous research reported preclinical anti-seizure effects of magnolol and honokiol. *Magnolia officinalis* extract suppressed behavior seizures alongside a reduction of seizure susceptibility in EEG findings following kainic acid-induced status epilepticus in immature rats.²⁴⁹ Further study characterized that magnolol and honokiol protected mice against psychomotor seizures in a 6-Hz induced focal seizure model.²⁵⁰ This same study also confirmed the anti-seizure effects of magnolol and honokiol, which reduced seizure activity in PTZ-induced seizures in larval zebrafish.²⁵⁰ Another study also observed compatible results in which magnolol delayed seizure onset and decreased the spike discharges in EEG recording in mice with seizures induced by PTZ. Although robust evidence indicates the anti-seizure effects of magnolol and honokiol, their efficacy in treating intractable epilepsies has not yet been investigated.

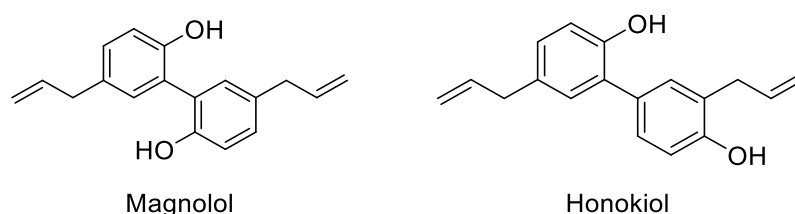


Figure 16. The chemical structures of magnolol and honokiol

Magnolol and honokiol are a pair of isomers that are classified as neolignans based on the link between their two C6–C3 units (Figure 16). The SAR of magnolol and honokiol is influenced by the arrangement of the lead biaryl structure and the position of hydroxyl groups. To further investigate the SAR of these compounds and their analogs, Fuchs et al. synthesized novel derivatives and demonstrated that modifications to the side chains of these derivatives retained comparable efficacy at CB1 receptors.²⁵¹ They concluded that the potency and efficacy at CB1 and CB2 receptors were altered by methylation of one of the phenolic hydroxyl groups, which tetrahydromagnolol enhanced 230-fold binding affinity at CB1 receptors and 17-fold at CB2 receptors.²⁵¹ These compounds provide accessible chemical scaffolds to develop analogs targeting intractable epilepsies. However, detailed characterization of the SAR in magnolol and honokiol in preclinical intractable epilepsy model remains unexplored. An aim of the current thesis was then to assess these compounds in mouse models of Dravet syndrome and Lennox-Gastaut syndrome.

1.10.9 Synthetic cannabinoids

Researchers have long focused on cannabinoid agonists as potential treatments for epilepsy. The phytocannabinoid scaffold of including Δ^9 -THC has provided the inspiration for medicinal chemists in developing a now long-list of synthetic cannabinoids targeting cannabinoid receptors. Common synthetic cannabinoids, such as CP 55,940, WIN 55,212-2, and HU-210, have been extensively studied in rodent seizure and epilepsy models.²⁵²⁻²⁵⁴ CP 55,940 is a high-efficacy synthetic agonist of CB1 receptors lacking the central pyran ring of Δ^9 -THC. Bilaterally acute intracollicular microinfusion of CP 55,940 (53 nmol/ μ l at a rate of 0.2 μ l/min for 1 min) suppressed audiogenic seizures in genetically epilepsy-prone rats.²⁵² Additionally, CP 55,940 (2.5 mg/kg i.p.) demonstrated anti-seizure effects against cocaine-induced seizures

in mice.²⁵³ However, repeated administration of daily CP 55,940 (0.4 mg/kg i.p.) failed to reduce seizure susceptibility to PTZ-induced generalized seizures in rats, possibly due to insufficient dosing compared to other studies.²⁵⁴

The synthetic CB1 receptor agonist WIN 55,212-2 is an aminoalkylindole structured cannabinoid that has demonstrated promising anti-seizure effects in preclinical models. Pretreatment with WIN 55,212 (2 mg/kg i.p.) prevented pilocarpine-induced status epilepticus in rats.²⁵⁵ Post-treatment of WIN 55,212-2 (2 mg/kg per day s.c.) for 15 days reduced the number of spontaneous seizures and the seizure duration following pilocarpine-induced status epilepticus in rats.²⁵⁶ In addition, adjunctive treatment of WIN 55,212-2 (5 mg/kg i.p.) with traditional ASMs had synergistic anti-seizure effects by increasing the proportion of protected mice against both 6 Hz-induced psychomotor seizures²⁵⁷ and MES-induced GTCS.²⁵⁸ Repeated dosing with WIN 55,212-2 also reduced atypical absence seizures in the *Gabrb3*^{+D120N} mouse model of Lennox-Gastaut syndrome.¹²³

However, the adverse on-target psychoactive effects of CB1 receptor agonists have limited their clinical applications. The psychoactive effects can increase abuse liability and psychosis in vulnerable individuals. Repeated exposure to CB1 receptor agonists is also associated with tolerance, a reduced drug effect that comes with repeated exposure, which is suboptimal.^{259, 260} These limitations restrict the translational potential of CB1 receptor agonists in clinical settings. To overcome the limitations, alternative strategies have focused on targeting the allosteric site of CB1 receptors to improve therapeutic effects whilst minimizing adverse effects associated with orthosteric CB1 receptor agonism. The orthosteric site is the site of action of classical CB1 receptor agonists but also the endocannabinoids such as AEA and 2-AG. Allosteric modulation describes ligands that either enhance the actions of endocannabinoids (positive allosteric modulators; PAMs), inhibit their action (negative allosteric modulators; NAMs), or modulate the receptor at the allosteric site without orthosteric agonism (allosteric agonists).^{259,}

²⁶⁰ Additionally, ago-PAMs refer to a subtype of PAMs that exhibit both allosteric agonist activity and positive allosteric modulation.²⁶¹ PAM ligands bind to a distinct topographical site to orthosteric agonists on the receptor and modify the conformation of the receptor to indirectly potentiate CB1 receptor activation and may enhance the binding affinity and potency of orthosteric ligands.^{262, 263}

Allosteric modulation at CB1 receptors have been argued to have several advantages over orthosteric activation, including a better safety profile and a lack of orthosteric agonist activity. In mice and rats, the tetrad test is used to gauge orthosteric or cannabimimetic activity, as CB1 receptor agonists like Δ^9 -THC and CP 55,940: 1) reduce body temperature (hypothermia), 2) reduce locomotor activity, 3) promote catalepsy, and 4) produce analgesia (often using the tail-flick test).^{260, 264} Studies have confirmed a lack of cannabimimetic tetrad effects of allosteric modulators - for example, Org27569, the first characterized NAM at CB1 receptors,²⁶⁰ did not affect body temperature or tail flick latency in rats compared to treatment with the orthosteric agonist CP 55,940.²⁶⁴ Similarly, GAT229, a PAM at CB1 receptors, was shown to have no significant effects on locomotor activity, body temperature, or catalepsy after acute dosing.²⁶⁵ These findings suggest that allosteric modulations minimize on-target adverse effects of CB1 receptors. Another advantage of allosteric modulators involves their pharmacological mechanism that acts exclusively in the presence of orthosteric ligands, therefore this *probe dependence* phenomenon enables allosteric modulators to provide more selective modulation of CB1 receptor signaling, especially in a disease context in which endocannabinoids are augmented to combat the pathophysiology of the disease. It is now well-established that endocannabinoid signaling is dynamically regulated characterized by upregulation during acute seizures and downregulation in chronic or drug-resistant epilepsy. Thus, therapeutic strategies that enhance endocannabinoid concentrations may help reduce neuronal

hyperexcitability and support seizure control. CB1 receptor PAMs then provide a means to tune-up this activity in a brain region- and temporally-specific manner.

Beyond epilepsy, CB1 receptor ago-PAMs have demonstrated promising efficacy in treating CNS disorders. In a mouse model of paclitaxel-induced pain, GAT211 (5 mg/kg i.p.), a CB1 ago-PAM, demonstrated synergistic antinociceptive effects with morphine to suppress hypersensitivities to mechanical and cold stimulation.²⁶⁶ Notably, co-treatment of daily GAT211 (5 mg/kg i.p.) prevented the development of tolerance from morphine over 20 days.²⁶⁶ In addition, GAT229, a pure CB1 receptor PAM derived from GAT211, improved cell viability in a Huntington's disease cell model.²⁶⁷ The same study also reported that an exogenous 21-day course of GAT229 at 10 mg/kg i.p. per day improved motor coordination and delayed symptom onset in the R6/2 HD mouse model of Huntington's disease compared to vehicle-treated mice.²⁶⁷

Few studies have examined the effects of CB1 receptor PAMs in seizure of epilepsy models. GAT211 and GAT229 reduced absence seizures in genetic absence epilepsy rats from the Strasbourg (GAERS) model.²⁶⁵ To further characterize the anti-seizure effects in Dravet syndrome, our group used *Scn1a*^{+/-} mice to examine the anti-seizure effects of GAT229.¹⁸⁴ In this study we first showed ABX-1431, a MAGL inhibitor that prevents 2-AG degradation, displayed anti-seizure effects against GTCS. GAT229 was then tested and shown to elevate the temperature threshold for thermally-induced GTCS consistent with an anti-seizure effect. This research highlights that CB1 receptor PAMs have anti-seizure efficacy in a drug-resistant model of epilepsy, thus it would be of interest to probe additional CB1 receptor PAMs related to this scaffold. GAT229 has suboptimal physiochemical properties and pharmacokinetics, and additional molecules have been developed by our collaborator Ganesh Thakur (Northeastern University, USA). The current thesis will test some of these molecules in mouse models of drug-resistant epilepsy.

To improve the physiochemical properties of GAT211, structural modifications were used to synthesize novel analogs. GAT211, the first ago-PAM derived from 2-phenylindole structural class, provides an active scaffold to design anti-seizure agents.²⁵⁹ Fluorine or nitrogen substitution by systematic approaches hopping around GAT211 identified functional analogs through electrophysiological assessments and radioligand binding experiments (Figure 17).²⁶⁸ Among many ago-PAM analogs, GAT591 was identified with an improvement on the inhibition of excitatory postsynaptic currents and metabolic stability.²⁶⁸ Subsequent studies confirmed that GAT591 decreased the number of absence seizures in a genetic rat model of absence epilepsy.²⁶⁹ Another suggested ago-PAM GAT1102 was synthesized with an addition of butanoic acid group which has structural novelty predicted to improve solubility and absorption.

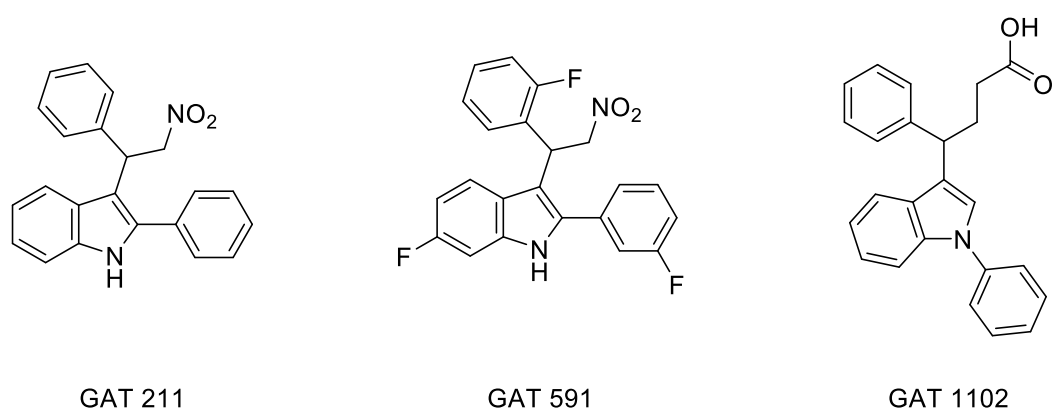


Figure 17. The chemical structures of GAT211, GAT591 and GAT1102

1.10.10 Pro-resolving mediators an extension therapeutic molecule from the endocannabinoid system

The examination of the neurochemical changes that occur in response to seizures may unlock novel therapies for epilepsy. There is now emerging interest in the role of lipid signaling molecules in epilepsy, which is exemplified by the endocannabinoids, which are augmented in brain tissue in response to acute seizures as a measure to limit neuronal hyperexcitability, a presynaptic CB1 receptor activation limits the release of excitatory neurotransmitters such as glutamate.^{270, 271} However, downstream of these endocannabinoids, other lipid signaling molecules are enhanced such as the pro-inflammatory prostaglandins.^{272, 273}

Increasing evidence supports that ongoing neuroinflammation facilitates seizure generation and epilepsy progression.^{274, 275} Numerous clinical studies have observed activated inflammatory pathways in epilepsy, including upregulated proinflammatory molecules, reactive astrocytosis and activated microglia.²⁷⁶⁻²⁷⁸ Elevated concentrations of tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β), and IL-6, pro-inflammatory cytokines, have been identified in patients with temporal lobe epilepsy compared to non-epilepsy controls.²⁷⁶⁻²⁷⁸ Furthermore, hippocampal tissue from patients with drug-resistant epilepsy shows the presence of reactive astrocytes and activated microglia.^{279, 280} Febrile seizures are common in Dravet syndrome and are closely associated with neuroinflammation. Our previous study showed that *Scn1a*^{+/-} mice with a high seizure burden had increased an hippocampal concentration of prostaglandins (PGF2 α , PGE2, PGD2, and 6-K-PGF1A) compared to *Scn1a*^{+/-} mice with a low seizure burden or WT mice.²⁷² Additionally, we demonstrated that the higher seizure burden increased the Cox-2 transcript expression compared to WT mice, suggesting the prostanoid inflammatory mediators might be augmented in the hippocampus of *Scn1a*^{+/-} mouse of Dravet syndrome.^{272, 281}

An emerging therapeutic option is to reduce inflammation during epileptogenesis to disrupt the inflammatory reinforcement that exacerbates seizure activity. Anti-inflammatory treatments with corticosteroids like adrenocorticotrophic hormone being an example for managing epileptic spasms²⁸² and intravenous immunoglobulin therapy for autoimmune-induced seizures.²⁸³ However, these therapies often induce moderate to severe adverse effects such as hyperglycemia and heart failure.^{284, 285} Long-term treatment with corticosteroids activity is inadvisable, as the long-term suppression of the immune system can increase the risk of infection and cancer despite their anti-inflammatory properties.^{286, 287} Consequently, alternative therapeutic options are needed to offer a safer way to limit neuroinflammation. A new and exciting endogenous system has been discovered recently that resolves inflammation, particularly in the context of inflammatory disorders such as asthma, allergy, cystic fibrosis, and acute respiratory distress syndrome.²⁸⁸ This system involves a collection of lipid-signaling molecules called the specialized pro-resolving mediators that clear inflammatory cells and promote tissue repair, a process known as resolution (Figure 18).²⁸⁹ The role of these mediators in CNS disorders is emerging given the recognition of the importance of neuroinflammation to pathophysiology,²⁹⁰ however there is scant research on these mediators in the field of epilepsy research.²⁹¹⁻²⁹³

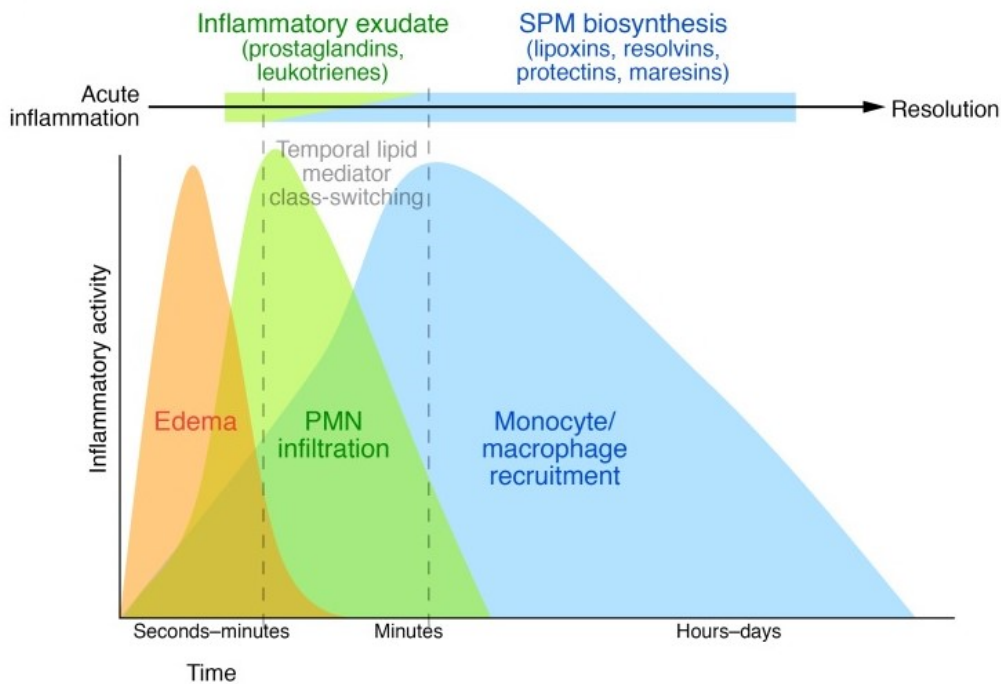


Figure 18. The mechanism of innate response from inflammation to resolution. The diagram represents lipid mediator class-switching initiates active resolution from polymorphonuclear leukocytes (PMNs) produced pro-inflammatory cytokines to specialized pro-resolving mediators (SPMs). Serhan CN, Levy BD. Resolvins in inflammation: emergence of the pro-resolving superfamily of mediators J Clin Invest. 2018 Jul 2;128:2657-2669.

Specialized pro-resolving mediators are endogenous lipid mediators synthesized from the downstream metabolism of polyunsaturated fatty acids, including arachidonic acid, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Interestingly, arachidonic acid is derived from the endocannabinoids 2-AG and AEA, and further synthesizes specialized pro-resolving mediators, while DHA has been shown to upregulate CB1 receptor expression in the hippocampal neurons.²⁹⁴ Additionally, DHA and EPA derivatives act as CB1 and CB2 receptor agonists.²⁹⁵ These findings suggest a potential link between specialized pro-resolving mediators and the endocannabinoid system. Specialized pro-resolving mediators are classified into classes with distinctive structures that are still expanding, including leukotrienes, lipoxins, resolvins (D-series and E-series resolvins), protectins, and maresins (Figure 19).²⁹⁶ The specialized pro-resolving mediators interact with corresponding receptors, for example lipoxin

at A4 receptors²⁹⁷ and resolvin at resolving D2 receptors²⁹⁸ as well as orphan GPCRs, including GPR18²⁹⁸ and GPR32 receptor²⁹⁹. Although the detailed pharmacological actions have not been established, one study found that resolvin D2 activated human GPR18 expressed in CHO cells to increase cAMP signaling to trigger apoptosis in human macrophages.²⁹⁸ These systems are therefore druggable via the development of small molecules that could target these cognate receptors.

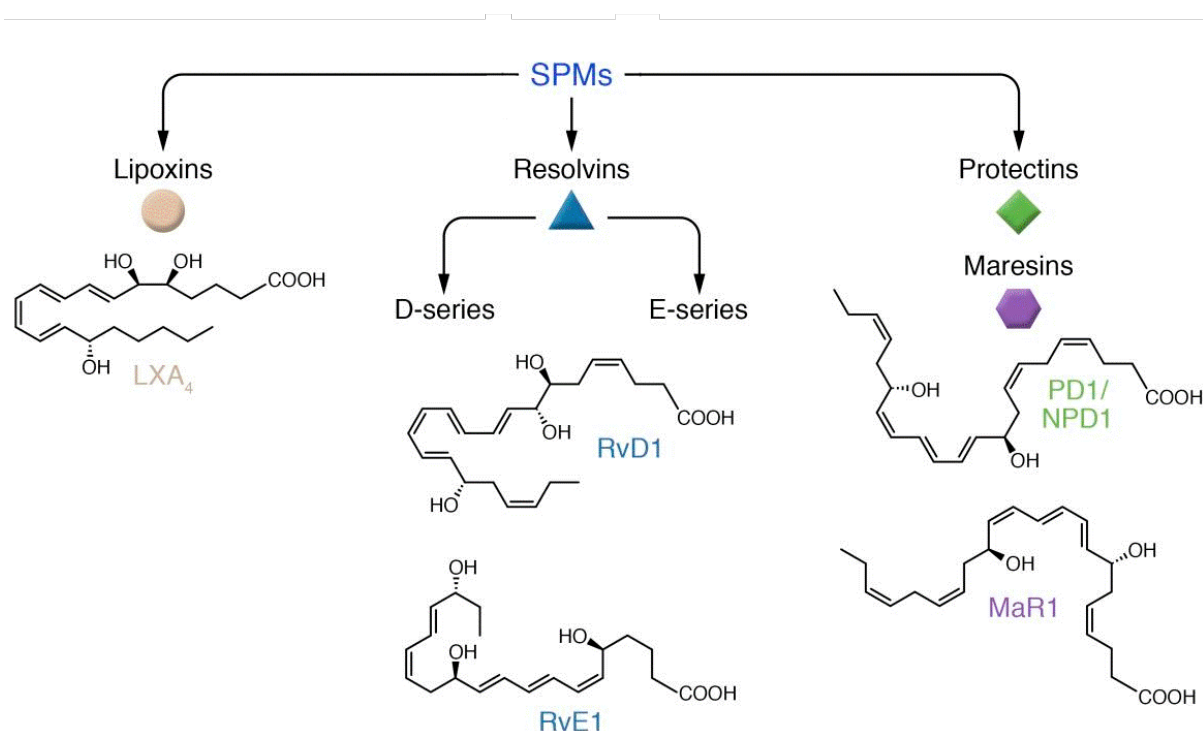


Figure 19. Structural representatives in lipoxin, D-series of resolvins, protectins and Maresins. Serhan CN, Levy BD. Resolvins in inflammation: emergence of the pro-resolving superfamily of mediators J Clin Invest. 2018 Jul 2;128:2657-2669.

In the field of epilepsy research, only a handful of studies have investigated the effects of these mediators with some demonstrating anti-seizure effects. The first study found that kindling-induced seizures triggered the endogenous production of protectin D1 (also known as neuroprotectin D1) in the hippocampus.²⁹² In the same study, administering protectin D1 prior

to seizure induction inhibited after discharges and reduced the number of spikes in the dorsal hippocampus of mice and rats.²⁹² Another study reported similar anti-seizure effects for protectin D1.²⁹³ Histological examination revealed that protectin D1 treatment prevented the beading in hippocampal dendritic spines, which further suggested a neuroprotective role in preventing epileptogenesis.²⁹³ This study was the first to propose that a specialized pro-resolving mediator could reduce neuroinflammation and enhance protective signaling during epileptogenesis.²⁹³

A recent study demonstrated dysregulation of specialized pro-solving mediators during epileptogenesis.²⁹¹ Frigerio et al. confirmed that pilocarpine-induced status epilepticus led to neuroinflammation, accompanied by inconsistent expression of specialized pro-solving mediators.²⁹¹ Among these mediators, docosapentaenoic acid (DPA)-derived protectin D1 was markedly upregulated in the hippocampus. When DPA-derived protectin D1 was administered to mice, it resolved inflammatory response by reducing pro-inflammatory mediators, such as TNF- α and IL-1 β .²⁹¹ In addition, DPA-derived protectin D1 reduced spontaneous seizures and improved cognitive function.²⁹¹ In another study DPA-derived protectin D1 increased inhibitory synaptic input in presynaptic neurons of the hippocampus,³⁰⁰ the pharmacological activities of other mediators remain largely unexplored. It is unknown whether specialized pro-solving mediators are involved in drug-resistant epilepsy syndromes such as Dravet syndrome. Given our research that a high seizure burden increased of the pro-inflammatory prostaglandins in the *Scn1a*^{+/-} mouse model of Dravet syndrome²⁷², it is possible the specialized pro-solving mediators might also be augmented in the brain as a means to resolve this neuroinflammation.

1.11 Aims

The global objective of this thesis was to advance the development of novel therapies and better understand the neurochemistry of the intractable epilepsies such as Dravet syndrome and Lennox-Gastaut syndrome using preclinical mouse models. The thesis has three major aims:

- (1) to assess the anti-seizure effects of novel chemical scaffolds related to the phytocannabinoids that are found in non-cannabis plants;
- (2) to examine the anti-seizure effects of novel allosteric modulators of the cannabinoid CB1 receptors; and
- (3) to identify whether a high-seizure burden impacts novel lipid signaling molecules called the specialized pro-resolving mediators in the hippocampus.

1.11.1 Aim 1: Assessing the anti-seizure effects of novel chemical scaffolds related to the phytocannabinoids that are found in non-cannabis plants (Chapter 2)

SAR helps in the development of novel compounds in anti-seizure drug discovery. Amorfrutin 2, honokiol, and magnolol share structural similarities with phytocannabinoids such as CBGA and CBD that have promising anticonvulsant effects in preclinical models. In Chapter 2, we aimed to examine the anti-seizure effects of amorfrutin 2, magnolol and honokiol in genetic mouse models of Dravet syndrome and Lennox-Gastaut syndrome. We provided the pharmacokinetic profiles of these compounds in plasma and brain. To evaluate their anti-seizure potentials, we implemented a systematic screening approach to refine compound candidates and determine effective doses. We first assessed their anti-seizure effects against hyperthermia-induced GTCS in the *Scn1a*^{+/-} mouse model of Dravet syndrome and the MES test. The most promising compound magnolol was then tested for its effects on atypical absence

seizures in the *Gabrb3*^{+/*D120N*} mouse model of Lennox-Gastaut syndrome. In addition, to further explore their potential anti-seizure modes of action, amorfrutin 2, magnolol and honkiol were tested for their effects on the anti-seizure drug targets T-type calcium channels (Cav3.1, Cav3.2 and Cav3.3), which has not been previously characterized, and cannabinoid receptors (CB1 and CB2).

1.11.2 Aim 2: Examining the anti-seizure effects of novel allosteric modulators of the cannabinoid CB1 receptor (Chapter 3)

Cannabinoid CB1 receptors have emerged as a novel anti-seizure drug target. Orthosteric CB1 receptor agonists like Δ^9 -THC have anti-seizure effects, however these compounds have significant on-target liabilities such as intoxication, sedation, tolerance and dependence which limits their translational potential. Allosteric modulators have been developed that target CB1 receptors with much more limited side-effect profiles and the potential to magnify the actions of our endogenous defense against seizures through the release of endocannabinoids in a cell, brain-region and temporally-specific manner. In Chapter 3, we aimed to determine whether two novel allosteric modulators of the cannabinoid CB1 receptor GAT591 and GAT1102, which have more drug-like physiochemical properties have anti-seizure effects in mouse models of intractable epilepsy. To this end, we used a similar approach to Chapter 2 where the anti-seizure activities of GAT591 and GAT1102 were examined in the *Scn1a*^{+/-} and *Gabrb3*^{+/*D120N*} mouse models of Dravet syndrome and Lennox-Gastaut syndrome respectively. Further, we also determined the brain/plasma pharmacokinetics of these compounds and their effects on body temperature, one measure of cannabimimetic activity.

1.11.3 Aim 3: Identifying whether a high-seizure burden impacts novel lipid signaling molecules called the specialized pro-resolving mediators in the brain (chapter 4)

Better understanding the neurochemistry of intractable epilepsy may unlock novel therapeutic approaches. Neuroinflammation is believed to be the pathophysiology of seizure recurrence, but also long-term cognitive and behavioral disturbances in intractable epilepsy. Our team recently showed that a high seizure burden increased the concentrations of pro-inflammatory prostaglandins mediators in the hippocampus of *Scn1a*^{+/-} mice. This raised the possibility that a high seizure burden might also trigger the accumulation of specialized pro-resolving mediators that facilitate the resolution of neuroinflammation and brain repair. Chapter 4 therefore aimed to examine whether a high seizure burden increased hippocampal concentrations of various specialized pro-resolving mediators in the *Scn1a*^{+/-} mouse model of Dravet syndrome, as these mediators had not been previously examined in a genetic drug-resistant epilepsy model.

Chapter 2

**Cannabinoid-like compounds found in non-cannabis plants
exhibit anti-seizure activity in genetic mouse models of drug-
resistant epilepsy**

RESEARCH ARTICLE

Cannabinoid-like compounds found in non-cannabis plants exhibit antiseizure activity in genetic mouse models of drug-resistant epilepsy

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Abstract

Objective: The cannabinoid cannabidiol has established antiseizure effects in drug-resistant epilepsies such as Dravet syndrome and Lennox–Gastaut syndrome. Amorfrutin 2, honokiol, and magnolol are structurally similar to cannabinoids (cannabis-like drugs) but derive from non-cannabis plants. We aimed to study the antiseizure potential of these compounds in various mouse seizure models. In addition, we aimed to characterize their molecular pharmacology at cannabinoid CB₁ and CB₂ receptors and at T-type calcium channels, which are known targets of the cannabinoids.

Methods: Brain and plasma pharmacokinetic profiles were determined. Antiseizure activity was assessed against hyperthermia-induced seizures in a *Scn1a*^{+/-} mouse model of Dravet syndrome. We then elaborated on the most promising compounds in the maximal electroshock (MES) test in mice and the *Gabrb3*^{+/-D120N} mouse model of Lennox–Gastaut syndrome. Fluorescence-based assays were used to examine modulatory activity at cannabinoid CB₁ and CB₂ receptors and T-type calcium channel subtypes Ca_v3.1, Ca_v3.2, and Ca_v3.3 overexpressed in mammalian cells. Automated patch-clamp electrophysiology was then used to confirm inhibitory activity on Ca_v3.1, Ca_v3.2, and Ca_v3.3 channels.

Results: Magnolol and honokiol had high brain-to-plasma ratios (3.55 and 7.56, respectively), unlike amorfrutin 2 (0.06). Amorfrutin 2 and magnolol but not honokiol significantly increased the body temperature threshold at which *Scn1a*^{+/-} mice had a generalized tonic–clonic seizure. Both amorfrutin 2 and magnolol significantly decreased the proportion of mice exhibiting hindlimb extension in the MES test. Furthermore, magnolol reduced the number and duration of atypical absence seizures in *Gabrb3*^{+/-D120N} mice. The three compounds inhibited all T-type calcium channel subtypes but were without specific activity at cannabinoid receptors.

Significance: We show for the first time that amorfrutin 2 and magnolol display novel antiseizure activity in mouse drug-resistant epilepsy models. Our results justify future drug discovery campaigns around these structural scaffolds that aim to develop novel antiseizure drugs for intractable epilepsies.

KEYWORDS

antiseizure drugs, cannabinoids, Dravet syndrome, Lennox–Gastaut syndrome, natural products, T-type calcium channels

1 | INTRODUCTION

Bioactive compounds derived from natural products have a long history of providing new leads in the development of novel pharmaceutical agents. In the treatment of epilepsy a recent example is cannabidiol (CBD), a molecule found in the cannabis plant that is now a registered medicine in the treatment of intractable epilepsies including Dravet syndrome and Lennox–Gastaut syndrome.^{1,2} Cannabis contains >100 cannabis-like compounds (cannabinoids), and recent research has uncovered additional cannabinoids with antiseizure effects in animal models.^{3–6} One of these plant cannabinoids is cannabigerolic acid (CBGA; Figure 1), which is a major biosynthetic precursor molecule in the cannabis plant.⁴ This compound displayed anticonvulsant effects against hyperthermia-induced seizures in an *Scn1a*^{+/-} mouse model of Dravet syndrome.⁴ Interestingly, CBGA resembles compounds called amorfrutins which are found in plants unrelated to cannabis and can be classified as phytocannabinoids based on structural similarity (Figure 1).⁷ Amorfrutin 2 is found in the legumes *Glycyrrhiza foetida* and *Amorpha fruticosa*, one of which is a licorice species.⁸ Amorfrutin 2 has not been previously examined for antiseizure activity.

Other molecules from natural products that resemble cannabinoids are the neolignans magnolol and honokiol (Figure 1). These compounds, found in the *Magnolia officinalis* plant, are structurally similar to the synthetic cannabinoid receptor agonist, CP55,940, an analogue of the main psychoactive constituent of cannabis, Δ^9 -tetrahydrocannabinol.⁹ Moreover, these compounds resemble biaryl cannabinoids found in the liverwort plant.^{9–11} We aimed to determine whether these structurally related molecules also display antiseizure properties. Prior research showed that a *Magnolia officinalis* extract reduced susceptibility to seizures following kainic acid-induced status epilepticus in immature rats.¹² This was accompanied by a reduction in neuronal damage and reactive gliosis.¹² Furthermore, both magnolol and honokiol reduced pentylenetetrazole (PTZ)-induced seizures in larval zebrafish, and magnolol protected against 6 Hz-induced psychomotor seizures in mice.^{13,14} Another study confirmed that magnolol

Key points

- Amorfrutin 2, honokiol, and magnolol are structurally similar to cannabinoids and have antiseizure potential.
- Amorfrutin 2 and magnolol displayed antiseizure activity against hyperthermia-induced seizures in the *Scn1a*^{+/-} mouse model of Dravet syndrome.
- Amorfrutin 2 and magnolol had antiseizure effects in the MES test.
- Magnolol reduced the number and duration of atypical absence seizures in the *Gabrb3*^{+/-D120N} knockin mouse model of Lennox–Gastaut syndrome.
- All compounds inhibited T-type calcium channels (Ca_v3.1, Ca_v3.2, or Ca_v3.3) but did not affect cannabinoid receptors (CB₁ and CB₂).

increased the latency to seizure onset and decreased the number of spikes on electroencephalographic (EEG) recordings in response to PTZ in mice.¹⁵ Magnolol also blunted epileptiform activity triggered by 4-aminopyridine in induced pluripotent stem cell-derived neurons.¹⁴ However, no study has yet examined the effects of magnolol and honokiol in mouse models of intractable epilepsy.¹⁵

Approximately 80% of Dravet syndrome patients carry pathological de novo mutations in the *SCN1A* gene.^{16,17} Heterozygous deletion of *Scn1a* in mice (*Scn1a*^{+/-} mice) produces a behavioral phenotype reflective of the human condition, such as hyperthermia-induced generalized tonic-clonic seizures (GTCSs) and afebrile spontaneous GTCSs that are manifest early in life.^{18,19} Furthermore, *Scn1a*^{+/-} mice have a reduced lifespan and exhibit behavioral abnormalities.^{18,19} Lennox–Gastaut syndrome is another childhood epilepsy characterized by drug-resistant seizures, most commonly tonic, atonic, and atypical absence seizures.²⁰ There are multiple proposed causes of Lennox–Gastaut syndrome; one genetic cause identified is a mutation in the *GABRB3* gene that encodes the $\beta 3$ subunit of the γ -aminobutyric acid

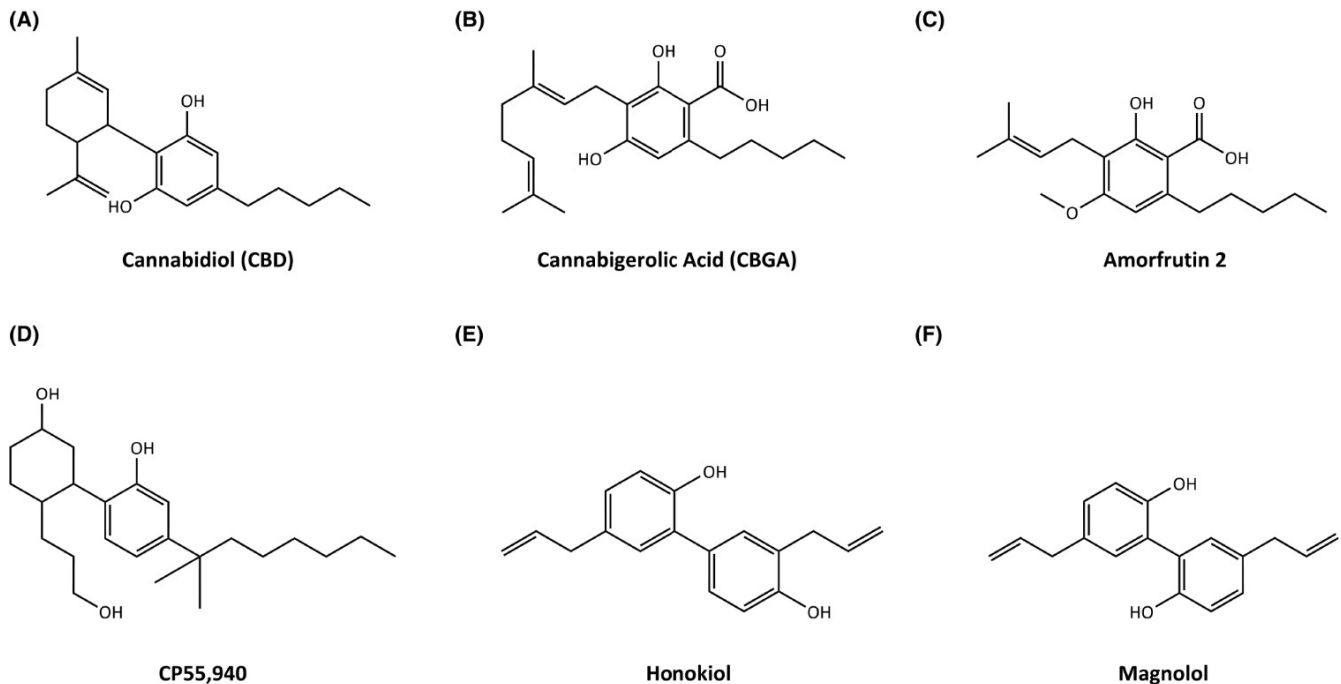


FIGURE 1 Chemical structures. (A) cannabidiol, (B) cannabigerolic acid, (C) amorfrutin 2, (D) CP55,940, (E) honokiol, and (F) magnolol.

type A (GABA_A) receptor.²¹ Heterozygous knockin of this *Gabrb3* mutant in mice (*Gabrb3*^{+/*D120N*} mice) provides a disease model for Lennox–Gastaut syndrome.²¹ The model recapitulates some of the clinical features of the syndrome; for example, the mice display infantile spasms, atypical absence seizures, GTCs, and behavioral abnormalities.²¹

The current study aimed to assess whether amorfrutin 2, magnolol, and honokiol have antiseizure effects on hyperthermia-induced GTCs in the *Scn1a*^{+/*−*} mouse model of Dravet syndrome. We also examined whether any positive effects extended to other models, including the maximal electroshock seizure (MES) test of GTCs and the *Gabrb3*^{+/*D120N*} mouse model of atypical absence seizures in Lennox–Gastaut syndrome. In addition, molecular pharmacology studies were employed to examine whether the compounds affect candidate antiseizure drug targets, that is, cannabinoid receptors and T-type voltage-gated calcium channels. Cannabinoids tend to be multimodal drugs that affect numerous targets. Some cannabinoids engage cannabinoid receptors such as CB₁ and CB₂, which are thought to yield antiseizure activity.²² Notably, the terpene beta-caryophyllene, which is also found in non-cannabis plants, is a CB₂ agonist and exerts antiseizure effects in rodent models.^{23,24} Phytocannabinoids with antiseizure effects also inhibit T-type voltage-gated calcium channels, with the amorfrutin 2-like compound CBGA showing particular potency at these channels.^{25,26} We therefore additionally assessed whether amorfrutin 2, magnolol, and honokiol affect human T-type calcium channels and cannabinoid receptors.

2 | MATERIALS AND METHODS

All animal experiments were approved by the University of Sydney Animal Ethics Committee in accordance with the Australia Code of Practice for the Care and Use of Animals for Scientific Purposes. The breeding protocols of *Scn1a*^{+/*−*} mice and *Gabrb3*^{+/*D120N*} knockin mice are detailed in [Supporting Information Methods](#) and in previous studies.^{21,27} In vivo experiments of the pharmacokinetic studies in wild-type (WT) mice, hyperthermia-induced seizures in *Scn1a*^{+/*−*} mice, MES test in WT mice, and EEG recordings for atypical absence seizures in *Gabrb3*^{+/*D120N*} knockin mice are detailed in the [Supporting Information Methods](#).^{4,21,27} In vitro recording of fluorescent imaging plate reader calcium flux assay, membrane potential assay, and automated patch clamp studies are also outlined in the [Supporting Information Methods](#).^{28,29}

3 | RESULTS

3.1 | Brain and plasma pharmacokinetic profiles of amorfrutin, honokiol, and magnolol in mice

We first examined the pharmacokinetic parameters of amorfrutin 2, honokiol, and magnolol, as summarized in [Table 1](#). After an intraperitoneal (ip) administration, absorption of honokiol and magnolol was rapid,

TABLE 1 Pharmacokinetic parameters in mouse plasma and brain.

Parameter	Amorfrutin 2		Honokiol		Magnolol	
	Plasma	Brain	Plasma	Brain	Plasma	Brain
C _{max} , µg/mL	17 ± .9	1.3 ± .1 ^a	.4 ± .0	2.1 ± .4 ^a	.4 ± .2	1.4 ± .7 ^a
t _{max} , min	30	30	15	30	15	30
t _{1/2} , min	115.5	83.4	19.3	15.7	18.4	20.8
AUC, µg × min/mL	2787	177	11.9	90	12	42.6
Brain-to-plasma ratio	0.064		7.56		3.55	

Abbreviation: AUC, area under the curve.

^aBrain concentrations converted from measured concentrations (ng/mg brain) assuming density of 1 g/mL.

with a plasma T_{max} of 15 min, whereas the absorption of amorfrutin 2 was slightly delayed, with a plasma T_{max} of 30 min (Figure 2). The peak brain concentrations for all three drugs (brain T_{max}) were 30 min post-administration (Figure 2). Regarding elimination rates, amorfrutin 2 was the most persistent, with greater brain and plasma half-lives than magnolol and honokiol (Table 1). Drug exposure (area under the curve, AUC) of honokiol and magnolol was higher in the brain than in plasma (Table 1), and the brain-to-plasma ratio was 7.56 for honokiol and 3.55 for magnolol, suggesting both displayed excellent brain penetration. Amorfrutin 2 had greater drug exposure in plasma than in the brain (Table 1), with a brain-plasma ratio of 0.064, indicating poor brain uptake.

3.2 | Anticonvulsant activities of amorfrutin 2 and magnolol but not honokiol against hyperthermia-induced GTCSs in *Scn1a*^{+/-} mice

We next assessed the effect of amorfrutin 2, magnolol, and honokiol at their plasma T_{max} on hyperthermia-induced GTCSs in *Scn1a*^{+/-} mice (Figure 3). Amorfrutin 2 dose-dependently displayed antiseizure activity against hyperthermia-induced seizures (Figure 3A), and significantly increased the body temperature threshold at which the *Scn1a*^{+/-} mice had a GTCS at 30 mg/kg ($p = .011$) and 100 mg/kg ($p < .0001$) compared to vehicle. The 10 mg/kg dose was ineffective. Magnolol also displayed antiseizure effects, but only at the 100 mg/kg dose, which significantly elevated the body temperature threshold compared to vehicle ($p < .0001$), but not at 10 or 30 mg/kg (Figure 3B). Honokiol (10–100 mg/kg) did not influence hyperthermia-induced seizures in *Scn1a*^{+/-} mice (Figure 3C). We then determined the brain and plasma concentrations of amorfrutin 2, magnolol, and honokiol in *Scn1a*^{+/-} mice after hyperthermia-induced seizures in experimental mice

(Table S1). There was a clear dose-dependent increase in brain concentrations of all compounds tested. The following brain concentrations were attained at the active antiseizure doses: amorfrutin 2, 30 and 100 mg/kg active doses = 21.4 and 45.2 µmol·L⁻¹, respectively; magnolol, 100 mg/kg active dose = 347.1 µmol·L⁻¹.

3.3 | Anticonvulsant effect of amorfrutin 2 and magnolol on the MES test

To further characterize the anticonvulsant properties of amorfrutin 2 and magnolol, we examined the effects of these compounds on the MES test of GTCSs (Figure 4A,B). In both experiments, mice treated with vehicle all had GTCSs that went to full hindlimb extension (Figure 4A,B). Both amorfrutin 2 and magnolol significantly protected against hindlimb extension compared to vehicle at 100 mg/kg ($p = .03$ and $p < .0001$, respectively) but not at 30 mg/kg. Amorfrutin 2 at 100 mg/kg protected 50% of mice, whereas magnolol at 100 mg/kg protected 90% of mice from hindlimb extension.

3.4 | Antiseizure effect of magnolol against atypical absence seizures in *Gabrb3*^{+/-D120N} mice

We decided to then elaborate further on magnolol regarding its potential antiseizure effect against atypical absence seizures in *Gabrb3*^{+/-D120N} mice (Figure 4C). We focused on magnolol for two reasons: (1) magnolol's brain penetration is optimal for an antiseizure drug (brain-to-plasma ratio = 7.56, whereas amorfrutin 2's = 0.064) and (2) magnolol displayed more robust effects in the MES model than amorfrutin 2. We assessed the effect of magnolol on atypical absence seizures for 30 min, which coincided with its presence in brain tissue. Atypical absence seizures were chosen over GTCSs because atypical absence seizures are sufficiently abundant in *Gabrb3*^{+/-D120N} mice to enable

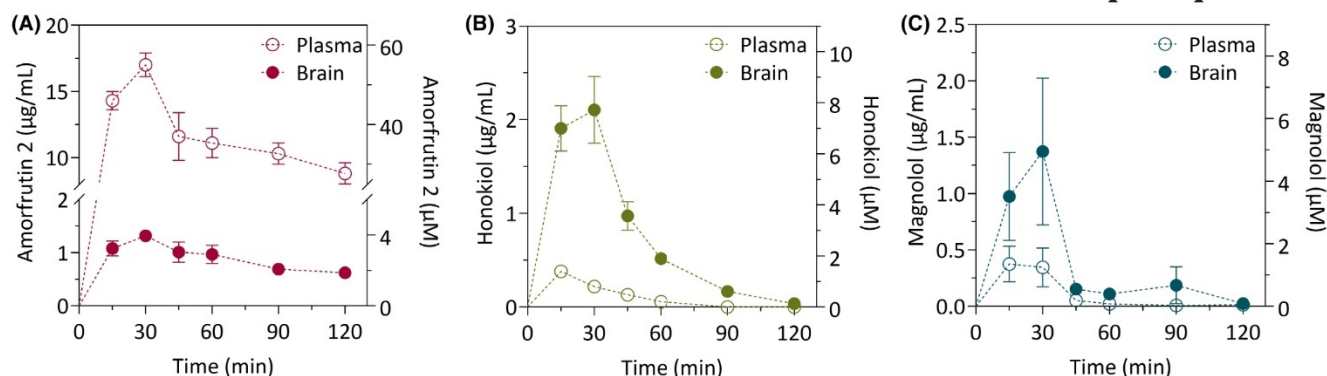


FIGURE 2 Brain and plasma pharmacokinetic analysis of amorfrutin 2, honokiol, and magnolol. Plasma and brain concentration–time curves are shown for (A) amorfrutin 2, (B) honokiol, and (C) magnolol following 10 mg/kg ip injections in mice. Compounds concentrations are depicted in both mass concentrations (left y-axis) and molar concentration (right y-axis). Data are expressed as mean $\pm$ SEM, with $n = 3$ –5 per time point.

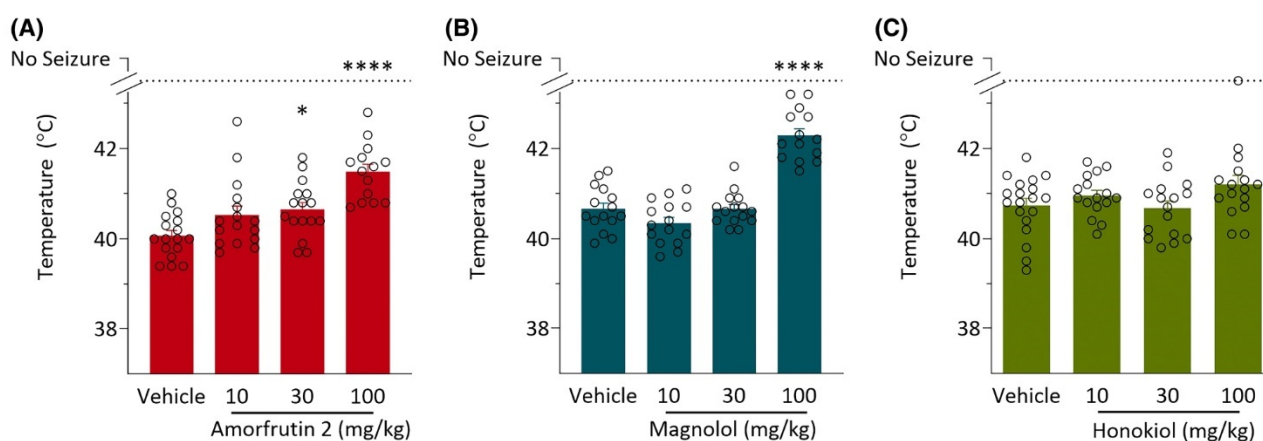


FIGURE 3 Anticonvulsant effects of amorfrutin 2 and magnolol but not honokiol against thermal-induced seizures in *Scn1a*^{+/-} mice. Body temperature threshold is shown for inducing a generalized tonic–clonic seizure in mice administered (A) amorfrutin 2, (B) magnolol, and (C) honokiol. Data are expressed in mean $\pm$ SEM, with $n = 14$ –19 per group (* $p = .011$ or **** $p < .0001$; log-rank Mantel–Cox compared to vehicle control).

pharmacological studies, whereas GTCSs are highly infrequent in this line.²¹ Atypical absence seizures have been shown to be sensitive to ethosuximide, a purported T-type calcium channel blocker, and the synthetic cannabinoid agonist WIN55,212-2.^{30,31} Although there were no overall main effects of magnolol treatment or treatment by time interactions, planned comparisons revealed acute magnolol administration reduced the number and duration of atypical absence seizures at various timepoints consistent with an antiseizure effect (Figure 4D,E).

3.5 | Amorfrutin 2, magnolol, and honokiol inhibit T-type calcium channels but do not influence cannabinoid receptors

T-type calcium channels are an emerging target for antiseizure drugs, and phytocannabinoids with antiseizure

effects such as CBD and CBGA inhibit T-type calcium channels.^{4,25,26} Therefore, we investigated whether magnolol, amorfrutin 2, or honokiol inhibit these voltage-gated ion channels. We commenced with a preliminary screen of the inhibitory effects of 10 $\mu\text{mol}\cdot\text{L}^{-1}$ of each compound on the change in fluorescence caused by 10 $\text{mmol}\cdot\text{L}^{-1}$ Ca^{2+} in human embryonic kidney 293 (HEK293) cells expressing $\text{Ca}_v3.1$, $\text{Ca}_v3.2$, or $\text{Ca}_v3.3$ channels. We found that all three compounds demonstrated appreciable and sometimes strong inhibitory effects on T-type calcium channel subtypes (Figure S1).

Next, we investigated tonic inhibition of T-type channels using single-cell electrophysiology, a gold standard for studying voltage-gated ion channels. All three compounds inhibited T-type channel currents in a concentration-dependent manner with comparable potencies, although honokiol was notably more potent at inhibiting $\text{Ca}_v3.1$ (Figure 5A). Specifically, honokiol exhibited an IC_{50} value

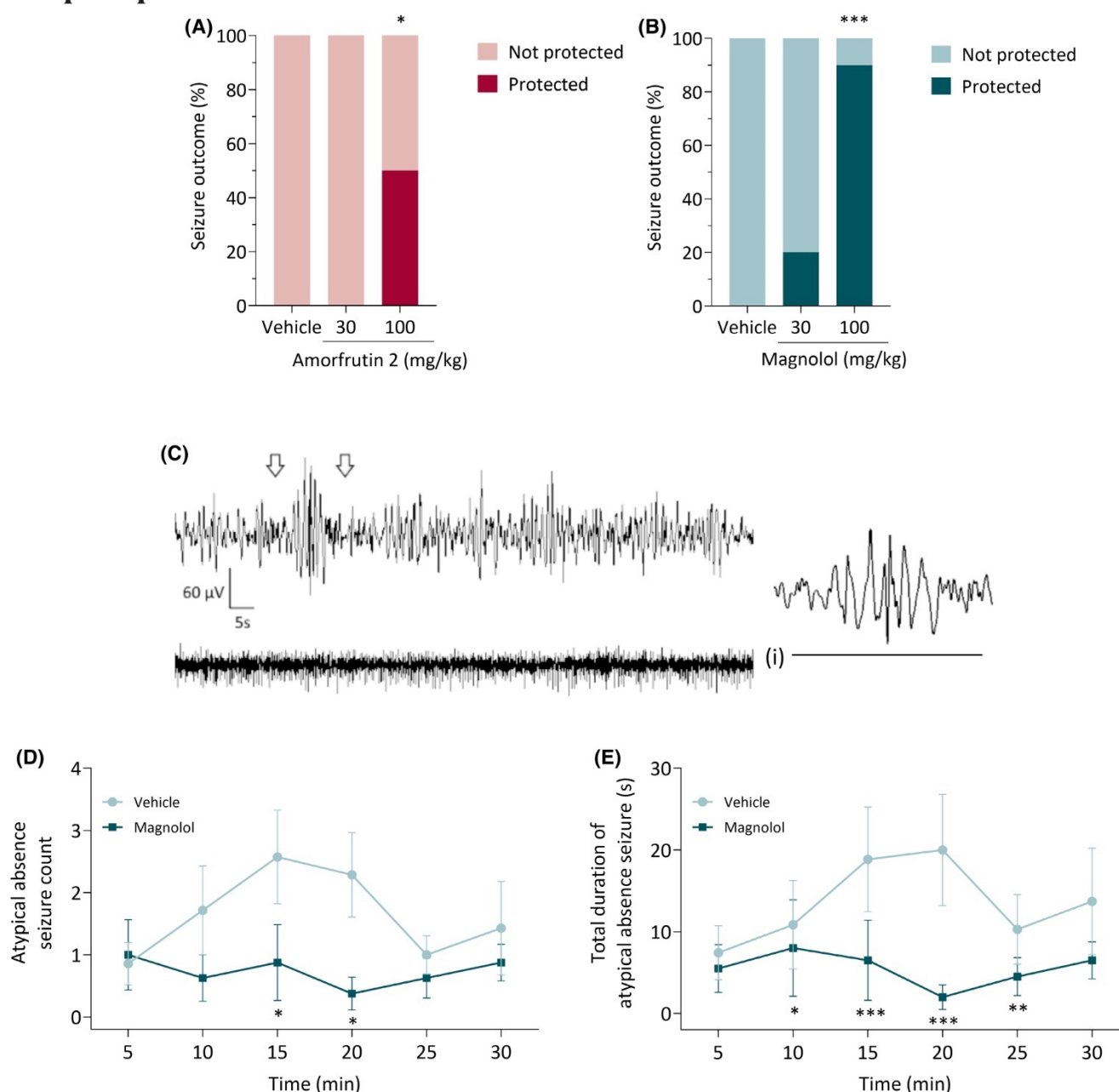


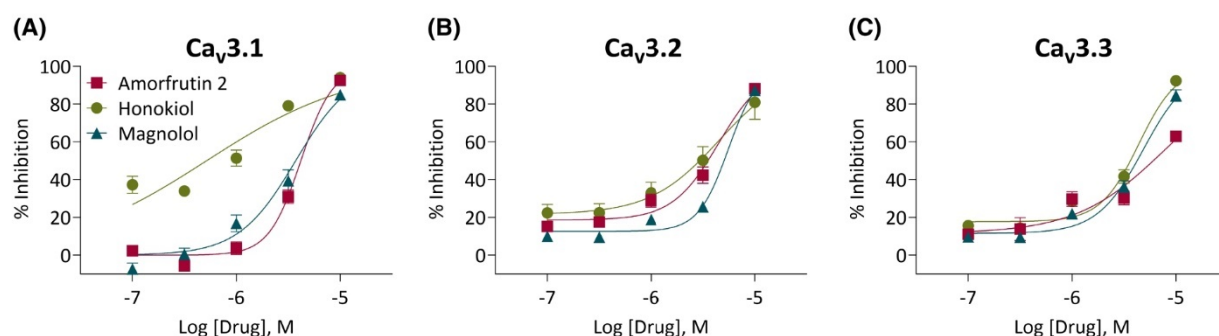
FIGURE 4 Antiseizure effects of amorfrutin 2 and magnolol in the maximal electroshock seizure test and an antiseizure effect of magnolol against atypical absence seizures in *Gabrb3^{+/-D120N}* mice. (A, B) Whereas all vehicle-treated mice exhibited hindlimb extension, mice treated with (A) amorfrutin 2 and (B) magnolol showed protection against hindlimb extension. Data are expressed in percentage, with $n = 10$ per group ($*p = .03$ or $***p < .0001$; Fisher exact test), compared to the vehicle group. (C) Representative electroencephalographic and electromyographic traces for atypical absence seizure in adult *Gabrb3^{+/-D120N}* mice. The arrows indicate the start and the end of an episode of (i) an atypical absence seizure. (D, E) Magnolol reduced the total count of atypical absence seizures (D) and the total duration of atypical absence seizures (E) at various timepoints. The seizure number and duration were counted in 5-min bins over 30 min of recording time. Data are presented as mean $\pm$ SEM, with $n = 7-8$ ($*p < .05$, $**p < .01$, and $***p < .001$; generalized linear mixed model with Sidák correction test compared to vehicle control).

of $787 \text{ nmol} \cdot \text{L}^{-1}$ at $\text{Ca}_v3.1$, which is markedly more potent than magnolol and amorfrutin 2, which both showed an IC_{50} of $4 \mu\text{mol} \cdot \text{L}^{-1}$. At $\text{Ca}_v3.2$, honokiol and amorfrutin 2 presented similar IC_{50} values of $3 \mu\text{mol} \cdot \text{L}^{-1}$, whereas magnolol had an IC_{50} of $5 \mu\text{mol} \cdot \text{L}^{-1}$ (Figure 5B). For $\text{Ca}_v3.3$ channels, the IC_{50} values were $3 \mu\text{mol} \cdot \text{L}^{-1}$ for honokiol,

$6 \mu\text{mol} \cdot \text{L}^{-1}$ for amorfrutin 2, and $4 \mu\text{mol} \cdot \text{L}^{-1}$ for magnolol (Figure 5C).

Given that magnolol, honokiol, and amorfrutin 2 share structural similarities with cannabinoids such as CP55,940, we investigated their activities at human CB_1 and CB_2 receptors stably expressed in AtT20 cells using

Automated patch-clamp



Membrane potential assay

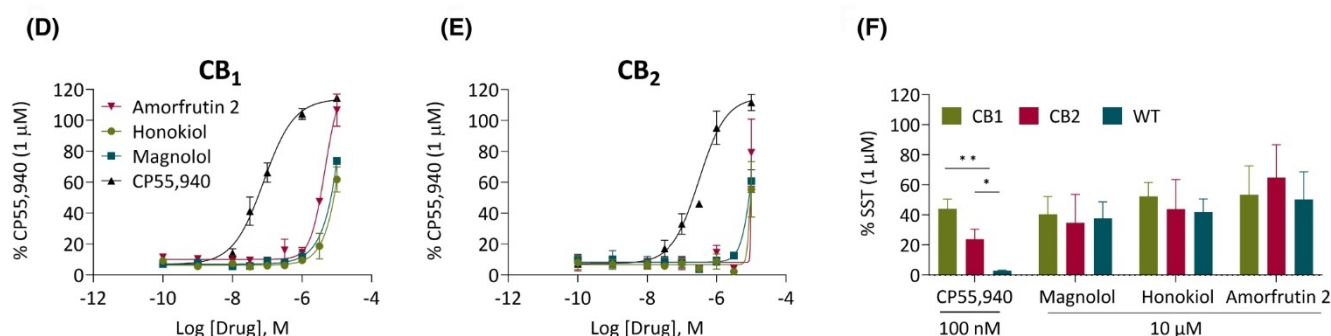


FIGURE 5 Amorfrutin 2, honokiol, and magnolol inhibit T-type calcium channels but do not affect cannabinoid CB₁ and CB₂ receptors. (A–C) Concentration–response curves for amorfrutin 2, honokiol, and magnolol inhibition of (A) Ca_v3.1, (B) Ca_v3.2, and (C) Ca_v3.3 using high throughput in vitro electrophysiology. Single-cell patch clamp recordings were made from HEK293 cells expressing Ca_v3.1, Ca_v3.2, or Ca_v3.3 channel current. At least 20 sweeps at 5s intervals were collected as a baseline before the drug was added, which was then followed by a further 50 sweeps of recording to reach a steady state of drug inhibition (% inhibition of peak current of vehicle control; $n = 11–21$ cells per concentration). (D, E) Concentration–response curves of CP55,940, amorfrutin 2, honokiol, and magnolol in AtT20-CB₁ cells (D) and AtT20-CB₂ cells (E). There was no clear concentration-dependent cellular hyperpolarization in response to amorfrutin 2, magnolol, or honokiol at concentration $\leq 1 \mu\text{mol}\cdot\text{L}^{-1}$, but an abrupt increase was observed at $3 \mu\text{mol}\cdot\text{L}^{-1}$ and $10 \mu\text{mol}\cdot\text{L}^{-1}$. (F) Summary data of CP55,940 ($100 \text{ nmol}\cdot\text{L}^{-1}$), and $10 \mu\text{mol}\cdot\text{L}^{-1}$ amorfrutin 2, magnolol, and honokiol induced cellular hyperpolarization of AtT20-CB₁, AtT20-CB₂, and nontransfected wild-type (WT) cells. Responses are normalized to $1 \mu\text{mol}\cdot\text{L}^{-1}$ somatostatin (SST), which activates endogenous SST receptors. Data presented are mean $\pm$ SEM of changes in fluorescence after normalization to SST ($n = 3–5$). * $p < .05$, ** $p < .01$.

the membrane potential assay.³² As expected, CP55,940 displayed potent concentration-dependent activation of CB₁ and CB₂ receptors (Figure 5D,E). We found that amorfrutin 2, honokiol, and magnolol all increased cellular hyperpolarization at concentrations $> 1 \mu\text{mol}\cdot\text{L}^{-1}$ suggesting agonist activity (Figure 5D,E). We then tested whether the responses were specific to the cannabinoid receptors by comparing the effects of the three compounds at $10 \mu\text{mol}\cdot\text{L}^{-1}$ across CB₁, CB₂, and WT nontransfected AtT20 cells. Unlike CP55,940, which was inactive in WT cells, magnolol, honokiol, and amorfrutin 2 produced a comparable response in WT cells to that observed in the CB₁ and CB₂ transfected cells (Figure 5F). This highlights that the three compounds were behaving nonspecifically in the AtT20 cells and cannot be deemed CB₁ and CB₂ agonists.

In addition, we examined whether amorfrutin 2, magnolol and honokiol could antagonize CB₁ or CB₂ receptors

by testing whether the compounds inhibited the response to submaximal concentrations of CP55,940. Testing was conducted using $1 \mu\text{mol}\cdot\text{L}^{-1}$ concentrations of the three compounds, which did not induce cellular hyperpolarization at either receptor. As expected, the CB₁ receptor inverse agonist/antagonist SR141716 inhibited the response to $100 \text{ nmol}\cdot\text{L}^{-1}$ CP55,940 at CB₁ receptors, and the CB₂ receptor inverse agonist AM630 reduced the activity of $300 \text{ nmol}\cdot\text{L}^{-1}$ CP55,940 at CB₂ receptors (Figure S2A,B). In contrast, magnolol, honokiol, and amorfrutin 2 had no effect on CP55,940-induced responses at either CB₁ or CB₂ receptors (Figure S2A,B). We also examined whether the compounds potentiated EC₂₀ responses of CP55,940 at CB₁ or CB₂ receptors, which would provide preliminary evidence for positive allosteric modulation. However, $1 \mu\text{mol}\cdot\text{L}^{-1}$ magnolol, honokiol, and amorfrutin 2 did not alter the EC₂₀ response to CP55,940 at CB₁ or CB₂ receptors (Figure S2C,D).

4 | DISCUSSION

Molecules exist in nature with structural and pharmacological commonalities to cannabinoids that are not found in cannabis. These non-cannabis-derived compounds may provide a source for novel therapeutics. Here, we examined the antiseizure potential of several of these compounds, namely amorfrutin 2, honokiol, and magnolol. We found for the first time that both amorfrutin 2 and magnolol displayed antiseizure activity against hyperthermia-induced seizures in the *Scn1a*^{+/-} mouse model of Dravet syndrome. These effects were corroborated by results from the mouse MES test, where amorfrutin 2 and magnolol protected against hindlimb extension following a GTCS. Furthermore, we found that magnolol reduced the number and duration of atypical absence seizures in the *Gabrb3*^{+D120N} mouse model of Lennox–Gastaut syndrome. These results thereby provide the first evidence that these compounds might be further leveraged as novel treatments for intractable epilepsies. We also provided brain and plasma pharmacokinetic data for these compounds, which highlight that both magnolol and honokiol are highly brain penetrant, an important characteristic for neuropharmacological agents. However, amorfrutin 2 displayed very low brain penetrance, which would need to be addressed in its further drug development. We discovered that amorfrutin 2, honokiol, and magnolol inhibited T-type calcium channel subtypes that are similarly impacted by other cannabinoids found in cannabis that display antiseizure activity.^{25,26,33,34,35} The compounds were without any activity at cannabinoid CB₁ and CB₂ receptors.

Amorfrutin 2 is a bioactive molecule found in two different species of plant, *Glycyrrhiza foetida* and *Amorpha fruticosa*, which are native to Africa and America, respectively. We were motivated to explore the antiseizure activity of this molecule, as it has structural similarity to CBGA (Figure 1), a major biosynthetic molecule in cannabis that displays antiseizure activity.⁴ We found that amorfrutin 2 displayed equivalent antiseizure potency to CBGA against hyperthermia-induced seizures in *Scn1a*^{+/-} mice, being effective at both 30 mg/kg and 100 mg/kg doses.⁴ This highlights that amorfrutin 2 is effective at a lower dose than CBD, which was only effective at 100 mg/kg in this model.^{6,36} The antiseizure effect of amorfrutin 2 in the MES test of GTCSs was also consistent with what was previously found with CBGA.⁴ The geranylated side-chain of CBGA appears to be important for antiseizure activity, as the biosynthetic precursor to CBGA, olivetolic acid, which lacks this side chain, displayed less potent antiseizure effects against hyperthermia-induced seizures in *Scn1a*^{+/-} mice.³⁷ The geranylated side chain is shorter in amorfrutin 2, although this did not appear to cause a loss in antiseizure activity. One major limitation of amorfrutin

2, which is common to compounds containing a carboxylic acid group such as olivetolic acid and CBGA, is that it displayed very low brain penetration.^{3,37} This major limitation of amorfrutin 2 would need to be overcome if it is advanced as a novel antiseizure agent.

We examined magnolol and honokiol, which are two molecules that derive from *Magnolia officinalis* and have been used in traditional Chinese medicine to treat anxiety, insomnia, and epilepsy. Whereas this is the first report of antiseizure activity of amorfrutin 2, magnolol has been characterized as an antiseizure agent in preclinical models.^{12–14} Notably, magnolol showed promising antiseizure effects in both PTZ and kainic acid seizure models.^{12,14,38} However, here we report the novel finding that magnolol's antiseizure activity extends to intractable epilepsy models, as it had antiseizure effects against hyperthermia-induced seizures in the *Scn1a*^{+/-} mice and reduced atypical absence seizures in *Gabrb3*^{+D120N} mice (albeit only in female mice here). Amorfrutin 2 was more potent than magnolol in the *Scn1a*^{+/-} mice; however, magnolol had more robust antiseizure effects than amorfrutin 2 on MES-induced seizures. Surprisingly, despite honokiol attaining equivalently high brain concentrations to magnolol, it was ineffective against hyperthermia-induced seizures in *Scn1a*^{+/-} mice. Magnolol and honokiol are structural isomers, and our data suggest that movement of the hydroxy group on the phenolic ring is sufficient to switch activity from active to inactive. Notably, both honokiol and magnolol exhibited comparable antiseizure potency in other preclinical seizure models such as the PTZ test in zebrafish¹³ and the intravenous N-methyl-D-aspartate seizure test in mice.³⁹ The differential effects of magnolol and honokiol presented here then appear to be specific to febrile seizures in *Scn1a*^{+/-} mice, which have a unique drug-resistant pathophysiology due to Nav1.1 dysfunction in hippocampal GABAergic inhibitory interneurons.¹⁹ Future studies are needed to reconcile the differential effects of these structural isomers in *Scn1a*^{+/-} mice.

Atypical absence seizures in Lennox–Gastaut patients persist into adulthood and may contribute to ongoing developmental deficits.⁴⁰ Here, we provide the first evidence that magnolol (100 mg/kg ip) could be advanced to target these drug-resistant forms of seizures, because its administration reduced the number and duration of atypical absence seizures in the *Gabrb3*^{+D120N} mouse model of Lennox–Gastaut syndrome. The *Gabrb3*^{+D120N} mouse model is a relatively new model and has only undergone a preliminary pharmacological validation.²¹ It was shown that ethosuximide (200 mg/kg ip) and clobazam (2.5 mg/kg ip) but not topiramate (30 mg/kg ip) diminished atypical absence seizures in this model.²¹ Interestingly, one established mode of action of ethosuximide is T-type calcium channel inhibition,³⁰ and clobazam is a positive allosteric

modulator (PAM) of GABA_A receptor, both pharmacological features shared by magnolol.⁴¹ Our results highlight that future studies could use the novel *Gabrb3*^{+D120N} mouse model of Lennox–Gastaut syndrome to develop analogues of magnolol with improved pharmacokinetic properties and enhanced antiseizure potency.

A limitation of the current study is that we did not comprehensively examine the effects of amorfrutin 2, honokiol, and magnolol in *Scn1a*^{+/-} mice or *Gabrb3*^{+D120N} mice. *Scn1a*^{+/-} and *Gabrb3*^{+D120N} mice exhibit additional seizure types such as spontaneous GCTSs, although these seizures are infrequent and difficult to quantify. We did not examine these endpoints in the present study. We chose not to because we utilize oral delivery for such studies to ensure steady-state drug exposure, but also to avoid the stress of repeated intraperitoneal injections. Such studies are not feasible because amorfrutin 2 is likely to have low oral bioavailability, and magnolol and honokiol have experimentally confirmed low oral bioavailability in rodents.^{42–44} To address this issue, medicinal chemists could synthesize analogues of amorfrutin 2 or magnolol with structural modifications to improve oral bioavailability. Moreover, bioisosteric replacement strategies could be applied to substitute the carboxylic acid moiety of amorfrutin 2 as a means to improve stability and brain penetration. Both amorfrutin 2 and magnolol provide scaffolds that are tractable to synthetic analogue development.⁴⁵ For example, one paper designed magnolol derivatives to increase the pharmacological actions at human cannabinoid CB₁ and CB₂ receptors.⁴⁶

Phytocannabinoids and synthetic cannabinoids with antiseizure activity inhibit T-type calcium channels.^{4,25,26,33} Here, we report for the first time that amorfrutin 2, magnolol, and honokiol, which are structurally similar to the cannabinoids, inhibit Ca_v3.1, Ca_v3.2, and Ca_v3.3 channel subtypes. These channels are known targets for antiseizure drugs, including the clinically utilized agents ethosuximide and zonisamide.^{47,48} Moreover, preclinical research has shown that novel pan T-type channel inhibitors reduce absence seizures in preclinical rat models,⁴⁹ and genetic mutations in T-type channel genes have been linked to idiopathic generalized epilepsies in patients.^{50,51} Within the specific context of Dravet syndrome, genetic knockout of *Cacna1g*, which encodes the Ca_v3.1 channel, reduced spontaneous seizure occurrence and improved survival in *Scn1a*^{+/-} mice.⁵² It is therefore possible that T-type channels could contribute to the antiseizure effects of magnolol and amorfrutin 2 observed here, especially when one considers that the inhibitory potencies of the compounds at the T-type channels are well below the brain concentrations reached in the experimental *Scn1a*^{+/-} mice. However, arguing against T-type inhibition being responsible, honokiol inhibited the

T-type channels without affecting hyperthermia-induced seizures in *Scn1a*^{+/-} mice. It could be that honokiol and magnolol have distinctive effects on other molecular targets beyond T-type channel inhibition.

Amorfrutin 2, honokiol, and magnolol have emerging polypharmacological effects, some of which are on known antiseizure drug targets. Both honokiol and magnolol are PAMs of inhibitory GABA_A receptors,⁵³ and amorfrutin 2 would be predicted to share this activity, given that the structurally similar molecule CBGA is a GABA_A receptor PAM.⁴ Another emerging antiseizure target of all these compounds are the peroxisome proliferator-activated receptors (PPARs), which are nuclear receptors originally exploited to treat metabolic disorders.^{54,55} Amorfrutin 2, magnolol, and honokiol inhibit PPAR α and PPAR γ .^{7,56,57} Magnolol is reportedly a low-potency and low-efficacy agonist of CB₁ and CB₂ receptors, whereas honokiol is reported to be a low-potency, high-efficacy agonist of CB₁ but behaves as an inverse agonist of CB₂ receptors in a cyclic adenosine monophosphate (cAMP) accumulation assay.⁹ However, in our evaluation of amorfrutin 2, magnolol, and honokiol as human CB₁ and CB₂ receptor agonists, we found that all three compounds were inactive at lower concentrations and produced responses that were nonspecific to the cannabinoid receptors at higher concentrations (>1 $\mu\text{mol}\cdot\text{L}^{-1}$). Additionally, we found no evidence that these compounds antagonize cannabinoid receptors in our assay. There are several potential reasons for these differences. Unlike cAMP assays, our membrane potential assay is not subject to strong signal amplification and requires significant receptor occupancy to produce detectable effects.⁵⁸ It is important to note that we did not test concentrations exceeding 10 $\mu\text{mol}\cdot\text{L}^{-1}$ at which nonspecific activities were observed, whereas the earlier study required much higher concentrations to elicit activity and did not assess for nonspecific effects in the WT Chinese hamster ovary cells used.⁹ Although our study provides some clues around potential additional molecular targets of these compounds, future studies are required to determine which target/s are specifically responsible for their antiseizure effects.

5 | CONCLUSIONS

The current study demonstrates for the first time that the non-cannabis-derived phytocannabinoids amorfrutin 2 and magnolol not only display antiseizure activity against febrile GTCs in a mouse model of Dravet syndrome but also against GTCs in the MES mouse model. Furthermore, magnolol was effective in reducing the number and duration of atypical absence seizures in a mouse model of Lennox–Gastaut syndrome. Adding to the likely

polypharmacological antiseizure actions of these compounds, we also discovered that amorfrutin 2, magnolol, and honokiol all inhibit T-type calcium channels; however, there was no activity at cannabinoid receptors using the membrane potential assay. Our results justify future drug discovery campaigns around the structural scaffolds of amorfrutin 2 and magnolol that aim to develop novel antiseizure drugs for intractable epilepsy, of which there is the greatest unmet clinical need.

AUTHOR CONTRIBUTIONS

Jonathon C. Arnold, Samuel D. Banister, and Ka Lai Yip conceived the current project. Ka Lai Yip contributed data acquisition of animal experiments and manuscript drafting. Michael Udoh contributed data acquisition of cell experiments and manuscript drafting. Laura A. Sharman contributed data acquisition of animal experiments and data analysis. Thomas Harman contributed to method validation and manuscript review. Miguel Bedoya-Pérez contributed data analysis of animal experiments. Jonathon C. Arnold and Lyndsey L. Anderson contributed to experimental design and data acquisition of animal experiments. Jonathon C. Arnold contributed to drafting the manuscript and finalizing it for submission.

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CONFLICT OF INTEREST STATEMENT

J.C.A. has served as an expert witness in various medico-legal cases involving cannabis and has received consulting fees from the World Health Organization, Medical Cannabis Industry Australia, and Haleon (consumer health care subsidiary of GSK). He reports research grants from the Australian National Health and Medical Research Council and from Lambert Initiative for Cannabinoid Therapeutics. J.C.A. and L.L.A. hold a patent on a novel anticonvulsant therapy for epilepsy (WO2019071302). The remaining authors have no conflicts of interest. We

confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Chapter 3

Assessment of novel ago-PAMs of the cannabinoid CB1 receptor in mouse models of intractable epilepsy

3.1 Introduction

Patients with epilepsy are highly reliant on anti-seizure medications (ASMs) to prevent recurrent seizures. These medications work by suppressing excessive excitability or enhancing inhibitory activities within neuronal networks.¹²⁵ Although there are over 30 ASMs with various pharmacological targets available on the market, drug resistance remains a major challenge in seizure management, underscoring the need for novel drug discovery programs to expand therapeutic options.^{301, 302} Both Dravet syndrome and Lennox-Gastaut syndrome are classified as childhood epilepsy syndromes characterized by treatment-resistant seizures and associated developmental deficits.^{13, 35, 80, 97} Dravet syndrome patients display febrile seizures and afebrile spontaneous generalized tonic-clonic seizures (GTCS),⁸⁰ while patients with Lennox-Gastaut syndrome commonly exhibit atonic and atypical absence seizures.⁹⁷ *De novo* mutations in the *SCN1A* gene, which encodes for the Na_v1.1 channel, account for 80% of cases in Dravet syndrome.⁴⁶ While Dravet syndrome is largely genetic in origin, Lennox-Gastaut syndrome aetiology is more complex and varied. Although, genetic mutations have been characterized in Lennox-Gastaut syndrome patients such as in the *GABRB3* gene, encoding the beta3 subunit of the GABA_A receptor.¹⁰²

The endocannabinoid system provides new targets for anti-seizure drug discovery. The endocannabinoid system is comprised of endogenous ligands, anandamide (AEA) and 2-arachidonoylglycerol (2-AG); their synthetic and degradative enzymes, for example fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL); and G-protein-coupled receptor targets cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptors. The endocannabinoids are lipid signaling molecules and undergo retrograde transmission in neuronal synapses, where they are liberated at the post-synaptic membrane to travel backwards to the presynaptic membrane to engage CB1 receptors.¹⁶⁹

This in turn inhibits release of other neurotransmitters including glutamate which dampens excessive excitatory neurotransmission. Seizures rapidly increase 2-AG concentrations in the hippocampus in mouse electrical kindling seizure model, coupling to neural activity by selectively reduce calcium signals to suppress seizures.²⁷³ However, clinical biopsies from patients with refractory epilepsy have revealed decreased concentrations of AEA and 2-AG, indicating that chronic seizure may impair endocannabinoid signaling that potentially further contributes to sustained hyperexcitability.^{171, 303} Exogenous AEA treatment has anti-seizure effects in preclinical models, including in the maximal electroshock (MES) test.¹⁷⁵ Further, the MAGL inhibitor JZL184, which augments brain 2-AG concentrations, suppressed epileptiform discharges and recurrent seizures in a kainic acid-induced seizure model.¹⁷⁹ Findings suggest endocannabinoids are released to inhibit neuronal hyperexcitability and that deficits in the system engenders enhanced susceptibility to seizures.³⁰⁴⁻³⁰⁶ Thus, the development of novel therapeutics that aim to enhance endocannabinoid signaling may provide a means to develop novel anti-seizure drugs.

Cannabinoid CB1 receptor orthosteric agonists have been explored in mouse models of the intractable epilepsies Dravet syndrome and Lennox-Gastaut syndrome. Heterozygous deletion of *Scn1a* gene in mouse (*Scn1a*^{+/-} mouse) replicates clinical phenotypes of Dravet syndrome such as hyperthermia-induced GTCS, afebrile recurrent seizures and premature death.^{184, 216} Mice with a heterozygous knock-in mutation in *Gabrb3* gene (*Gabrb3*^{3+/D120N} mouse) display atypical absence seizures and GTCS.¹²³ The major psychoactive constituent of cannabis Δ⁹-tetrahydrocannabinol (Δ⁹-THC), which is an orthosteric agonist of the CB1 receptor, displayed anticonvulsant effects against hyperthermia-induced seizures.¹⁹⁰ The synthetic orthosteric CB1 receptor agonist WIN 55,212-2 displayed anti-seizure effects in reducing atypical absence seizure in a *Gabrb3*^{3+/D120N} mouse model of Lennox-Gastaut syndrome.¹²³

An issue with CB1 receptor orthosteric agonists is that they have sedative and intoxicating effects, thus limiting their translational potential.^{141, 155, 307} Targeting the allosteric site of CB1 receptors offers pharmacological advantages as an alternative strategy to enhance CB1 signaling. As opposed to orthosteric agonists which bind the same binding pocket to the endogenous ligands of the receptor, allosteric agonists bind a distinct site that can magnify the actions of the endogenous ligands via increasing their binding affinity or efficacy.²⁶¹ These are referred to as positive allosteric modulators (PAMs). CB1 receptor PAMs hold great promise because they would further amplify the actions of the endocannabinoids which are released in response to seizures to dampen neuronal hyperexcitability. Moreover, their actions would be more targeted as they act in a cell, brain region and temporally-specific manner.²⁶¹ Several CB1 receptor PAMs have demonstrated therapeutic effects in preclinical models of neurological disorders. For instance, lipoxin A4 reduces neuroinflammation,³⁰⁸ ZCZ011 limits neuropathic pain,³⁰⁹ and GAT229 has anti-seizure activity.^{184, 265} The anti-seizure effects of GAT229 have been observed in different preclinical epilepsy models.^{184, 265} GAT229 inhibited spike-and-wave discharges in a Genetic Absence Epilepsy Rat from Strasbourg (GAERS) model.²⁶⁵ The same study also evaluated whether GAT229 produced similar side effects as direct agonists and found that GAT229 did not exhibit cannabis-like side effects.²⁶⁵ Our group showed GAT229 has anti-seizure effects against hyperthermia-induced seizures in *Scn1a*^{+/-} mice¹⁸⁴

At present only a handful of pure CB1 receptor PAMs have been developed. Some of the newly developed ligands in this chemical space also exhibit mixed allosteric agonist and PAM activity, which are referred to as ago-PAMs. Allosteric agonists activate the CB1 receptor via the allosteric site in the absence of the endogenous ligand. For example, GAT591 has been characterized as a potent ago-PAM with improved physicochemical properties and *in vitro* metabolic stability.²⁶⁸ GAT591 had analgesic effects as it reduced mechanical allodynia in mice that was absent in CB1 receptor knockout mice, and increased pain response latency in the tail

flick test.²⁶⁸ Consistent with potential as an anti-seizure agent it reduced excitatory post synaptic current in autaptic hippocampal neurons.²⁶⁸ It also lacked cannabimimetic effects, as it did not promote catalepsy or hypothermia at least up to 10 mg/kg in mice.²⁶⁸ The current study aimed to investigate the anti-seizure effects of two ago-PAMs GAT591 and GAT1102 (a newly developed compound) (Figure 1), in mouse seizure models including the *Scn1a*^{+/-} mouse model of Dravet syndrome and the *Gabrb*^{3+/D120N} mouse model of Lennox-Gastaut syndrome. Experiments were conducted in both male and female mice.

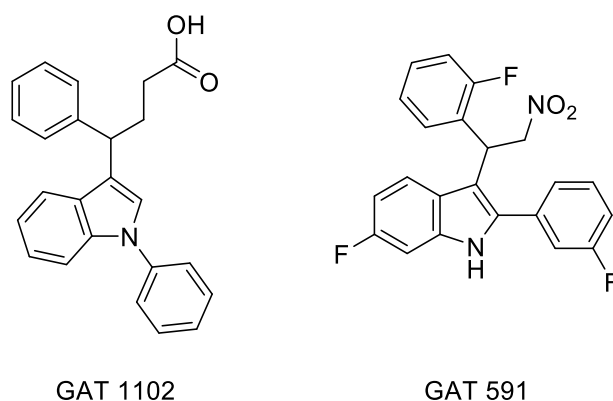


Figure 1. Chemical structures of GAT591 and GAT1102

3.2 Methods

3.2.1 Animal

All animal experiments were conducted following the ethics protocol approved by the University of Sydney Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All animals were group-housed in individually ventilated cages in an animal holding room with controlled temperature (22 ± 2 C) and 12 h light-to-dark cycles and had access to food and water *ad libitum*. All animals were habituated for one week upon arrival before any experimental procedures.

Male *Gabrb3*^{+/*D120N*} knock-in mice carrying a mutation on gene *Gabrb3*, c.G358A p.D120N, (stock 035437 in Jackson Laboratory, Bar Harbour, USA) were bred with female WT C57BL/6J mice to generate experimental *Gabrb3*^{+/*D120N*} mice.¹²³ Offspring mice were genotyped to determine whether they were carrying the heterozygous of *Gabrb3* genotype or WT as previously described.¹²³

A congenic *129.Scn1a*^{+/-} line was generated by crossing *Scn1a*^{+/-} mice from the Jackson Laboratory (stock 37107-JAX; Bar Harbor, USA) with 129S6/SvEvTac background strain from Australian BioResources (New South Wales, Australia).³¹⁰ For our experiment, *129.Scn1a*^{+/-} mice were bred with wild-type C57BL/6J (B6) to generate [129 x B6] F1.*Scn1a*^{+/-} mice (*Scn1a*^{+/-} mice) or F1 wild type (WT) mice. The heterozygous *Scn1a* genotype in *Scn1a*^{+/-} mice was determined as previously described. All litters were kept in their home cage and weaned at P21.¹⁸⁴ Adult male C57BL/6J mice, aged between 6 to 7 weeks, were purchased from Australian Bioresources (Mossvale, NSW) for the MES tests.

3.2.2 Compounds and chemicals

GAT591 and GAT1102 were synthesized and generously provided by Prof. Ganesh A Thakur (Northeastern University), with > 99% enantiomeric purity.³¹¹ For administration, these compounds were freshly dissolved in a sequence of dimethyl sulfoxide, tween 80 and 0.9% saline (1:1:18 ratio). GAT591 and GAT1102 in both doses of 10 and 30 mg/kg were fully dissolved as a colorless solution, while 100 mg/kg GAT591 and 60 mg/kg GAT1102 were suspensions. Acetonitrile, dimethyl sulfoxide, methanol and formic acid were obtained from Merck Millipore (Massachusetts, USA). Diazepam was supplied by Sigma-Aldrich (Missouri, USA). All chemicals achieved > 99% purity in HPLC standard.

3.2.3 Pharmacokinetic studies

A single i.p. injection of 10 mg/kg GAT591 or GAT 1102 was given to WT C57BL/6J female and male mice aged between 6 to 7 weeks. They were then anesthetized with 5% isoflurane at six selected time points (15 - 120 min) for whole blood and brain sampling. Whole blood was collected by cardiac puncture and then centrifuged at 10,000 g for 10 min at 4°C to isolate the plasma.¹⁸⁴ Whole brains were harvested and snap-frozen on dry ice. Both samples were stored at -80°C until analyzed. Compound concentrations were averaged at each time point (n = 4).¹⁸⁴ Pharmacokinetic parameters were calculated using the linear-long trapezoidal method and fitted to noncompartmental analysis as previously described.²¹⁶

3.2.4 Sample preparation and analytical chemistry

Compounds were extracted from plasma and brain samples according to our previous methods with minor modifications.^{184, 216} GAT591 and GAT1102 samples were reconstituted in 60% methanol and 40% 0.1% formic acid in water (v/v). Compound concentrations were determined by using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) using a Shimadzu Nexera ultra-high-performance liquid chromatograph coupled with a Shimadzu 8,030 triple quadrupole mass spectrometer (Kyoto, Japan). Alongside an eight-concentration standard curve for each compound, samples were injected into the system equipped with a Zorbax XDB-C18 (100 × 2.1 mm, 3.5 µm, Agilent) reverse-phase column and with a Zorbax C8 guard column (Santa Clara, USA) at a flow rate of 0.6 mL/min.¹⁸⁴ Fragmentation ions were detected using the following mass transition pairs for m/z 397.1 > 336.1, 397.1 > 229.1 and 397.1 > 122.1 for GAT591 and m/z 356.3 > 194.2, 356.1 > 117.2 and 356.1 > 163.2 for GAT1102. Compound concentrations were quantified against the standard curve (R² > 0.98).

3.2.5 *Hyperthermia-induced seizure*

Hyperthermia seizure was induced in male and female *Scn1a*^{+/-} mice aged P14 to P16 and had been described in our previous reports.^{184, 190} The experimental time point was targeted according to the brain's time to maximum concentration (T_{max}) from the pharmacokinetic study. A single dose of (10 or 30 or 100 mg/kg) GAT591 or (10 or 30 or 60 mg/kg) GAT1102 or vehicle was delivered by i.p. injection (n = 14 - 19 for each group). A RET-3 rectal temperature probe was applied to acclimate the body temperature measurement 15 minutes before the brain T_{max}. A heat lamp was turned on to increase 0.5°C their core body temperature every two minutes. The seizure endpoint was considered when the clonic convulsant onset, with loss of posture.¹⁸⁴ Seizure-free was considered when the body temperature reached 42.5°C and held for three minutes. The body temperature was recorded, and the mice were immediately anesthetized with isoflurane. Whole blood was collected via cardiac puncture, followed by 14,000 g for 10 min centrifugation at 4°C to isolate the plasma. The brain was also collected. All samples were kept at -80°C for further analysis.

3.2.6 *Maximal electroshock test (MES test)*

Male in C57BL/6J mice aged 7 – 8 weeks were randomized to a single dose (i.p.) of treatment (vehicle or GAT591 at 100 mg/kg or GAT1102 at 30 or 60 mg/kg, n = 10 per group). Based on the brain T_{max} from our pharmacokinetic study, a tonic hindlimb seizure was induced using a Ugo Basile electroconvulsive therapy unit (Model 57800, Gemonio, Italy). Maximal electroshocks were delivered at 46 mA, 299 Hz, 0.2 s duration with a 0.9 ms rectangular pulse width. These parameters were calibrated to obtain CC₉₇ which produced tonic hindlimb seizure in 97% of animals.^{312, 313} Mice received 0.5% tetracaine on both corneas 5-15 min prior to testing for anesthesia. A drop of saline solution was applied to both eyes before applying

electrodes to ensure adequate electrical conductivity. The presence of a tonic hindlimb extension was scored immediately within 30 second following the shock delivery. Mice who did not exhibit tonic hindlimb extension were scored as protected.

3.2.7 *Electroencephalogram recording and analysis*

The surgical protocol for EEG implantation was published previously.^{123, 314} In brief, *Gabrb^{3+/D120N}* female and male mice aged 10 - 11 weeks received a subcutaneous injection of meloxicam (5 mg/kg) 15 minute before the surgery. They were anaesthetized in an induction chamber with up to 4% isoflurane in oxygen (1 L/min). They were then transferred to stereotactic instrument in the prone position and maintained anesthesia with 1 – 2.5% isoflurane in oxygen (1 L/min) throughout the surgery. Their body temperature (37°C) was maintained by using a heating pad (Homeothermic Monitoring System, Conduct Science, USA) placed under the animals. Ophthalmic ointment (LubriThal eye gel, Pet Chemist Pharmacy, Australia) was applied to the eyes to avoid irritation and a rectal probe was inserted to monitor body temperature. Hair was shaved in the surgical area and was disinfected the skin with 75% ethanol followed by povidone-iodine, three times. A scalpel blade was used to make a cranial incision along the midline between the eyes and the occiput. A cotton swab was used to clear the skull and expose lambda and bregma. A prefabricated headmount (Pinnacle Technology, In., USA) was mounted to the skull using cyanoacrylate glue. Stainless steel screws (0.10” in anterior and 0.12” in posterior) (Pinnacle Technology, In., USA) were then drilled via boreholes to contact the brain surface. Electromyography (EMG) wires were inserted into the neck muscle and sutured in place. Non-absorbable monofilament sutures were used to close the incision. The headmount was then secured and insulated with dental cement. Buprenorphine (0.05 mg/kg) was administered subcutaneously to the mice. They were housed

in a cage of four mice and monitored daily for one week. Meloxicam (5 mg/kg) was administered once daily for 48 hours after surgery. All mice were carefully monitored for one-week post-surgical recovery before any recording.

For the recordings, a mouse was placed into a recording chamber for 30 minute to habituate in a single house. A pre-amplifier was attached to the exposed headmount on the mouse. Mice was then randomized to vehicle or GAT1102 at 60 mg/kg treatment according to a crossover Latin-square design with a one-week washout period. Treatments were administrated (i.p) based on the correspondence brain Tmax with an adjustment to match the peak atypical absence seizure activity in the light-to-dark transition. EEG and EMG signals were recorded by Sirenia Acquisition software (Pinnacle Technology Inc. Version 2.2.2) for 3 hour following treatment administration. Recordings were visually scored for atypical absence seizures, which were characterized by diffuse slow spike wave discharge patterns that were high-voltage and low frequency (3 -7 Hz) episodes (Somnivore software).¹²³

3.3 Results

3.2.1 Pharmacokinetic features of GAT591 and GAT1102 in mouse brain and plasma

We assessed the pharmacokinetic parameters of GAT1102 and GAT591 in brain and plasma from wild-type male mice (n = 3 – 4 in each time point) following a dose of 10 mg/kg i.p. (Table 1). GAT1102 exhibited a rapid absorption in plasma and brain with a Tmax of 15 min and 30 min respectively, while absorption of GAT591 was slightly delayed with a plasma Tmax of 30 min and a brain Tmax of 60 min (Figure 2). We observed that the maximal brain concentration (Cmax) of the two compounds was comparable, while GAT1102 had a higher plasma Cmax than GAT591 (Table 1). Drug exposure measured by area under curve (AUC) indicated a higher concentration in plasma than in the brain for GAT1102. In contrast, GAT591

concentrations were higher in the brain than in plasma (Table 1). GAT591 had an optimal brain-plasma ratio (4.5) whereas GAT1102's brain-plasma ratio was inferior (0.2) (Table 1). Additionally, GAT591 was more persistent in brain and plasma with longer half-lives ($t_{1/2}$) than GAT1102 (Table 1).

Table 1. Pharmacokinetic parameters in plasma and brain samples from wild-type male mice (n = 19 – 20)

	GAT1102		GAT591	
	Plasma	Brain	Plasma	Brain
C_{max} (µg/mL)	11.9 ± 1.3	1.2 ± 0.2 ^a	0.3 ± 0.1	0.9 ± 0.3 ^a
t_{max} (min)	15	30	30	60
t_{1/2} (min)	20.6	38.5	43.2	53.4
AUC (µg* min/mL)	559.5	98.8	21.1	94
brain-plasma ratio	0.2		4.5	

Note: n = 3 – 4 per time point.

^aConverted from measured concentrations (ng/mg brain) assuming density of 1 g/mL

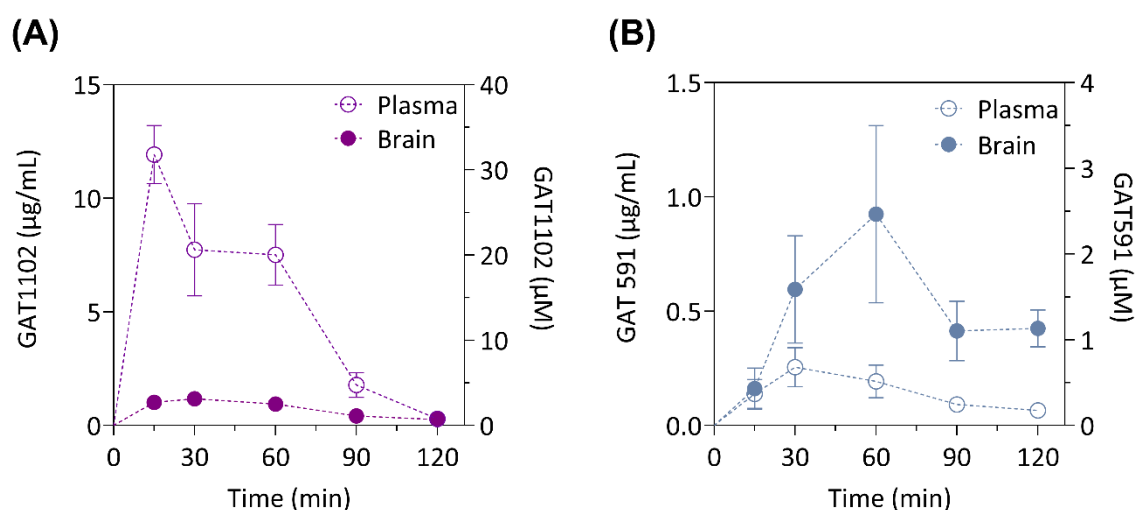


Figure 2. Pharmacokinetic profiles of GAT1102 and GAT591 in the brain and plasma from wild-type male mice. Concentration-time curves are presented for (A) GAT1102 and (B) GAT591 following a 10 mg/kg intraperitoneal (i.p.) injection in wild-type mice. Compounds concentrations are shown as both mass concentrations (left y-axis) and molar concentration (right y-axis). Data are expressed as mean $\pm$ SEM, with n = 3 – 4 per time point.

3.2.2 *GAT1102 but not GAT591 reduce baseline body temperature*

We initially planned to conduct experiments with both GAT1102 and GAT591 at 10, 30 and 100 mg/kg. However, administration of 100 mg/kg of GAT1102 to *Scn1a*^{+/-} mice, revealed anecdotal signs of sedation. We determined that 100 mg/kg was not a suitable dose for further experiments and subsequently selected 60 mg/kg as the highest dose for testing.

To evaluate potential hypothermic effects of the compounds prior to the hyperthermia protocol, we compared baseline body temperature across treatment groups (Figure 3). GAT1102 at 60 mg/kg significantly reduced baseline body temperature compared to the vehicle group ($p < 0.0001$, Figure 3A), whereas GAT591 did not affect body temperature even at doses up to 100 mg/kg (Figure 3B). These findings align with the known CB1 receptor pharmacology, where it is known that CB1 receptor activation decreases body temperature via actions on the hypothalamus.

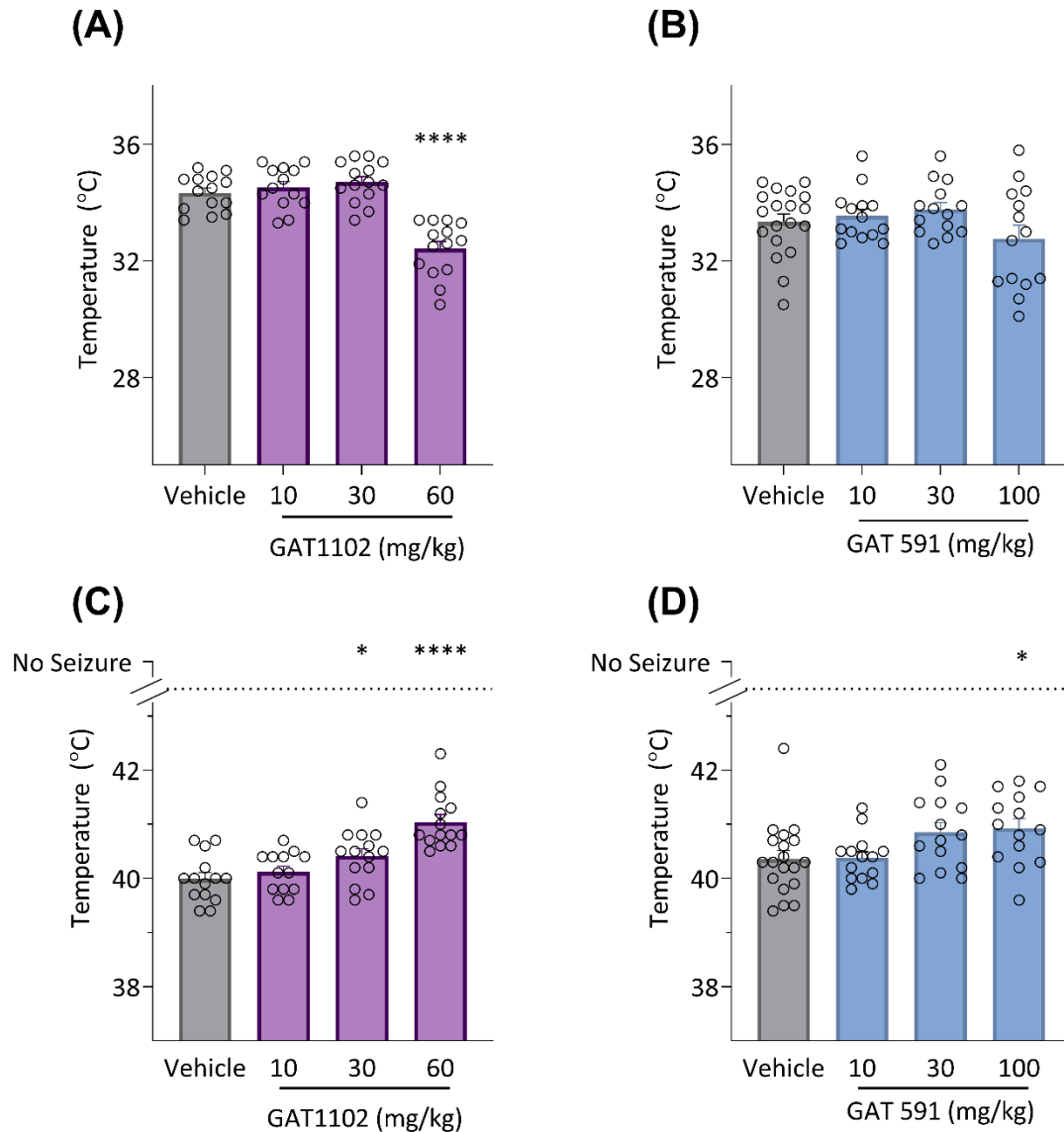


Figure 3. Hypothermic effects of GAT1102 and anticonvulsant effects of GAT1102 and GAT591 against hyperthermia-induced seizures in both male and female *Scn1a*^{+/-} mice. Baseline body temperature prior to the hyperthermia protocol following (A) GAT1102 administration at doses of 10, 30, and 60 mg/kg (*****p* < 0.0001 Dunnett's post-hoc test in one-way ANOVA compared with the vehicle group) and (B) GAT591 at doses of 10, 30, and 100 mg/kg. Samples from both sexes were analyzed, and no sex differences were identified so sexes were collapsed. Temperature threshold for generalized tonic-clonic seizures are shown for (C) GAT1102 at doses of 10, 30, and 60 mg/kg and (D) GAT591 at doses of 10, 30, and 100 mg/kg. The dashed line represents the threshold for no seizures. Data are expressed as mean ± SEM, with *n* = 14 - 19 (**p* < 0.05, *****p* < 0.0001 log-rank Mantel-Cox compared to vehicle control).

3.2.3 Both GAT509 and GAT1102 are anticonvulsant against hyperthermia-induced seizures in *Scn1a*^{+/-} mice

We evaluated the anticonvulsant effects of GAT591 and GAT1102 at their brain T_{max}. Both GAT591 and GAT1102 were anticonvulsant against hyperthermia-seizures in *Scn1a*^{+/-} mice, as the compounds dose-dependently increased the body-temperature threshold at which the mice had a GTCS compared to vehicle although GAT1102 was effective at lower doses (30 mg/kg and 60 mg/kg; *p* = 0.029 and *p* < 0.0001 respectively) than GAT591 (100 mg/kg; *p* = 0.041) (Figure 3C and D). The plasma concentration of GAT591 and GAT1102 following hyperthermia-induced seizures was determined in these experimental mice (Table 2). A positive correlation was observed between the doses and plasma concentrations for both compounds. The effective plasma concentration was 9.4 μM in 100 mg/kg of GAT591, 162.3 μM in 30 mg/kg of GAT1102 and 439.4 μM in 100 mg/kg of GAT1102. Although the effective plasma concentration of GAT591 was lower than that of GAT1102, further pharmacodynamic investigations are needed to fully elucidate their relative efficacy.

Table 2 Plasma concentrations of compounds in experimental *Scn1a*^{+/-} mice in subset of male and female (n = 3 – 4)

Compound	Dose (mg/kg)	Plasma Concentration	
		μg/mL	μM
GAT1102	10	13.1 ± 0.9	36.8
	30	57.7 ± 6.1	162.3
	60	156.2 ± 13.3	439.4
GAT591	10	0.3 ± 0.1	0.8
	30	1.6 ± 0.1	4.1
	100	3.7 ± 1.3	9.4

3.2.4 *GAT1102 but not GAT591 is anticonvulsant in maximal electroshock seizure (MES) test*

To further investigate the anticonvulsant activity against GTCS, we examined the effective dose of GAT591 and GAT1102 in the MES test of GTCS (Figure 4). GAT1102 treatment displayed anti-seizure effects at 60 mg/kg ($p = 0.0195$) by protecting 80% of mice against hind limb extension compared to vehicle which had a 10% protection (Figure 4A). 30 mg/kg GAT1102 showed a 40% protection but did not significantly differ from vehicle. GAT591 at 100 mg/kg displayed no significant protection against hindlimb extension compared to vehicle (Figure 4B).

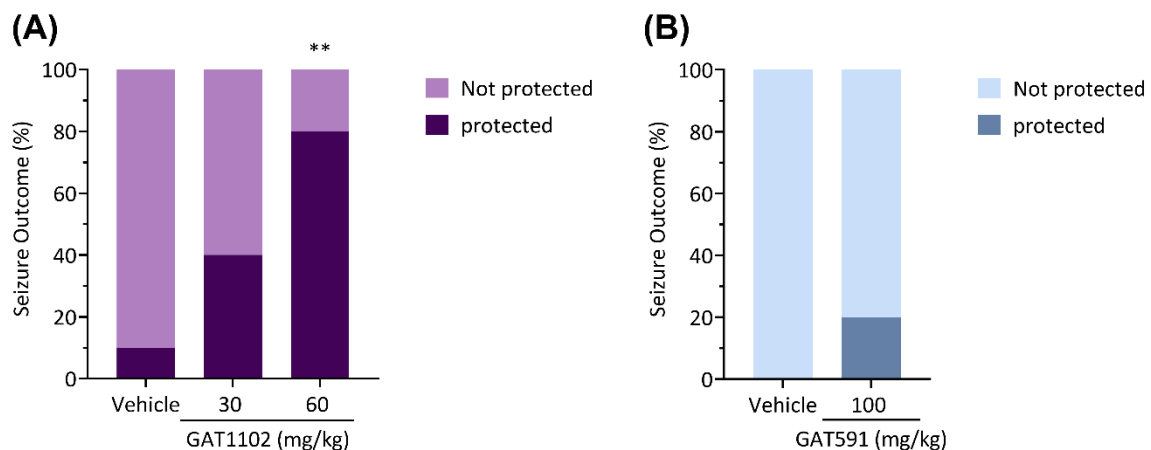


Figure 4. Anticonvulsant seizure of GAT1102 but not GAT591 in the maximal electroshock seizure test. Seizure outcomes are shown for (A) GAT1102 at doses of 30 and 60 mg/kg and (B) GAT591 at a dose of 100 mg/kg, indicating whether these treatments protected mice against hindlimb extension. Data are expressed in percentage, with $n = 10$ per group (* $p = 0.0055$ Fisher exact test compared to vehicle control).

3.2.5 *GAT1102 increased atypical absence seizures in Gabrb3^{+/-D120N} mice*

Given GAT1102 appeared a more robust anti-seizure agent, we then sought to assess whether it affected atypical absence seizures in *Gabrb3^{+/-D120N}* mice at 60 mg/kg (Figure 5A). We

analyzed seizure count and total seizure duration over a 60-minute therapeutic window, corresponding with the peak concentration in the brain (Figure 5B, C). There was no significant overall treatment effect or treatment by time interaction effect on the total seizure count, however planned comparisons indicated that GAT1102 increased the seizure counts at several time points (Figure 5B). GAT1102 did not affect the duration of seizures.

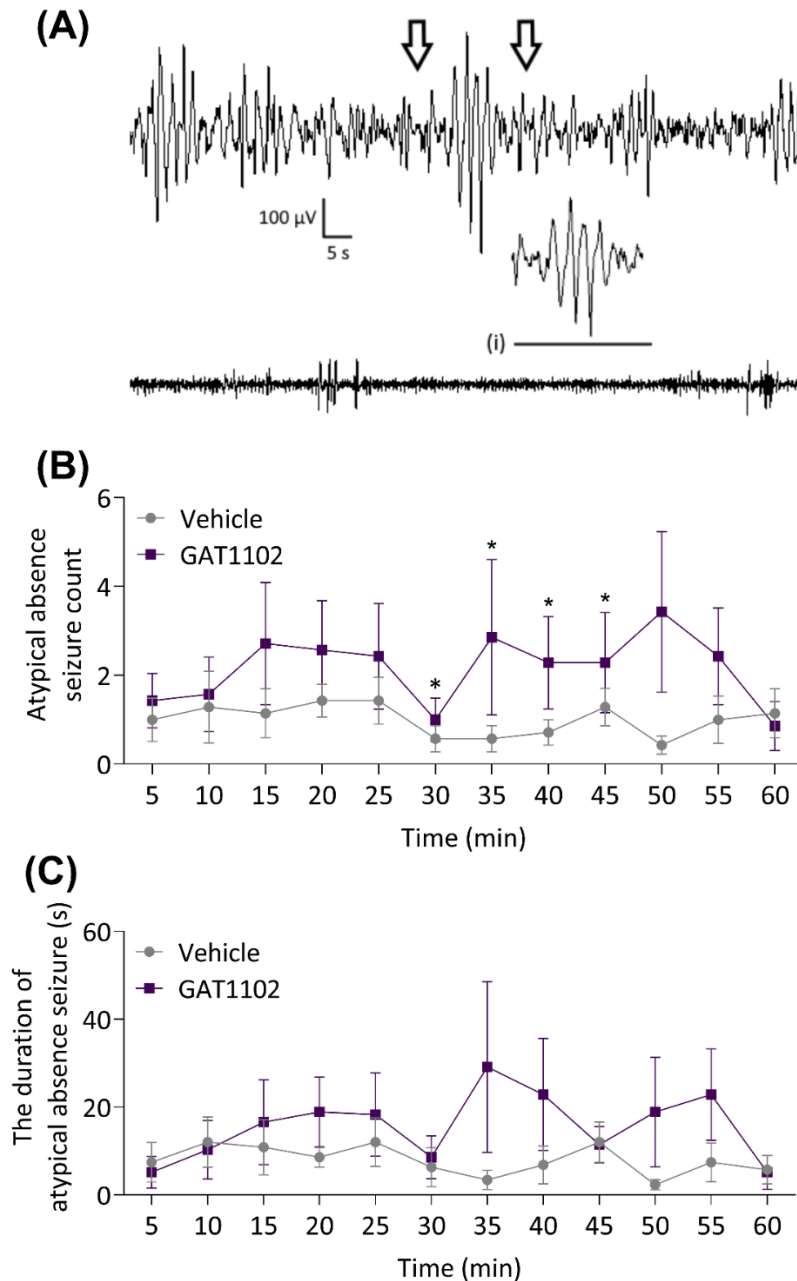


Figure 5. Proconvulsant effects of GAT1102 on atypical absence seizures in *Gabrb3*^{+/D120N} mice (A) Representative electroencephalography (EEG) and electromyography (EMG) traces illustrating (i) atypical absence seizures in adult *Gabrb3*^{+/D120N} mice, with arrows marking the start and end of a seizure. (B) A high dose of GAT1102 increases the number of atypical absence seizures during the 30- to 45-minute recording window. (C) GAT1102 does not alter the duration of atypical absence seizures. Seizures were quantified in 5-minute bins over a 60-minute recording period. Data are presented as mean $\pm$ SEM, with $n = 7$ (* $p < .05$ generalized linear mixed model with Šidák correction test compared to vehicle control).

3.4 Discussion

Dravet syndrome and Lennox-Gastaut syndrome are two forms of intractable epilepsy that are drug-resistant. There is therefore an urgent need to develop better ASMs for intractable epilepsies by leveraging drugs with novel anti-seizure mechanisms. Allosteric modulators of cannabinoid CB1 receptors have recently emerged as novel anti-seizure agents. In this study, we explored the anti-seizure effects of two CB1 receptor ago-PAMs, GAT591 and GAT1102, in mouse models of intractable epilepsies. GAT591 and GAT1102 exhibited anti-seizure effects against hyperthermia-induced GTCS in *Scn1a*^{+/-} mice, with GAT1102 displaying greater potency than GAT591 at a lower effective dose. GAT1102 induced hypothermia at higher doses than that which produced anti-seizure effects, and GAT591 did not produce any measurable hypothermic effect. This indicates that seizure suppression is at least partially independent of thermoregulatory changes. This distinction is consistent with the known CB1 receptor pharmacology, where both neuronal modulation and temperature regulation are separable outcomes.^{190, 315} Additionally, GAT1102 was also effective in protecting mice against MES-induced GTCS, whereas GAT591 did not show similar effects. Despite its robust anti-seizure activities against GTCS, GAT1102 increased the number of atypical absence seizures in the *Gabrb3*^{+/*D120N*} mice. Collectively, these results show that GAT591 and GAT1102 have treatment potential for febrile seizures in Dravet syndrome; however, only GAT1102 was assessed in the Lennox-Gastaut syndrome model, where it did not show efficacy against atypical absence seizures.

We provided brain and plasma pharmacokinetic data on GAT591 and GAT1102 in mice in the current study. GAT591 had a higher brain-plasma ratio than GAT1102, indicating better brain penetration. However, the brain concentration and AUC values of both compounds were comparable, exhibiting a similar brain exposure. Structurally, the carboxylic group in GAT1102 may limit brain uptake, which is similar to the limitation observed in other drugs including

phytocannabinoids such as CBDA and CBGA.²²¹ Improving brain uptake will be essential to optimize the therapeutic effects of GAT1102. The longer brain and plasma half-lives of GAT591 are consistent with its improved metabolic stability *in vitro*.²⁶⁸

Targeting CB1 receptors has been explored for anti-seizure effects in various preclinical models,^{186, 190, 255} however only a handful studies have characterized the anti-seizure potentials of CB1 receptor PAMs or ago-PAMs.^{184, 265, 269} Our group previously evaluated GAT229, another suggested CB1 receptor PAM, to have anti-seizure effects on hyperthermia-induced seizures in *Scn1l*^{+/-} mice. Here GAT1102 displayed similar potency to GAT229, being effect as low as a 30 mg/kg dose.¹⁸⁴ GAT591 was also effective and had comparable effects to CBD in this model which also has anti-seizure effects against hyperthermia-induced seizures at 100 mg/kg.¹⁹⁰ These results further underscore the potential of CB1 receptor PAMs as an additional therapeutic strategy for the treatment of Dravet syndrome. Previous work has demonstrated that CB1 receptor might be genetically modified in Dravet syndrome because *Scn1a*^{+/-} seizure-susceptible mice with a mixed B6/129 strain have significantly lower CB1 receptor expression than seizure-resistant 129 background strain mice¹⁸⁴. This suggests that reduced CB1 receptor expression increases the susceptibility to seizures. We have also observed that seizures increase several closely-related endocannabinoid molecules and prostaglandin species which are formed from endocannabinoids in *Scn1a*^{+/-} mice.^{272, 316} Further, others have shown that endocannabinoids are rapidly released in response to seizures.²⁷³ This highlights that CB1 receptor PAMs could hold great promise via the toning up of endocannabinoid signaling in the brain in a regional and temporal specific manner, and thus reducing brain hyperexcitability in a more nuanced way than orthosteric agonists. Future research could focus on developing novel compounds targeting CB1 receptor allosteric sites and evaluate their effects across various seizure models.

Here GAT1102 exerted anti-seizure effects against GTCS in both the MES test and *Scn1a*^{+/-} mouse model. However, GAT1102 exhibited proconvulsive effects in the *Gabrb3*^{+/*D120N*} mouse model of Lennox-Gastaut syndrome where it increased atypical absence seizures. GAT591 only displayed anti-seizure effects against hyperthermia-induced GTCS in *Scn1a*^{+/-} mice but not against MES-induced GTCS in mice. Consistent with our findings, a previous study demonstrated anti-seizure effects of GAT591 in the GAERs model of childhood absence epilepsy.²⁶⁹ Given the finding with GAT1102, it would be of interest to assess the effects of GAT591 on atypical absence seizures in *Gabrb3*^{+/*D120N*} mice. It would be anticipated that GAT591 would be effective in this model given its anti-absence seizure activity in the GAERs model and that the CB1 receptor agonist WIN 55-212-2 reduces atypical absence seizures in *Gabrb3*^{+/*D120N*} mice. Confirming GAT591's anti-seizure activity in the *Gabrb3*^{+/*D120N*} mice would then strongly imply off-target activity of GAT1102 that provokes seizures in this specific model. If GAT591 also increases absence seizures it might be due to on-target differential regulation of the endocannabinoid system across the models. It is well-established that one anti-seizure drug does not apply to all epilepsy and seizure types; notably, lamotrigine which is used to treat Lennox-Gastaut syndrome is contraindicated in Dravet syndrome and makes seizures worse. Future studies are then needed to better characterize CB1 receptors and endocannabinoid signaling in these mouse models to better illuminate mechanisms behind the divergent actions of GAT1102. CB1 receptors are expressed on excitatory pyramidal neurons and via retrograde signaling activated CB1 receptors to inhibit glutamate release.^{169, 317-319} However, CB1 receptors are also expressed on GABA interneurons and their activation causing neuronal hyperexcitability.^{192, 320} It is possible then that CB1 receptor expression may be differentially expressed across cell-types in the *Scn1a*^{+/-} and *Gabrb3*^{+/*D120N*} mice to divergently influence GTCS and atypical absence seizures respectively.

The use of CB1 receptor agonists is associated with on-target adverse effects, including psychoactivity and sedation.^{155, 307, 321} For example, CB1 receptor agonists produce phenotypic responses such as catalepsy, hypothermia, and hypolocomotion.³²²⁻³²⁵ In contrast, studies have shown CB1 receptor PAMs and ago-PAMs have the advantage of avoiding these on-target adverse effects.^{265, 309} For example, the CB1 receptor PAM GAT229 did not affect locomotor activity, body temperature or induce catalepsy in mice.²⁶⁵ Further, GAT591 did not produce these effects up to 10 mg/kg in mice. Our study further reinforces a lack of cannabimimetic effects with no effect of GAT591 on body temperature even as high as 100 mg/kg. However, for GAT1102 we observed strong signs of motor suppression at 100 mg/kg which is consistent with CB1 receptor orthosteric agonism and it promoted hypothermia at 60 mg/kg implying that GAT1102 may have orthosteric agonist properties. These findings suggest that while GAT591 may indeed be without any cannabimimetic effects, GAT1102 requires further examination and may actually have orthosteric agonist properties. A limitation of this study is that we did not block the anti-seizure effects with a CB1 receptor antagonist to prove CB1 receptor mediation by the compounds. This might be challenging as CB1 receptor antagonists may have intrinsic pro-seizure activity. Studies using CB1 receptor knockout mice might also be considered, notably, the antinociceptive effect of GAT591 was abolished in CB1 receptor knockout mice, suggesting that its mechanism of action relies on CB1 receptors.²⁶⁸

Another major limitation of this study was that we did not examine the anti-seizure activities under a longer-term dosing regimen for recurrent seizures in these animal models. For longer-term dosing we would usually deliver the drug orally in chow to avoid the stress of repeated IP injections that might influence seizure severity. The predicted low oral bioavailability of these compounds and lack of available test compound in multigram quantities precluded testing via the oral route on spontaneous seizures in the *Scn1a*^{+/-} and *Gabrb3*^{+/*DI20N*} mice. Future studies are needed to structurally modify GAT591 and GAT1102 to improve their oral bioavailability,

and GAT1102 to improve its brain uptake while preserving their efficacy. The benefits of conducting a more detailed appraisal of these compounds in additional seizure models appears premature given some of the conflicting results found here.

3.5 Conclusion

Here we report novel anti-seizure effects of the CB1 receptor ago-PAMs, GAT591 and GAT1102, against hyperthermia-induced seizures in the *Scn1a*^{+/-} mouse model of Dravet syndrome. The anti-seizure effects of GAT1102 extended to the MES test which protected mice against hind limb extension. However, GAT1102 increased the number of atypical absence seizures in the *Gabrb3*^{+/*D120N*} mouse model of Lennox-Gastaut syndrome. Our findings suggest that the scaffolds of GAT1102 and GAT591 could be further advanced in drug discovery programs developing anti-seizure agents for Dravet syndrome, but further medicinal chemistry work is needed to optimize their pharmacodynamic and pharmacokinetic properties.

Chapter 4

A high seizure burden increases brain concentrations of specialized pro-resolving mediators in the *Scn1a*^{+/-} mouse model of Dravet syndrome

4.1 Introduction

Neuroinflammation can be considered a dynamic mechanism that comprises two phases: inflammation and resolution.^{326, 327} In epilepsy, neuroinflammation occurs as a defensive response against the dysfunction of brain cells and neuronal network hyperexcitability,³²⁸ while resolution takes place to stop inflammation and repair brain tissue.^{289, 329} Persisting inflammation appears to reinforce not only seizure recurrence, but also cognitive and behavioral disruptions.³²⁸ It has been hypothesized that recurrent seizures may be driven by persistent inflammation that fails to adequately resolve. Consequently, steroidal and non-steroidal anti-inflammatory drugs have been explored as anti-seizure agents.³³⁰ However, these agents have major liabilities such as immunosuppression, cardiovascular³³¹ and gastrointestinal toxicity.³³²

Identifying specialized pro-resolving mediators that dampen inflammation and enhance host defense may provide the key to a new therapeutic approach.³³³ The resolution of inflammation is not simply the dilution of pro-inflammatory mediators, but an active process involving the liberation of specialized pro-resolving mediators that restore homeostasis.^{329, 333} Specialized pro-resolving mediators are a group of endogenous lipid signaling molecules that include lipoxins, resolvins, protectins, and maresins.²⁹⁶ These molecules are derived from polyunsaturated fatty acid precursors such as arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and initiate the resolution of inflammation and tissue repair.^{289, 331} While resolution therapeutics are at a clinical stage of development for inflammatory conditions including ocular and periodontal inflammation,³³⁴ only a handful of preclinical studies have characterised specialized pro-resolving mediators in epilepsy and seizure-related disorders.^{291-293, 335, 336}

There are several studies that showed the precursor molecules EPA and DHA decreased hippocampal neuronal excitability of mice to convulsant stimuli *in vivo* and *ex vivo*,^{335, 336}

suggesting specialized pro-resolving mediators might be enhanced in response to seizures and epilepsy. However, these studies did not confirm the specific involvement of specialized pro-resolving mediators. Musto et al. (2011) then showed that DHA and protectin D1 (PD1, also known as neuroprotection 1) reduced seizure severity and hippocampal hyperexcitability due to hippocampal kindling-induced epileptogenesis in rats.²⁹² In a follow up paper, the same group applied a lipidomic analysis to measure a broader number of specialized pro-resolving mediators and re-affirmed that DHA and PD1 reduced spontaneous epileptiform activity and seizures in a pilocarpine-induced status epilepticus model in mice.²⁹³ Another important study by Frigerio et al. (2018) showed DHA derived PD1 resolved inflammation and arrested epileptogenesis that was associated with improved cognitive function and reduced spontaneous seizure frequency in an intra-amgydala kainic acid model of status epilepticus.²⁹¹ While these studies collectively highlight a role of specialized pro-resolving mediators in acquired chronic epilepsy, it is unknown whether these mediators are recruited in intractable epilepsies involving genetic mutations.

Dravet syndrome is an infantile-onset intractable epilepsy characterised by febrile and afebrile seizures, developmental impairments, and premature mortality.³³⁷ The underlying cause of this syndrome in 70-80% of patients is *de novo*, loss of function mutations of the *SCN1A* gene that encodes the $\alpha 1$ subunit of the sodium channel (Nav1.1).³³⁸ Heterozygous deletion of *Scn1a* in mice (*Scn1a*^{+/-}) phenocopies key clinical features of Dravet syndrome, including hyperthermia-induced seizures, spontaneous generalized tonic-clonic seizures (GTCS) and a high mortality rate.³³⁹ We recently characterized neuroinflammation in the *Scn1a*^{+/-} mouse model of Dravet syndrome.²⁷² Whilst we could not find any evidence of heterozygous *Scn1a* deletion inducing neuroinflammation per se, we discovered that a high seizure burden led to a specific and marked induction of several prostaglandin species in the hippocampus of *Scn1a*^{+/-} mice.²⁷² Prostaglandins are lipid signaling derivatives of arachidonic acid that are structurally related to

specialized pro-resolving mediators. Given that a high seizure burden in *Scn1a*^{+/-} mice increased prostaglandin markers of neuroinflammation, we decided to address whether the high seizure burden similarly augmented concentrations of several specialized pro-resolving mediators in the hippocampus, a region known to be important to seizure propagation and cognitive impairment

4.2 Methods

4.2.1 Animals

Animal samples were obtained from the University of Sydney (Australia) and Northwestern University (USA). The University of Sydney Animal Ethics Committee approved all the animal care and experimental procedures conducted at the University of Sydney in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Northwestern University Animal Care and Use Committee approved experimental procedures conducted at Northwestern University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In brief, animal holding facilities operated on a 12 h light/dark cycle. *Scn1a*^{+/-} mice from the Jackson Laboratory (stock 37107-JAX; Bar Harbor, USA) were maintained by crossing with 129S6/SvEvTac background strain mice to generate a congenic 129.*Scn1a*^{+/-} line.³¹⁰ Experimental animals were bred by crossing 129.*Scn1a*^{+/-} mice with wild-type C57BL/6J (B6) mice, resulting in F1.*Scn1a*^{+/-} mice or F1 wild type (WT) mice.^{90, 216} The heterozygous *Scn1a* genotype was determined as previously described.^{219, 272} All litters were kept in their home cage and weaned at day P21 with *ad libitum* access to food and water in pathogen-free cages. Analyses of the brain tissue of these mice was recently described.²⁷² Both male and female mice were used in subsequent experiments.

4.2.2 *Hyperthermia-induced seizure priming*

The protocol of priming with a hyperthermia-induced seizure event was previously described.²⁷² Briefly, at P21 *Scn1a*^{+/-} mice were primed under a heat lamp to elevate body temperature at a rate of 0.5°C every 2 min, until the onset of clonic convulsion accompanied by loss of posture, at which point, the mouse was transferred to a 4°C cooling mat to rapidly return the core body temperature to 37°C.

4.2.3 *Spontaneous seizure monitoring*

All mice were video recorded for 24 h continuously at age P23 in observation cages. Recordings were made by an overhead camera with an infrared lens (Samsung SCB5003, Tamron 13F604IRSQ) from 12:00 on P23 to 12:00 on P24. Mice were then euthanized via cervical dislocation at the termination of the video recording.²⁷² Hippocampal brain tissue was dissected and stored at -80°C. Spontaneous GTCS frequency was scored by an observer blind to the experimental conditions.

4.2.4 *Experimental design*

Scn1a^{+/-} mice resemble the key characteristics of Dravet syndrome, including susceptibility to febrile seizures triggered by elevated body temperature and onset of spontaneous seizures around P16.²¹⁶ Inducing a febrile seizure event in these mice increases the proportion of mice developing spontaneous seizures.³⁴⁰ To explore the impact of seizure frequency on the brain concentrations of specialized pro-resolving mediators, a subset of *Scn1a*^{+/-} mice were primed with a single hyperthermia-induced seizure event (primed group), while another subset of *Scn1a*^{+/-} mice remained untreated (naïve group). Along with the WT group, all mice underwent

video monitoring to assess their seizure frequency for 24 h. The mice would be further classified according to whether they had a low seizure burden (<3) or a high seizure burden (≥ 3) over the monitoring period thus yielding the following 3 groups: 1) naïve WT mice; 2) naïve *Scn1a*^{+/-} mice with a low seizure burden (Naïve *Scn1a*^{+/-} LOW) and 3) primed *Scn1a*^{+/-} mice with a high seizure burden (Primed *Scn1a*^{+/-} HIGH). This is the same design used previously in our study which showed a high seizure burden increased hippocampal concentrations of an array of prostaglandin species in *Scn1a*^{+/-} mice.²⁷² Brain samples from these mice were collected on P24 for analysis of specialized pro-resolving mediators and gene expression.

4.2.5 Real-time quantitative PCR analysis

Total RNA was purified from whole hippocampus according to the manufacturer's protocol of the RNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration and purity of RNA was measured using a NanoDrop spectrophotometer (ThermoFisher Scientific, Massachusetts, USA) to determine the absorbance at 260 nm, and the ratio of the absorbance between 260 nm and 280 nm, respectively. Samples with an A260/280 ratio of > 2.0, and A260/230 of > 1.7 were considered pure without contamination. Reverse transcription produced single-stranded complementary DNA (cDNA) from 1 µg of RNA of each sample according to the manufacturer's instructions of the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems™, California, USA).

RT-qPCR was performed with a master mix consisting of a 1:15 dilution of cDNA using Taqman™ Universal Master Mix II, no UNG (Applied Biosystems™, California, USA) and PrimeTime qPCR Primer Assay (Integrated DNA Technologies, Iowa, USA) to quantify the target mRNA expression of interest, including Alox5 (ref. Mm.PT.58.30176779), Alox12 (ref.

Mm.PT.58.28734558), Alox15 (ref. Mm.PT.58.13020726), with TATA-binding protein (Tbp; ref. Mm.PT.39a.22214839) and hydroxymethylbilane synthase (Hmbs; Mm.PT.39a.22214827) as reference genes. The real time signal was determined by a StepOnePlus™ Real-Time PCR system (Applied Biosystems™, California, USA). Each RT-qPCR analysis was run in triplicate with no-RT and no-template negative controls in MicroAmp™ Optical 96-Well Reaction Plates (Applied Biosystems™, California, USA).

Relative gene expression was calculated by normalization to the average expression of the two reference genes, Tbp and Hmbs, using the $2^{-\Delta\Delta C_t}$ method. Normality was assessed by a Shapiro-Wilk normality test. Statistical analysis was performed by one-way ANOVA followed by Holm-Sidak multiple comparisons for normally distributed data or Kruskal-Wallis test followed by Dunn's multiple comparisons test for nonparametric data.

4.2.6 Sample preparation and LC-MS/MS analysis

Brain tissue sample preparation and LC-MS/MS analysis was performed by Ambiotis SAS (France). Hippocampal tissue was homogenized with methanol in Precellys apparatus and CK₁₄ tubes and beads (Bertin Instruments, Montigny-le-Bretonneux, France). Samples were centrifuged at 20,000 g for 20 min at 4 °C. The supernatants were then collected and subjected to solid-phase extraction using Oasis HLB 96-well plate (Waters, Massachusetts, USA). Samples were eluted with 5 mL of methyl formate and evaporated under nitrogen gas. The samples were then dissolved in 30 µL of methanol and stored at -80 °C until LC-MS/MS analysis.

Lipid separation was achieved using a UHPLC ExionLC AD LC system (Sciex, Massachusetts, USA). The instrument was equipped with a kinetex C18 column (California, USA). LC-

MS/MS was performed using a Sciex QTRAP 6500+ MS system (Massachusetts, USA) equipped with an electrospray ionization source operating in negative mode. The instrument parameters were set as described previously.³⁴¹ 5 μ L of each sample was subjected to LC-MS/MS system with a flow rate at 0.35 mL/min.

Normality of LC-MS/MS data was assessed by a Shapiro-Wilk normality test. Data were analyzed using a one-way ANOVA followed by Tukey's multiple comparisons for normally distributed data. A Kruskal-Wallis test followed by Dunn's multiple comparisons test was used for nonparametric data. Statistical comparisons were considered statistically significant with $p < 0.05$.

4.3 Results

4.3.1 *Primed $Scn1a^{+/-}$ mice with a high seizure burden had increased arachidonic acid derived pro-inflammatory mediators but not specialized pro-resolving mediators in the hippocampus*

The concentrations of arachidonic acid-derived inflammatory mediators (leukotrienes) and specialized pro-resolving mediators (Figure 1) were compared across the WT mice, primed $Scn1a^{+/-}$ mice with a high seizure burden (primed $Scn1a^{+/-}$ mice HIGH) and the naïve $Scn1a^{+/-}$ mice with a low seizure burden (naïve $Scn1a^{+/-}$ LOW) (Figure 2).

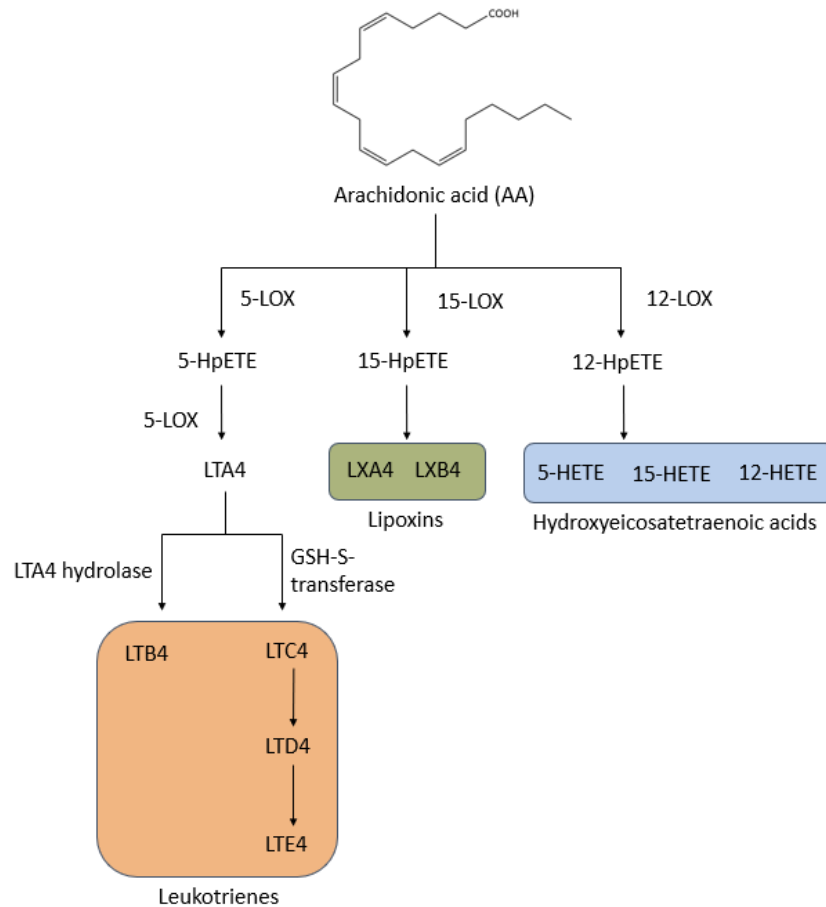


Figure 1. Metabolic pathway of the endogenous biosynthesis of specialized pro-resolving mediators derived from arachidonic acid. Lipoxygenases (5, 12 and 15 LOX) catalyze the conversion of arachidonic acid into hydroperoxyeicosatetraenoic acids (HpETEs) and are further converted to leukotrienes A4 (LTA4) followed by other leukotrienes, lipoxins and hydroxyeicosatetraenoic acids (HETEs).

There were significant differences in hippocampal concentrations of the arachidonic acid-derived leukotrienes, that is, leukotriene B4 (LTB4) ($F(2,15) = 5.8$, $p = 0.0137$, Figure 2A), leukotriene D4 (LTD4) ($H(2) = 7.4$, $p = 0.0181$, Figure 2C) and leukotriene E4 (LTE4) ($H(2) = 10.1$, $p = 0.0022$, Figure 2D). The primed *Scn1a*^{+/-} mice with a high seizure burden had significantly increased hippocampal LTB4 and LTE4 concentrations compared to the WT group ($p = 0.0137$ and $p = 0.0175$, respectively). Primed *Scn1a*^{+/-} mice with a high seizure burden also displayed significantly higher concentrations of LTE4 than naïve *Scn1a*^{+/-} with a

low seizure burden ($p = 0.0175$). There were no differences between groups in the lipoxin or hydroxyeicosatetraenoic acid specialized pro-resolving mediators.

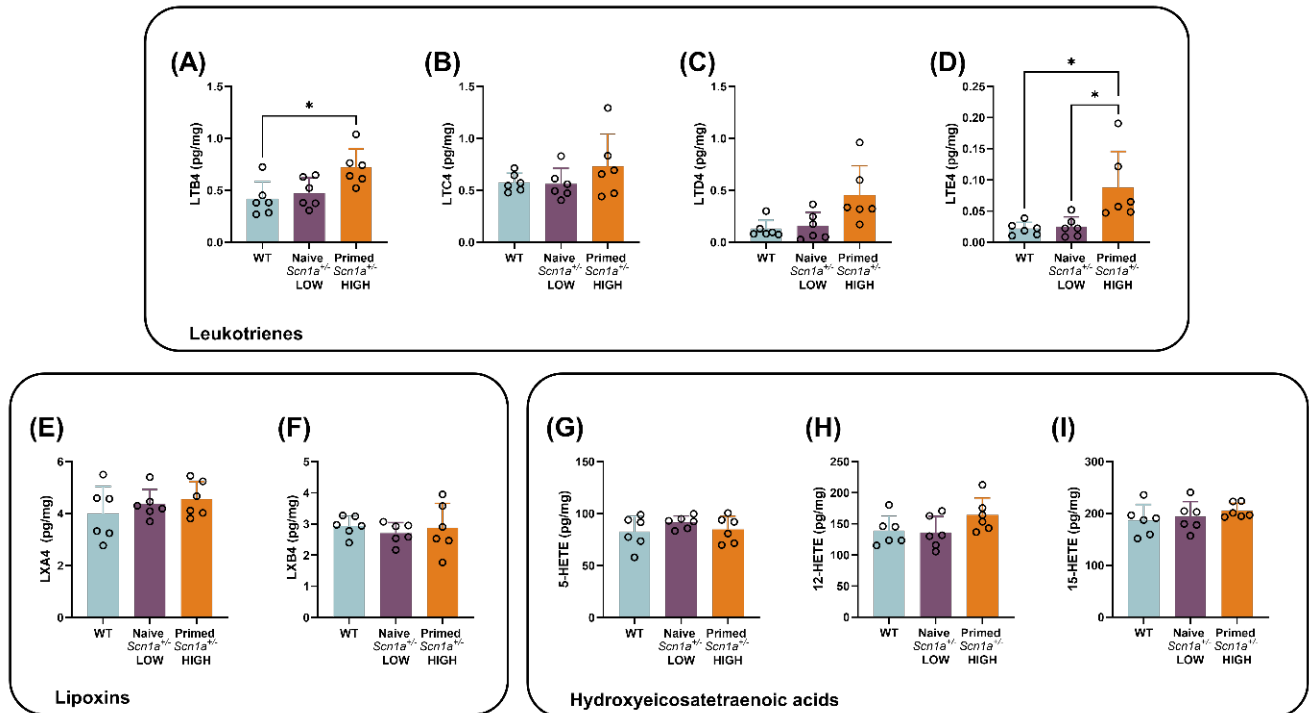


Figure 2. Primed *Scn1a*^{+/-} mice with a high seizure burden display increased arachidonic acid derived pro-inflammatory leukotrienes but not the specialized pro-resolving mediators lipoxin or hydroxyeicosatetraenoic acids (HETEs) in the hippocampus. Hippocampal concentrations of leukotrienes (LTs), lipoxins (LXs) and HETEs in naïve *Scn1a*^{+/-} with a LOW seizure burden, primed *Scn1a*^{+/-} with a HIGH seizure burden and wild-type (WT) mice for (A) LTB₄, (B) LTC₄, (C) LTD₄, (D) LTE₄, (E) LXA₄, (F) LXB₄, (G) 5-HETE, (H) 12-HETE and (I) 15-HETE. Data is presented as mean ± SEM (n= 6 biological replicates per group). * $p < 0.05$ (Tukey's test for LTB₄ or Dunn's post-hoc tests for LTE₄).

4.3.2 *Primed Scn1a*^{+/-} mice with a high seizure burden had increased docosahexaenoic acid (DHA) derived specialized pro-resolving mediators in the hippocampus

The concentration of DHA-derived specialized pro-resolving mediators (Figure 3) was compared (Figure 4 and Figure 5). We observed changes in mediators in the

hydroxydocosahexaenoic acid, maresin, D-series resolvins, protectin but not maresin conjugate classes.

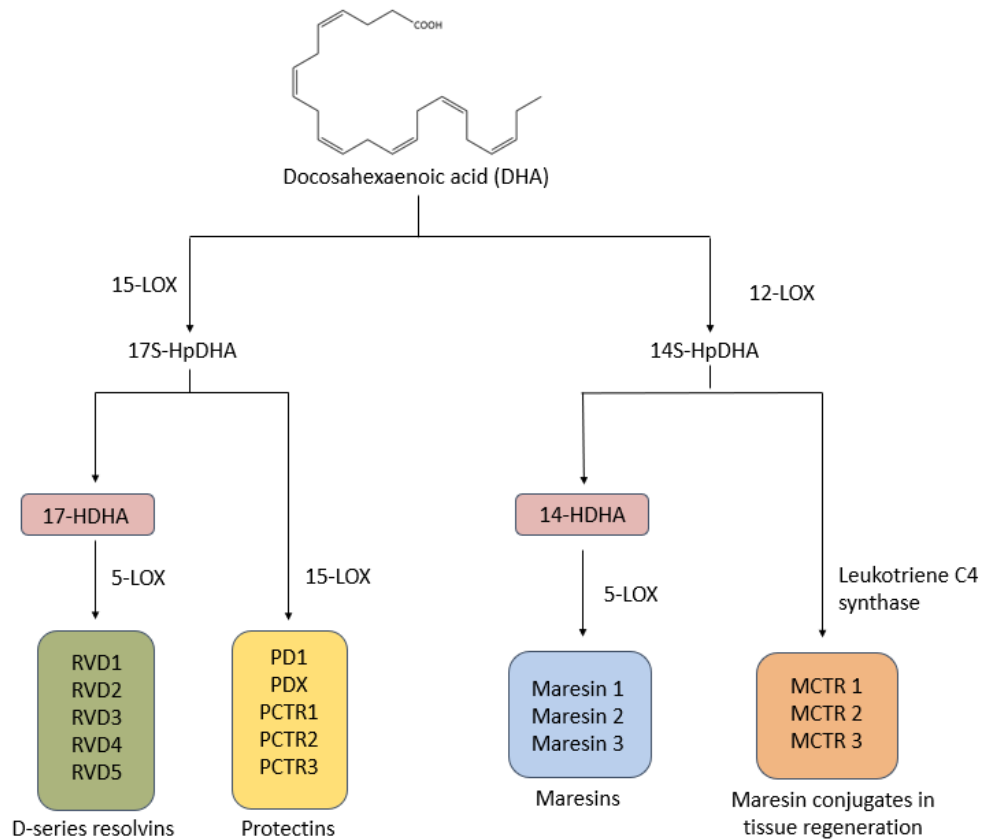


Figure 3. Metabolic pathway of the endogenous biosynthesis of specialized pro-resolving mediators derived from docosahexanoic acid (DHA). DHA is a precursor to hydroperoxy docosahexanoic acids (HpDHAs) and hydroxydocosahexanoic acids (HDHAs) which is catalyzed by lipoxygenases (LOXs). HpDHAs are converted to maresins, maresin conjugates in tissue regeneration, D-series resolvins and protectins.

For the maresins, there was only differences across treatment groups in maresin 1 ($F(2,15) = 15.8$, $p = 0.0002$, Figure 4B). The primed *Scn1a*^{+/-} mice with a high seizure burden had a higher hippocampal maresin 1 concentration than WT and naive *Scn1a*^{+/-} mice with a low seizure burden ($p = 0.0003$ and $p = 0.0015$, respectively).

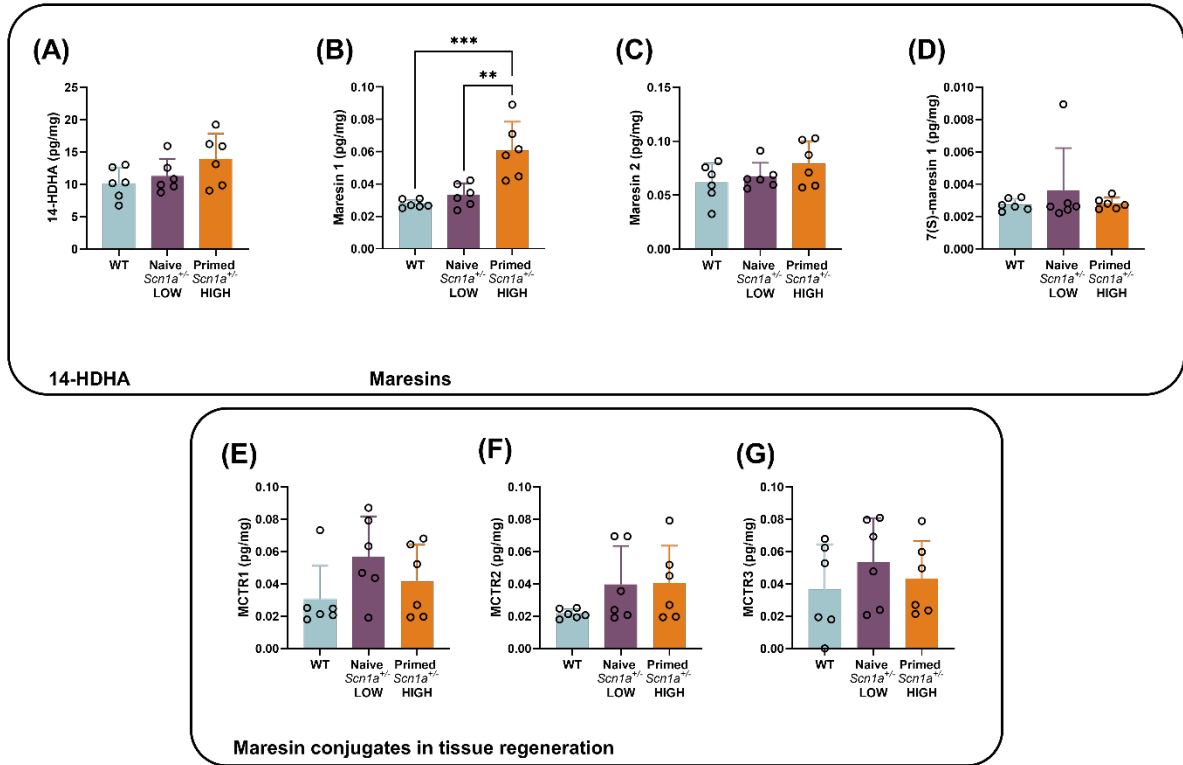


Figure 4. Primed *Scn1a*^{+/-} mice with a high seizure burden display increased docosahexaenoic acid (DHA)-derived maresins, but not hydroxydocosahexaenoic acid 14 (14-HDHA) or maresin conjugates in tissue regeneration (MCTR) specialized proresolving mediators in the hippocampus. Hippocampal concentrations of 14-HDHA, maresins and MCTR in naïve *Scn1a*^{+/-} with a LOW seizure burden, primed *Scn1a*^{+/-} with a HIGH seizure burden and wild-type (WT) mice. (A) 14-HDHA, (B) maresin 1 (**p = 0.0015 and ***p = 0.0003; Tukey's post-hoc tests), (C) maresin 2, (D) 7(S)- maresin 1, (E) MCTR1, (F) MCTR2 and (G)MCTR3. Data is presented as mean ± SEM (n= 6 biological replicates per group).

Concentrations of the hydroxydocosahexaenoic acid mediator 17-HDHA were significantly different across the three groups (F (2,15) = 6.1, p = 0.0117, Figure 5A). The primed *Scn1a*^{+/-} mice with a high seizure burden displayed significantly greater hippocampal 17-HDHA concentrations than WT mice (p = 0.0134) and the naïve *Scn1a*^{+/-} mice with a low seizure burden (p = 0.0432). For D-series resolvins, we found that there were significant differences across groups for RVD1 (F (2,15) = 6.7, p = 0.0082, Figure 5B) and RVD4 (F (2,15) = 10.8, p

= 0.012, Figure 5E). Primed *Scn1a*^{+/-} mice with a high seizure burden, had higher hippocampal concentrations of RVD1 and RVD1 when compared to WT mice ($p = 0.0082$ and $p = 0.0018$ respectively) and naive *Scn1a*^{+/-} mice with a low seizure burden ($p = 0.0434$ and $p = 0.0054$ respectively).

For the protectins, both PCTR1 and PD1 had a significantly different hippocampal concentrations across the experimental groups ($F(2,15) = 40$, $p < 0.0001$, Figure 5G; and $F(2,15) = 3.7$, $p = 0.048$, Figure 5J, respectively). Primed *Scn1a*^{+/-} mice with a high seizure burden had higher hippocampal concentrations of PCTR1 than WT mice ($p = 0.0436$) and the naive *Scn1a*^{+/-} mice with a low seizure burden ($p = 0.0324$). However, post-hoc analysis showed no significant differences between specific groups in hippocampal PD1 concentrations. There were no differences in hippocampal concentrations of 14-HDHA, maresin 2, 7(S)-maresin 1, RVD2, RVD3, RVD5 and all maresin conjugate species.

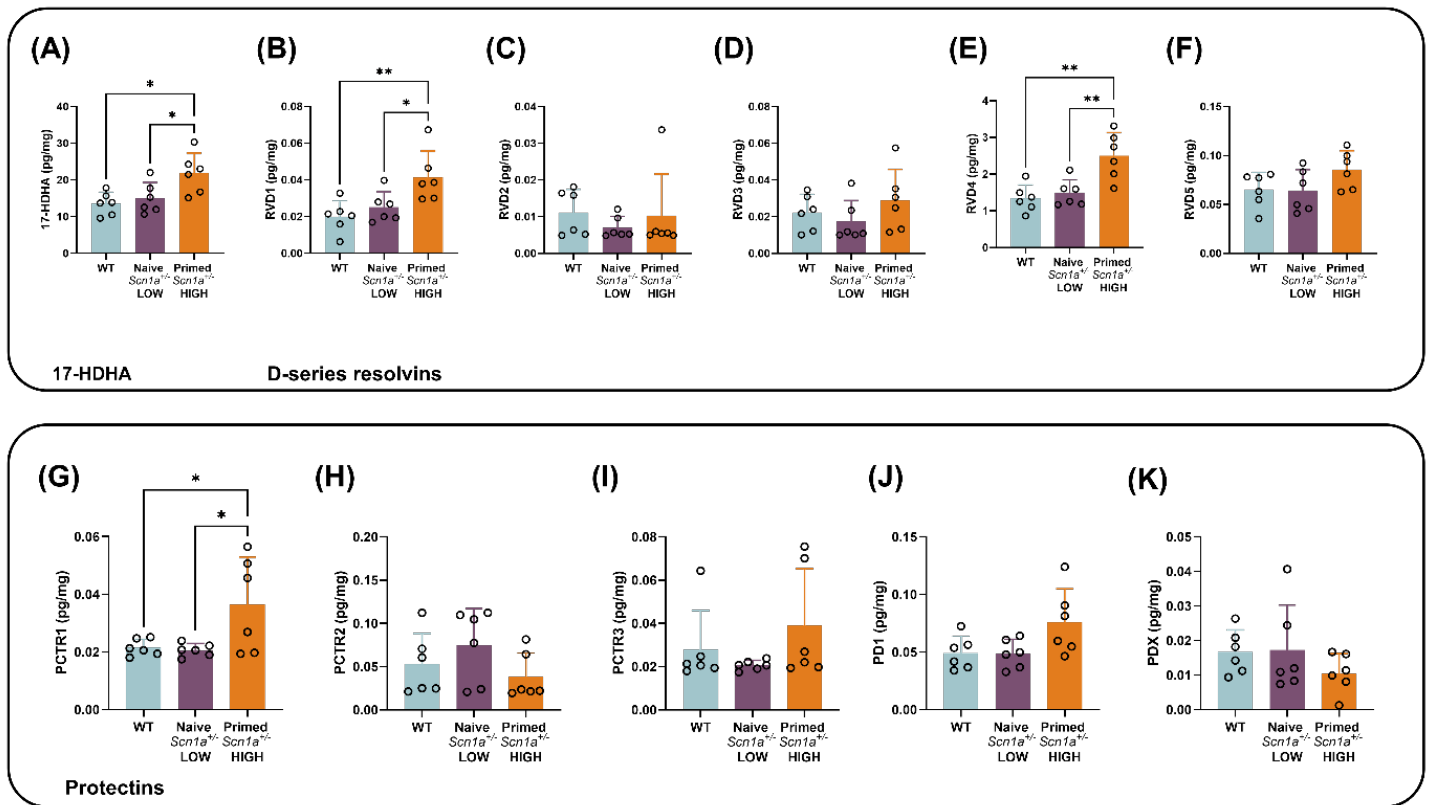


Figure 5. Primed *Scn1a*^{+/-} mice with a high seizure burden display increased docosahexaenoic acid (DHA)-derived hydroxydocosahexaenoic acid 17 (17-HDHA), DHA-derived D-series resolvins (RVDs) and protectins in the hippocampus. Hippocampal concentrations of 17-HDHA, RVDs and protectins (PCTR1-3, PD1 & PDX) in naïve *Scn1a*^{+/-} with a LOW seizure burden, primed *Scn1a*^{+/-} with a HIGH seizure burden and wild-type (WT) mice. (A) 17-HDHA (**p* < 0.005; Tukey's post-hoc tests), (B) RVD1 (**p* = 0.0434 and ***p* = 0.0082; Tukey's post-hoc tests), (C) RVD2, (D) RVD3, (E) RVD4 (***p* < 0.01; Tukey's post-hoc tests), (F) RVD5, (G) PCTR1 (**p* < 0.05; Tukey's post-hoc tests), (H) PCTR2, (I) PCTR3, (J) PD1 and (K) PDX. Data is presented as mean ± SEM (n= 6 biological replicates per group).

4.3.3 Primed *Scn1a*^{+/-} mice with a high seizure burden showed effects on eicosapentaenoic acid (EPA) derived specialized pro-resolving mediators in the hippocampus

The concentrations of eicosapentaenoic acid-derived specialized pro-resolving mediators (Figure 6) were compared (Figure 7). We observed changes in mediators in the hydroxyeicosapentaenoic acid, leukotriene but not E-series resolvins classes.

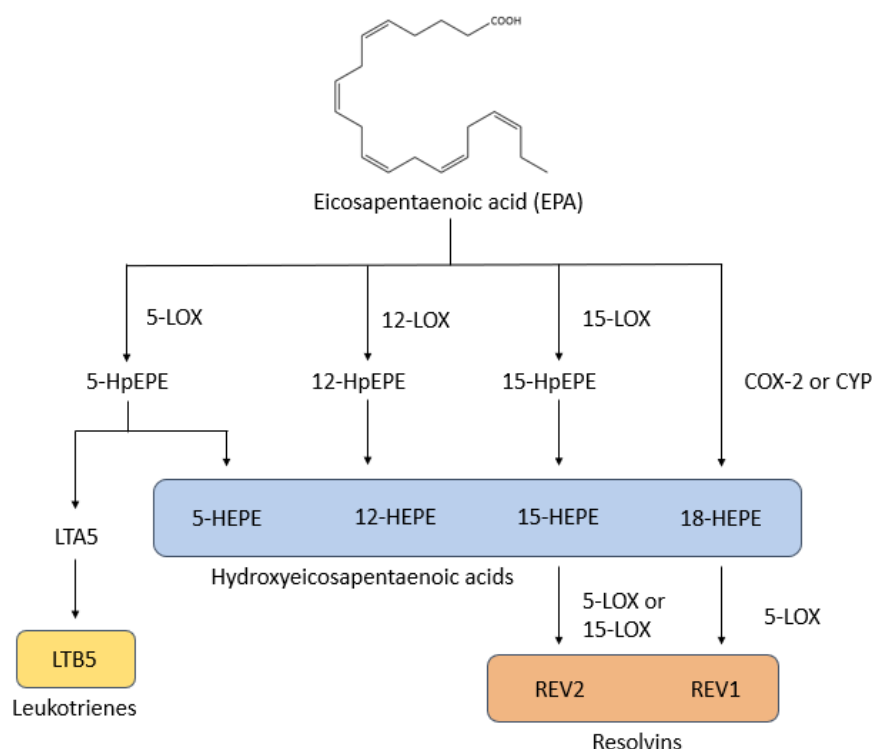


Figure 6. Metabolic pathway of the endogenous biosynthesis of specialized pro-resolving mediators derived from eicosapentaenoic acid (EPA). LOXs and cytochromes P450 (CYP450) convert eicosapentaenoic acid (EPA) into hydroperoxyeicosapentaenoic acids (HpEPEs) and are further converted to hydroxyeicosapentaenoic acids (HEPEs), leukotrienes A₄ and E-series resolvins.

For the hydroxyeicosapentaenoic acids (HEPEs), all species measured displayed statistical differences across groups; that is, 5-HEPE ($F(2,15) = 11.1$, $p = 0.0011$, Figure 7A), 12-HEPE ($F(2,15) = 19.7$, $p < 0.0001$, Figure 7B), 15-HEPE ($F(2,15) = 13.7$, $p = 0.0004$, Figure 7C) and 18-HEPE ($F(2,15) = 14.6$, $p = 0.0003$, Figure 7D). Primed *Scn1a*^{+/-} mice with a high seizure burden had significantly higher hippocampal concentrations of 5-HEPE, 12-HEPE, 15-HEPE and 18-HEPE than both WT mice ($p = 0.0011$; $p = 0.0001$; $p = 0.0003$; and $p = 0.0003$ respectively) and naive *Scn1a*^{+/-} mice with a low seizure burden ($p = 0.0107$; $p = 0.0004$; $p = 0.0102$; and $p = 0.0034$ respectively).

There were also group differences in hippocampal concentrations of leukotrienes B5 (LTB5) (F (2,15) = 5.9, p = 0.0132, Figure 7E). Primed *Scn1a*^{+/-} mice with a high seizure burden displayed lower hippocampal LTB5 concentrations compared to both WT mice and naive *Scn1a*^{+/-} mice with a low seizure burden (p = 0.0237 and p = 0.0257, respectively).

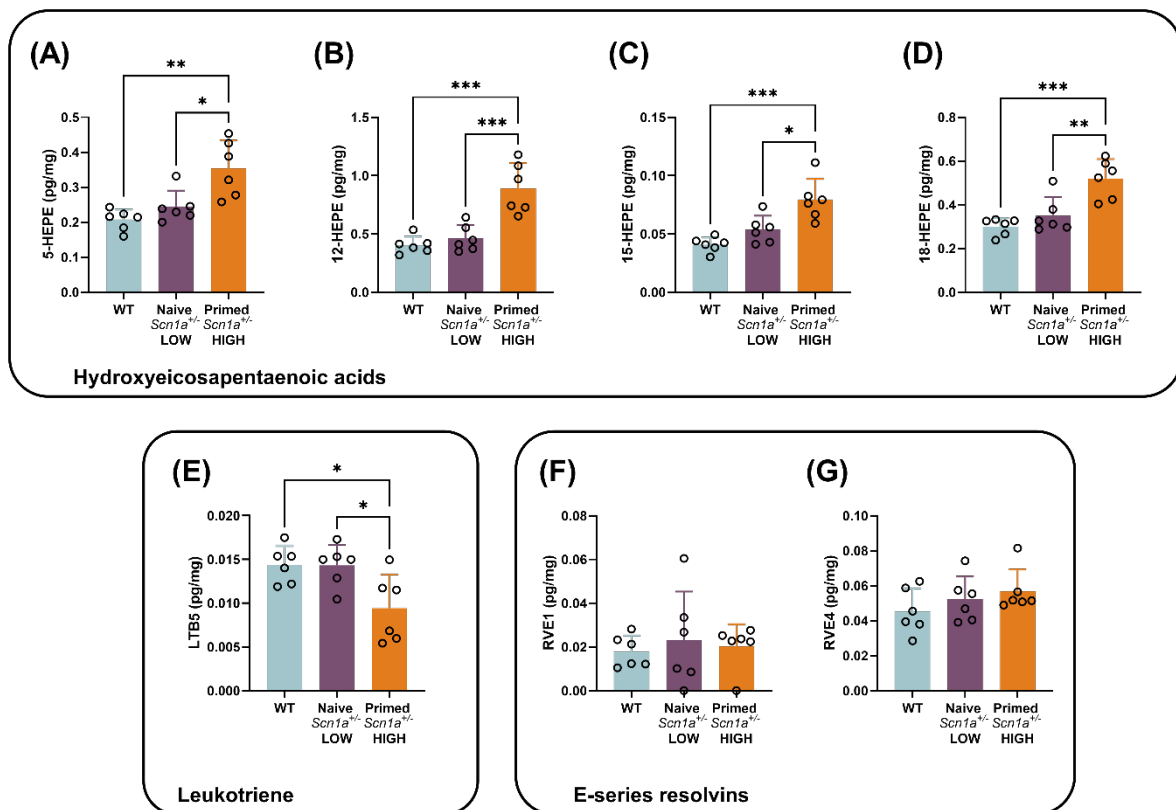


Figure 7. Primed *Scn1a*^{+/-} mice with a high seizure burden display both increased eicosapentaenoic acid (EPA)-derived hydroxyeicosapentaenoic acids (HEPEs) and decreased leukotriene B5 (LTB5) but not EPA-derived E-series resolvins (RVEs) specialized pro-resolving mediators in the hippocampus. Hippocampal concentrations of HEPEs, LTB5 and RVEs in naïve *Scn1a*^{+/-} with a LOW seizure burden, primed *Scn1a*^{+/-} with a HIGH seizure burden and wild-type (WT) mice for (A) 5-HEPE (*p = 0.0107 and **p = 0.0011; Tukey's post-hoc tests), (B) 12-HEPE (***p < 0.0005; Tukey's post-hoc tests), (C) 15-HEPE (*p = 0.0102 and ***p = 0.0003; Tukey's post-hoc tests), (D) 18-HEPE (**p = 0.0034 and ***p = 0.0003; Tukey's post-hoc tests), (E) LTB5 (*p < 0.05; Tukey's post-hoc tests), (F) RVE1 and (G) RVE4. Data is presented as mean ± SEM (n= 6 biological replicates per group).

4.3.4 No group effects on lipoxygenase mRNA expression in the hippocampus

We then sought to determine whether high seizure burden impacted mRNA expression of various lipoxygenases in the hippocampus, as these synthetic enzymes are common across classes of specialized pro-resolving mediators (Figure 8). However, there was no group differences in mRNA expression of Alox5, Alox12 and Alox15.

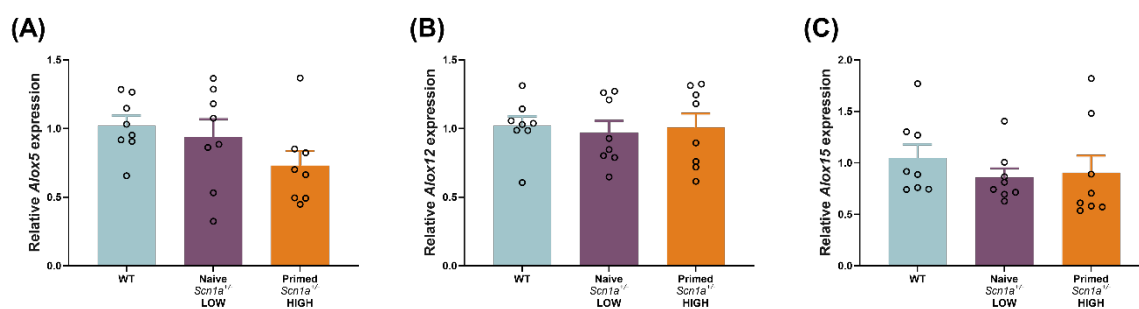


Figure 8. Lipoxygenase gene expression in the hippocampus does not differ between the experimental groups. Relative mRNA expression of (A) *Alox5*, (B) *Alox12* and (C) *Alox15* (n = 8 biological replicates per group) in the hippocampus of wild-type (WT), naïve *Scn1a*^{+/-} with a LOW seizure burden, and primed *Scn1a*^{+/-} with a HIGH seizure burden. Data are presented as mean ± SEM.

4.4 Discussion

Here we report for the first time that an array of specialized pro-resolving mediators is augmented in hippocampal tissue of *Scn1a*^{+/-} mice with a high seizure burden. Primed *Scn1a*^{+/-} mice with a high seizure burden displayed higher concentrations of the hydroxydocosahexaenoic acid mediator 17-HDHA, maresin 1, the D-series resolvins (RVD1 and RVD4) and the protectin PCTR1 compared to controls. We also observed an increase of all hydroxyeicosapentaenoic acid species (5-HEPE, 12-HEPE, 15-HEPE and 18-HEPE).

We recently identified that a high seizure burden in *Scn1a*^{+/-} mice promoted neuroinflammation in the hippocampus as highlighted by increased COX-2 expression and concentrations of arachidonic acid-derived pro-inflammatory prostaglandin mediators.²⁷² This appeared quite specific to prostaglandins, as there was no evidence of increased mRNA expression of other cytokines such as IL-1 β and TNF α . Here we extend on our prior findings by showing that a high seizure burden in *Scn1a*^{+/-} mice also increased hippocampal concentrations of the pro-inflammatory arachidonic acid-derived leukotrienes LTB₄ and LTE₄, but not LTC₄ and LTD₄, although LTC₄ and LTD₄ did show mean increases and could be biologically relevant; the small sample size may have precluded observing statistical differences for these mediators. LTB₄ is a non-cysteinyl leukotriene (it does not contain the cysteine amino acid group), whereas LTE₄ is a cysteinyl leukotriene. Most studies thus far have identified an association between cysteinyl LTC₄ and LTD₄ and seizures. For example, kainic-acid induced seizures in rats increased concentrations of LTC₄ in the cortex, hippocampus and hypothalamus.³⁴² Further, in a PTZ-induced seizure model in mice, intracerebroventricular injection of LTD₄ increased seizure susceptibility, suggesting a pathological role of this molecule in seizure generation.³⁴³ Interestingly, recent studies found that montelukast, a cysteinyl leukotriene receptor antagonist, reduced recurrent seizures and prevented blood brain barrier disruption in various rodent epilepsy models.^{343, 344} Future studies are required to further understand the functional implications of the increase in LTB₄ and LTE₄ observed following a high seizure burden in the context of Dravet syndrome.

Here we observed that a high seizure burden increased hippocampal concentrations of 17-HDHA in *Scn1a*^{+/-} mice. This is the first demonstration that this mediator is augmented in the brain in response to seizures. Recent research has examined whether 17-HDHA administration resolves inflammation in preclinical models. In a mouse model of colitis, systemic 17-HDHA treatment decreased the expression of inflammatory mediators including IL-1 β and TNF- α and

reduced colon damage through the regulation of the NF- κ B pathway.³⁴⁵ Further, 17-HDHA treatment reduced nociception in a monosodium iodoacetate model of osteoarthritis pain,³⁴⁶ which correlated with reduced astrocyte reactivity in their dorsal horn of the spinal cord.³⁴⁶ No study has yet characterised the effect of 17-HDHA treatment in preclinical seizure and epilepsy models. Given the results here, a future study could examine whether 17-HDHA treatment facilitates the resolution of neuroinflammation to reduce seizure phenotypes in the *Scn1a*^{+/-} mouse model of Dravet syndrome.

Formed from DHA, 17-HDHA is a precursor to the D-series resolvins,³⁴⁷ two of which were also increased in this study (RVD1 and RVD4), thus highlighting an upregulation of a DHA/17-HDHA/D-series resolvins pathway. Our results differ from Frigerio et al. (2018) who found no change in hippocampal RVD1 concentrations and decreased RVD4 concentrations in response to intra-amygdala kainic-acid induced status epilepticus. The resolvins are classified into three structurally similar subtypes: DHA-derived D-series, docosapentaenoic acid (DPA)-derived D-series, and EPA-derived E series resolvins.³⁴⁷⁻³⁴⁹ Consistent with Frigerio et al. (2018), EPA-derived E-series resolvins were also unaffected in our study. DPA-derived resolvins were not measured in the present study. Interestingly, epileptic foci of focal cortical dysplasia type IIb and tuberous sclerosis complex patients had lower concentrations of RVD1 and its target receptor FPR2, that negatively correlated with seizure frequency.³⁵⁰ This suggests that reduced RVD1-FPR2 signaling might increase seizure severity. Future studies are needed to confirm whether the manipulation of the D-series resolvins protects against seizures in preclinical epilepsy models. Studies in preclinical models of inflammatory pain and thrombosis showed these mediators have analgesic and anti-thrombotic effects^{351, 352} that are due to their pro-resolving activity in reducing inflammation and neutrophil recruitment.

Our study reports for the first time that the DHA-derived protectin PCTR1 was increased in the hippocampus of primed *Scn1a*^{+/-} mice with a high seizure burden. Notably, treatment with

PCTR1 promoted tissue regeneration and healing following ischemia-induced injury in mice.³⁵³ Removal of the vagus nerve and acetylcholine signaling reduced peritoneal concentrations of PCTR1 that was associated with less resolution of inflammation and increased bacterial loads following *E. coli* infection.³⁵⁴ This provides evidence that specialized pro-resolving mediators are regulated by cholinergic neurotransmission, thus future studies could explore whether additional neurotransmitter systems influence these mediators in brain diseases such as epilepsy. While the role of the protectin PCTR1 in epilepsy remains unexplored, the DHA and DPA-derived protectins have been identified in preclinical epilepsy models. Electrical kindling-induced seizures in rats increased DHA-derived protectin D1 concentrations in the hippocampus,²⁹² and intra-amygdala kainic acid-induced seizures increased DPA-derived protectin D1 concentrations in mouse hippocampus.²⁹¹ We did not measure DPA-derived specialized pro-resolving mediators, which could be examined in future studies in *Scn1a*^{+/-} mice. In other models of epilepsy, treatment with DHA-derived protectin D1 had an anti-seizure effect by arresting epileptogenesis.²⁹¹⁻²⁹³ Further, DPA-derived protectin D1 enhanced inhibitory drive in pyramidal neurons via actions on perisomatic synapses.³⁰⁰ Future studies could examine whether the administration of various protectins ameliorate seizures in *Scn1a*^{+/-} mice.

Here we report the novel observation that *Scn1a*^{+/-} mice with a high seizure burden displayed increased brain hippocampal concentrations of maresin 1. This differed from Frigerio et al. who reported no change in hippocampal concentrations of maresin 1 post-status epilepticus.²⁹¹ Maresin 1 has well-established pro-resolving activity in preclinical models of chronic inflammation.³⁵⁵⁻³⁵⁷ Sánchez-Fernández et al. (2022) showed maresin 1 is important to resolve neuroinflammation in a mouse experimental autoimmune encephalomyelitis (EAE) model. They showed that maresin 1 treatment in EAE mice suppressed pro-inflammatory cytokines and protected against neurological impairments including demyelination.³⁵⁶ In another study,

maresin 1 treatment promoted axon regeneration and functional recovery in a mouse model of sciatic crush injury.³⁵⁷ These studies collectively demonstrate neuroprotective effects^{356, 357} of maresin 1. Our results here suggest future studies could examine whether maresin 1 exposure decreases seizures and behavioural impairments in *Scn1a*^{+/-} mice.

Another novel finding of the present study is that hippocampal concentrations of the hydroxyeicosapentaenoic acids (5, 12, 15 and 18-HEPE) were all elevated in *Scn1a*^{+/-} mice with a high seizure burden. Hydroxyeicosapentaenoic acids are the critical metabolites of EPA, which are precursors to leukotrienes (LTA5 and LTB5) and the E-series resolvins (REV1 and REV2). Notably, whilst the hydroxyeicosapentaenoic acids were elevated, rather than observing a corresponding increase in concentrations of the downstream mediators, we observed a decrease in the hippocampal concentration of LTB5, and there was no change in REV1 and REV2. One limitation of this study is that we only measured the mediators at one experimental time-point, thus it is possible that increased concentrations of the downstream mediators might be observed at a latter sampling time. There is accumulating evidence that EPA-supplementation has anti-inflammatory and antidepressant effects via increasing hydroxyeicosapentaenoic acids.^{358, 359} For example, 5-HEPE and 18-HEPE treatment decreased the ability of inflammatory cytokines to reduce neurogenesis and induce apoptosis in cultured human hippocampal neuron progenitor cells.³⁵⁸ Our results highlight the need for more research on the hydroxyeicosapentaenoic acids in epilepsy and seizure-related disorders.

One limitation of this research is that we cannot differentiate between the influence of priming alone from the impact of the high seizure burden alone on the brain concentrations of the specialized pro-resolving mediators. However, to the best of our knowledge, there is no evidence that increased body temperature alone increases these mediators. Further, in our previous work we only observed an increase in cyclooxygenase 2 (COX2) gene expression in the primed group with a high seizure burden, not in the primed group with a low seizure

burden.²⁷² Moreover, the *Scn1a*^{+/-} mice that were not primed but had a high-seizure burden similarly displayed an increase in COX2 gene expression compared to WT mice. This suggests that the neuroinflammation was specific to the high seizure burden. It then follows that the specialized pro-resolving mediators would also be influenced by the high-seizure burden, as these mediators accumulate as a consequence of inflammation. Finally, the closer proximity of the brain sampling to the behavioral seizures rather than the priming event, which occurred 24 hours earlier, further argues for the high seizure burden being the most important factor in triggering the elevated hippocampal concentrations of the specialized pro-resolving mediators.

4.5 Conclusion

Our results show that a high seizure burden in a mouse model of Dravet syndrome increased concentrations of various specialized pro-resolving mediators in the hippocampus. Our results provide a foundation for future research that aims to further explore the biology of these mediators in seizure control and brain repair mechanisms. These mediators and their cognate receptors might then be exploited in the development of novel treatments for Dravet syndrome based on a novel approach that seeks to resolve neuroinflammation and enhance brain repair.

Chapter 5

General Discussion

5.1 Summary of findings

The original research presented in this thesis examined novel candidate molecules for anti-seizure effects and characterized novel lipid signaling pathways that respond to seizures in preclinical models of intractable epilepsies. The investigation into cannabinoid-related compounds for intractable epilepsies was inspired by the promising effects of CBD, which is approved as adjunctive treatment for Dravet syndrome and Lennox-Gastaut syndrome. The first two studies presented in Chapter 2 and 3 presented the novel anti-seizure effects of these cannabinoid-related compounds with different therapeutic rationales. Additionally, the final study presented in Chapter 4 reported the impact of seizure frequency on the regulation in hippocampal concentrations of specialized pro-resolving mediators, novel mediators that are involved in the resolution of neuroinflammation and brain repair.

Chapter 2 examined the anti-seizure effects of three non-cannabis derived natural compounds. The study revealed key findings that: (1) magnolol and amorfrutin 2 demonstrated anticonvulsant effects against hyperthermia-induced GTSC in a *Scn1a*^{+/-} mouse model of Dravet syndrome with amorfrutin 2 showing a greater potency than magnolol; (2) in the MES test, magnolol exhibited more robust anti-seizure effects against electroshock-induced GTCS; (3) magnolol reduced the number of atypical absence seizures and their duration in the *Gabrb3*^{+/*D120N*} mouse model of Lennox-Gastaut syndrome; and (4) all compounds inhibited T-type calcium channel subtypes (Cav1.1, Cav1.2 and Cav1.3) but showed no activity at cannabinoid receptors. These findings suggested some novel scaffolds for future anti-seizure drug discovery and development campaigns.

The work in Chapter 3 demonstrated the anti-seizure effect of two cannabinoid CB1 receptor ago-PAMs, that is, GAT591 and GAT1102. The major findings were that: (1) both compounds exhibited anti-seizure effects against hyperthermia-induced GTCS in *Scn1a*^{+/-} mice, with GAT1102 being effective at a lower dose than GAT591; (2) GAT1102 extended its anti-seizure

effects to protect the mice against GTCS in the MES test in mice but not GAT591; and (3) GAT1102 displayed pro-seizure effects as it increased the number of atypical absence seizures in *Gabrb3^{+/-D120N}* mice. These findings thus highlighted mixed effects of GAT591 and GAT1102 on seizures in different models. Overall, the results again highlight these scaffolds could be useful in anti-seizure drug discovery and development programs but at this stage might be better targeted to Dravet syndrome than Lennox-Gastaut syndrome.

Chapter 4 reported that a high seizure burden in *Scn1a^{+/-}* mice increased hippocampal concentrations of several specialized pro-resolving mediators. Previous research from our group demonstrated that a high seizure burden in *Scn1a^{+/-}* mice increased various prostaglandin species in the hippocampus, which suggested these mice had neuroinflammation. Consistent with these findings, this chapter found that a high seizure burden in *Scn1a^{+/-}* mice: (1) increased hippocampal concentrations of the pro-inflammatory arachidonic acid-derived LB4 and LTE4; and (2) increased hippocampal concentrations of specialized pro-resolving mediators, including the hydroxydocosaehaenoic acid mediator 17-HDHA, maresin 1, the D-series resolvins (RVD1 and RVD4) and the protectin PCTR1 compared to controls. We also observed an increase of all hydroxyeicosapentaenoic acid species (5-HEPE, 12-HEPE, 15-HEPE and 18-HEPE). Importantly, these findings support the hypothesis that a high seizure burden impacts not only inflammatory signaling pathways but also the resolution mechanisms in the *Scn1a^{+/-}* mice of Dravet syndrome. This result provides a foundation for future studies to further interrogate whether targeting resolution pathways and receptors have anti-seizure effects.

5.2 Broader potential implications and further directions

5.2.1 Chapter 2

Chapter 2 demonstrated the anti-seizure effects of amorfrutin 2, magnolol and honokiol in preclinical models of intractable epilepsies. These compounds are non-cannabis derived natural cannabinoid-like compounds that refer to the characteristics of structural similarities with cannabinoids or pharmacological interaction with cannabinoid receptors. Phytocannabinoids are natural derivatives found in many plants for example liverworts (*Amorpha fruticosa* and *Glycyrrhiza acanthocarpa*)³⁶⁰ and magnolias (*Magnolia officinalis*).²⁴⁸ Amorfrutins can be classified to CBGA-type cannabinoids²⁴³ that was found in *Amorpha fruticosa* and *Glycyrrhiza acanthocarpa* while magnolol and honokiol in *Magnolia officinalis* exhibited agonistic effects in cannabinoid receptors.²⁴⁸

The results of the present thesis add to the known effects of amorfrutin 2, honokiol and magnolol on different seizure types (summarized in Table 1). Magnolol not just displayed anti-seizures effects in acute seizure models, such as PTZ-induced symptomatic generalized seizure²⁵⁰ but also exerted long-term anti-seizure effects following 10 days of treatment against repeated kainic acid-induced status epilepticus.²⁴⁹ Further, our study is the first evidence to show anti-seizure potential of amorfrutin 2. Experiment in Chapter 2 clearly demonstrated the anti-seizure effects of amorfrutin 2 and magnolol in both symptomatic and disease-specific based GTCS, further study can investigate the effects of these compounds in long-term use, for examples in refractory status epilepticus which has been considered as risk factor that is closely associated with SUDEP in children with Dravet syndrome and Lennox-Gastaut syndrome.^{60-62,}

Table 1. Summary of the anti-seizure effects of compounds examined in the thesis

	<i>Scn1a</i> ^{+/-} mice	MES test	<i>Gabrb3</i> ^{+/<i>D120N</i>} mice
Amorfrutin 2	✓	✓	NT
Honokiol	NE	NT	NT
Magnolol	✓	✓	✓
GAT1102	✓	✓	✕
GAT591	✓	NE	NT

Anti-seizure effects = ✓;

Pro-convulsant effects = ✕;

No effect = NE and

Not tested = NT

To briefly highlight the potential implications of the findings, it is worth to consider the polypharmacological effects of amorfrutin 2, honokiol and magnolol on some known anti-seizure targets. This thesis characterized for the first time the inhibitory effects of all compounds on T-type calcium channels, which regulate neuronal excitability. These channels are also validated targets for CNS drug discovery. For example, T-type calcium channels antagonist Z944 displayed antinociceptive effects against inflammatory pain and anti-seizure effects on absence seizures both of which hold significant translational potential.^{361, 362} Compared to our findings, Z944 had greater potency at T-type calcium channels than amorfrutin 2, honokiol and magnolol (Table 2). Interestingly, honokiol exhibited subtype selectivity at Cav3.1 ($IC_{50} = 787$ nmol), which was more potent than at Cav3.2 and Cav3.3 ($IC_{50} \sim 3$ μ M). In contrast, amorfrutin 2 and magnolol demonstrated comparable inhibitory potencies ($IC_{50} = 2 - 5$ μ M) across three subtypes of T-type channels (Table 2).

Table 2. Comparison of mechanism of action across compounds

	Amorfrutin 2	Honokiol	Magnolol	Z944
Cav3.1 (IC ₅₀)	4 μ M	787 nM	4 μ M	50 nM
Cav3.2 (IC ₅₀)	3 μ M	3 μ M	5 μ M	160 nM
Cav3.3 (IC ₅₀)	2 μ M	3 μ M	4 μ M	110 nM
GABA receptor	NT	PAM at GABA _A (100 μ M)	PAM at GABA _A (100 μ M)	NT
GPR55 (IC ₅₀)	NT	~10 μ M	>10 μ M	NT
PPAR γ (EC ₅₀)	1.2 μ M	3.9 μ M	17.7 μ M	NT

Not tested = NT

Our findings here with T-type channels add to the known pharmacological actions of these compounds on anti-seizure targets but it is still unclear whether one target is responsible for their anti-seizure effects, or a combination of targets. For example, magnolol modulated neuronal excitability by acting as a PAM at GABA_A receptors at 100 μ M,^{363, 364} which is an established mechanism of many ASMs. In addition, tetrahydro-magnolol is an antagonist of GPR55²⁴⁸ at 13.3 μ M which shares the mode of action with CBD. Interestingly, amorfrutin 2, honokiol and magnolol share similar mode of action on activating PPAR γ at 1.2 μ M,³⁶⁵ 3.9 μ M³⁶⁶ and 17.7 μ M,³⁶⁷ respectively, which are an emerging target for anti-seizure drugs. Collectively, these results highlight many potential anti-seizure mechanisms of these compounds which could be explored further in future studies.²⁴⁸

Beyond their anti-seizure effects, it is interesting to consider the potential applications of these compounds in other neurological diseases, for example in the treatment of sleep disorders and pain.³⁶³ The activity of T-type calcium channels affects sleep rhythms in thalamocortical network.^{368, 369} Mice with T-type calcium channels knockout exhibited sleep deficits characterized by lacking delta oscillations and sleep spindles.³⁷⁰ Notably, a prescribed medicine trazodone for treating insomnia is a T-type calcium channel blocker with IC₅₀ = 43 μ M at

Cav3.1, 45 μ M at Cav3.2 and 23 μ M at Cav3.3.³⁷¹ In comparison, our findings demonstrated that amorfrutin 2, honokiol and magnolol had higher inhibitor potencies to trazodone across three subtypes of T-type channels. This suggests that these compounds could have therapeutic potential in sleep disorders. Supporting this hypothesis, one study discovered that magnolol shortened the sleep latency and increased both non-rapid eye movement (NREM) and rapid eye movement (REM) sleep.³⁶³ In the area of pain, honokiol and magnolol produced antinociceptive effects against formalin-induced acute inflammatory pain in mice,³⁷² while only magnolol attenuated chronic neuropathic pain in preclinical mice models after two week of treatment.³⁷³ Another study in rats showed that amorfrutins increased the pain withdrawal threshold and thermal latency, indicating a reduction of chronic constriction injury-induced neuropathic pain.³⁷⁴ Collectively these findings highlight that amorfrutin 2, honokiol and magnolol might be explored in future studies examining their effects on sleep in preclinical models and further elaborating on their effects in various pain models.

Chapter 2 also provided important brain/plasma pharmacokinetic information around amorfrutin 2, honokiol and magnolol which is useful for their advancement as neuropharmacological agents. These corresponding findings justify the therapeutic window that proposed structural modifications are needed to address the low brain uptake of amorfrutin 2 and rapid metabolism of magnolol and honokiol to improve their drug-like qualities and potential development as anti-seizure drugs. Indeed, whilst not necessarily to improve their pharmacokinetics per se, structural analogs have already been developed. For example, two analogs of magnolol (methylnonokiol and tetrahydromagnolol) also inhibited the PTZ-induced seizure activity in zebrafish.²⁵⁰ Additionally, another set of analogs of magnolol (tetrahydromagnolol and *trans*-isomagnolol) exhibited greater selectivity and potency at CB1 receptors compared to magnolol. Tetrahydromagnolol also displayed stronger antagonistic activity at GPR55 than magnolol.²⁴⁸ These findings highlight the potential of structural modifications to

enhance the therapeutic efficacy and receptor specificity. One advantage of using these natural products is that even in nature their exist structural variants of these compounds (e.g. amorfrutin 1, 2 and 3) which could be used to develop more SAR information in target-based drug design but also for phenotypic drug discovery for new ASMs.

5.2.2 Chapter 3

While Chapter 2 utilized a phenotypic drug discovery approach with compounds derived from natural products, Chapter 3 presented evidence as part of a target-based drug discovery approach using novel chemical entities. The larger program of research this thesis contributes to aims to develop novel therapeutics that target allosteric sites rather than orthosteric sites on the cannabinoid CB1 receptors which is an emerging anti-seizure drug target.^{173, 270, 368} This thesis presented novel anti-seizure properties of two ago-PAMs of the CB1 receptor GAT591 and GAT1102 in various seizure models (Table 1). These molecules appeared to show promise as anti-seizure agents against GTCS including in a mouse model of Dravet syndrome, however there was evidence of pro-seizure effects against atypical absence seizures in a mouse model of Lennox-Gastaut syndrome. This discrepancy is commonly seen for anti-seizure drugs because one drug may not necessarily be effective against all seizure types and indeed can also be contraindicated due to seizure precipitation. This might be explained by the ago-PAM in question GAT1102 having off-target effects or on-target issues specific to the pathobiology of atypical absence seizures.

The seizure modulating effects of CB1 receptors depend on whether they are expressed on glutamatergic neurons or GABAergic neurons. Our previous study observed decreased expression of CB1 receptors in the hippocampus of *Scn1a*^{+/-} mice with heightened seizure severity.¹⁸⁴ This evidence aligns with findings from studies showing CB1 receptor knock-out

mice exhibit increased seizure susceptibility.¹²⁹ Therefore, enhancing CB1 receptor signaling with GAT1102 may compensate for the downregulated CB1 receptor expression, producing anti-seizure effects in the mouse model of Dravet syndrome. Some studies have demonstrated anti-seizure effects through activation the CB1 receptors, while other have reported that high doses of CB1 receptor agonists can produce dose-dependent proconvulsant activities.^{188, 191, 252-254} The most accepted hypothesis to explain these conflict observations attributes the proconvulsant effects to non-specific activation of CB1 receptors in GABAergic neurons at high doses, leading to a reduction of inhibitory neurotransmitter release. Given that the *Gabrb3*^{+/-D120N} mouse model carries a mutation in a gene encoding a subunit of the GABA_A receptor, which reduces the inhibitory postsynaptic currents, it is therefore plausible that the effects of GAT1102 on CB1 receptors exacerbate this reduction in inhibitory currents at GABAergic neurons, thereby contributing to proconvulsant activity. Although, conflicting with this viewpoint is that the CB1 receptor agonist WIN55-212-2 reduces atypical absence seizures in this model.¹²³ WIN 55-212-2 is not a particularly selective CB1 receptor agonist with activity at other targets including transient receptor potential channels which might also contribute to its anti-seizure effects.^{375, 376} Future studies are needed to examine the exact role of the endocannabinoid system and CB1 receptors in the *Gabrb3*^{+/-D120N} mouse model of Lennox-Gastaut syndrome including an evaluation of the function of CB1 on specific neuronal populations.

The key feature of positive allosteric modulation but not for allosteric agonists is the ligand-dependence of their pharmacological actions and the required presence of orthosteric ligands.³⁷⁷ For example, GAT591 exhibits ligand-dependence as it enhanced the binding infinity of the orthosteric agonist CP 55,940.²⁶⁸ However, GAT591 also displays allosteric agonist properties meaning it can activate the CB1 receptor via a topographically distinct site without the requirement of the presence of endogenous or exogenous orthosteric ligands.

Indeed, a recent study showed that a variety of suggested pure PAMs like GAT229 were actually all ago-PAMs, and that the PAM activity involved potentiation of anandamide but not 2-AG.³⁷⁸ This calls into question whether the promise of PAM-mediated potentiation of spatiotemporal endocannabinoid signaling would apply *in vivo*. Future studies could attempt to discern whether the anti-seizure effects of the ago-PAMs like GAT591 involve any requirement for endocannabinoid signaling by testing their effects in mice with genetically inducible knockout of endocannabinoid synthetic enzyme genes.^{379, 380} If the effects were absent it would suggest that the PAM effects of the endogenous agonist is required, whereas if the anti-seizure effects were retained it would show the molecules work through allosteric agonism alone.

A lack of cannabimimetic effects with allosteric ligands at CB1 receptors is another important implication of our study. Prior research initially explored CB1 receptor PAMs, such as ZCZ011 and GAT229, as potential antinociceptive drug for neuropathic pain.^{309, 381} In a mouse model of chronic neuropathic pain, ZCZ011 increased the pain threshold while avoiding the inherent adverse effects typically observed with CB1 orthosteric activation such as catalepsy and hypothermia.³⁰⁹ Notably, the antinociceptive effects of ZCZ011 were blocked by rimonabant, a CB1 antagonist, highlighting CB1 receptor mediation. Although our results did not demonstrate a reduction in body temperature following the administration of GAT591 even at a high dose of 100 mg/kg (10-fold higher than in a previous study). This bodes well with the further development of GAT591 as an ago-PAM of CB1 receptors with anti-seizure effects. However, GAT1102 displayed anecdotal sedative effects at 100 mg/kg, and promoted hypothermia at 60 mg/kg. The pharmacological characterization of GAT1102 as an ago-PAM might then be called into question, and perhaps at high concentrations it might display orthosteric agonism of the CB1 receptor *in vitro*. Based on the plasma concentration in experimental mice at 60 mg/kg and the brain-plasma ratio of 0.2 we would expect

concentrations of approximately 90 μ M, which is in clear excess of those tested *in vitro* for PAM activity (usually up to 10 μ M maximum).²⁶⁸ This also highlights the potential for off-target engagement at such high concentrations. The present thesis study did not systematically investigate as the effects of these ago-PAMs at the doses tested on other phenotypic markers of cannabimimetic activity, that is catalepsy and hypolocomotion, which would strengthen this research.

Looking beyond the horizon of the present thesis, it would be of interest to assess the effects of emerging CB1 receptor modulators such as biased allosteric modulators (BAM) that act on cannabinoid receptors to selectively influence signaling pathways linked to the G-protein-coupled receptor.³⁸² Within this class, BAMs are further categorized into pure BAMs and ago-BAMs, the latter exhibiting allosteric agonistic activity, such as CB-02 and CB-05.³⁸³ Notably, one recent study demonstrated analgesic effects of CB-05 against inflammatory pain and chronic neuropathic pain in mouse models.³⁸³ The same study further observed a favorable safety profile of CB-05, without cannabimimetic side such as drug tolerance and hypothermia.³⁸³ Given the pharmacological effects between the analgesic agents and anti-seizure drugs, particularly their sedative properties, it would be worth evaluating BAMs like CB-05 in preclinical model of seizures. Such investigations could advance anti-seizure drug discovery by determining whether this mode of action confers anti-seizure efficacy. The development of positive BAMs would be a natural next-step, and it would be of interest to apply these in the area of anti-seizure drug discovery. Considering that CB1 receptors have multiple downstream signaling pathways, functional selectivity might allow these BAMs to activate one signaling pathway over another to produce more specific therapeutic effects. For example, selectively targeting CB1-linked inhibition of voltage-gated calcium channels may hold anti-seizure effects, as calcium channel inhibition is a well-established mechanism of many current anti-seizure medications.

Further allosteric modulators of the CB2 receptor are now emerging as a new therapeutic modality for anti-seizure drug design. Recently, CB2 receptor PAMs were reported to exhibit anti-seizure effects. For instance, the CB2 receptor PAM EC21A delayed the onset latency of PTZ-induced GTCS and protected mice against 6 Hz-induced psychomotor seizures in both WT mice and *Scn1a*^{RH/+} mutant mouse model of Dravet syndrome.³⁸⁴ The effects of EC21A were confirmed to be mediated by the CB2 receptor as the anti-seizure effects were abolished by the CB2 receptor antagonist AM630.³⁸⁴ The expression of CB2 receptor in the brain is quite controversial. While it is well accepted that CB2 receptors are involved in neuroinflammation through their expression on the brain's immune cells, the microglia, their expression on neurons themselves is hotly contested.^{157, 194, 196} Nonetheless, there is some evidence that CB2 receptors themselves regulate neurons and might dampen neuronal electrophysiological activity. For example, microinjections of CB2 receptor agonists JWH133 inhibited neuronal excitability in dopaminergic neurons.³⁸⁵ These effects were validated in *CB2*^{-/-} knockout mice and were abolished by coadministration of the inverse agonist of CB2 receptors AM630.³⁸⁵ A follow-up study consolidated these findings and revealed that the underlying mechanism involved the enhancement of K⁺ channel currents, which reduced neuronal excitability.³⁸⁶ Interestingly, while JWH133 did not alter seizure susceptibility, administration of the CB2 receptor antagonist SR144528 increased susceptibility to PTZ-induced seizures and 6 Hz-induced seizures in mice.²⁰³ Research is needed to address this discrepancy and understand the direct or indirect role of CB2 receptors in seizure treatment. The time-frame of effects of EC21A was argued to make more sense with respect to direct modulation of neurons than any effect on brain inflammation.

In any case, the development of novel pharmacological probes of both CB1 and CB2 receptors remains an exciting area of seizure and epilepsy drug discovery and development as supported by the finding of the present thesis. Moving forward, further characterization of CB1 and CB2

receptors pharmacology is needed in seizure models. For example, studies could test other CB2 receptor agonists such as GW405833, SER601, and CB65 in different seizure models including those with a significant inflammatory component.

5.2.3 Chapter 4

Specialized pro-resolving mediators have emerged as a new area of scientific exploration in the pathobiology of epilepsy. The research in Chapter 4 advances our understanding of this area by providing the first analysis of how a high seizure burden influences these mediators in the *Scn1a*^{+/-} mouse model of Dravet syndrome. This study discovered that a high seizure burden increased a variety of specialized pro-resolving mediator species including mediators that had not been previously characterized (e.g. maresins, D-series resolvins and protectin). Chapter 4 not only confirms the elevation of neuroinflammation via leukotrienes but also highlights the elevation of resolution of inflammation mechanisms.

Unlike other neurological conditions, the role of specialized pro-resolving mediators in epilepsy remains largely unexplored. One recent hypothesis suggests that persistent neuroinflammation without an active pro-resolving brain response may further facilitate seizure generation.³²⁸ Clinical evidence has consistently reported that the inflammation occurs in different forms of epilepsy, including Dravet syndrome²⁷² and Lennox-Gastaut syndrome.³⁸⁷ Notably, our findings revealed an elevation of pro-resolving mediators in *Scn1a*^{+/-} mice with a high seizure burden, however we cannot be sure based on our experimental design whether the magnitude of this effect is optimal to resolve inflammation or whether it represents a suboptimal pathological response to epilepsy. To bridge this gap, time-specific investigations should address the dynamic nature of the resolution processes in different stages of Dravet syndrome. Further, the magnitude of the response of the brain in elevating the specialized pro-

resolving mediators could be evaluated by comparing it to responses observed to other brain challenges that do not involve epilepsy pathophysiology (e.g. stress or brain trauma).

The first research on specialized pro-resolving mediators in epilepsy by Musto et al. demonstrated that protectin D1 reduced the epileptic discharges in an electrical kindling model.²⁹² A subsequent hallmark study by Frigerio et al. further confirmed the anti-seizure effects of protectin D1, showing reductions in both the number and duration of recurrent seizures, along with a decrease in inflammatory responses in kainic-induced seizure models.²⁹¹ Furthermore, the same study found that protectin D1 treatment improved the cognitive function which was measured by the novel object recognition test for non-spatial recognition memory in mice.²⁹¹ These encouraging findings highlight the therapeutic potential of specialized pro-resolving mediators not only for seizure management but also for addressing cognitive and behavioral deficits, which are common in Dravet syndrome and Lennox-Gastaut syndrome. Given the limited treatment options available for these aspects of epilepsy, future research could explore the broader effects of specialized pro-resolving mediators on behavior and cognition in these syndromes.

Immunosuppression therapy is considered a relatively aggressive approach for seizure control commonly used in patients with autoimmune or infection-related epilepsy but also against infantile spasms in Wests syndrome, a precursor to Lennox-Gastaut syndrome. Recent research interest has focused on targeting chronic inflammation as a therapeutic strategy, particularly for drug-resistant epilepsy and refractory status epilepticus.^{388, 389} In the treatment of Dravet syndrome, CBD is a currently approved drug exhibiting promising effects not only in reducing seizures but also in displaying anti-inflammatory properties. A recent study has shown that CBD allosteric activates 15-LOX and switches macrophage phenotypes to stimulate the production of specialized pro-resolving mediators.³⁹⁰ These findings suggest that CBD actively promotes pro-resolving responses.³⁹⁰ Future studies could investigate involvement of

specialized pro-resolving mediators in the anti-seizure actions of CBD, particularly in seizure and epilepsy types involving significant neuroinflammation. Furthermore, this might help explain additional benefits of CBD in addition to seizure control, as it might facilitate brain repair and assist in minimizing the long-term cognitive and behavioral deficits of epilepsy.

A potential therapeutic advantage of using specialized pro-resolving mediators lies in their ability to minimize adverse effects typically associated with immunosuppression. Anti-inflammatory therapies like corticosteroids have major side-effect liabilities such as increasing susceptibility to infection and potentially cancer. Unlike anti-inflammatory agents, specialized pro-resolving mediators resolve inflammation without impairing immune response. Structural analogs of specialized pro-resolving mediators which makes use of these molecules as templates for drug discovery could be explored.³⁹¹ In particular, analogs can be developed to increase receptor potency and improve therapeutic efficacy. For example, protectin D1 has been shown to selectively bind and signal through GPR37, a receptor known to be expressed in the brain.³⁹² Two synthetic analogs of protectin D1 (3-oxa n-3 DPA³⁹³ and 3-oxa PD1_{n-3} DPA³⁹⁴) have been synthesized. Notably, 3-oxa PD1_{n-3} DPA displayed a more potency than PD1_{n-3} DPA in reducing diabetic-induced neuropathic pain in a mouse model.

However, the number of known receptors for specialized pro-resolving mediators remains limited. Resolvin D2, acts as an agonist of GPR18/DRV2, a receptor involved in anti-inflammatory processes such as regulating neutrophil infiltration and enhancing macrophage phagocytosis.^{298, 395, 396} Our study observed an increased concentration of resolvin D2, highlighting that resolvin D2 might be targeted to develop new anti-seizure treatments or those that assist in brain repair.

Recent research has demonstrated the translational potential of SPM analogs, exemplified by Resolvyx, a resolvin E1 analog, which has progressed to phase I/II clinical trial for dry eye

syndrome that underlines the translational potential of these compounds.³⁹⁷ Despite these promising applications, research into the specialized pro-resolving mediators for epilepsy remains scarce and so the present thesis advances our understanding by providing evidence of their recruitment in response to a high seizure burden in a mouse model of drug-resistant epilepsy.

5.2 Conclusion

The research presented in this thesis provides novel preclinical evidence of anti-seizure efficacy for four compounds—amorfrutin 2, magnolol, GAT591, and GAT1102—in two genetic mouse models of intractable epilepsies. The study is the first to demonstrate the anti-seizure properties of non-cannabis-derived phytocannabinoid-like natural product compounds, but also novel chemical entities that allosterically modulate cannabinoid CB1 receptors in intractable epilepsy models. Collectively, the findings of the present thesis help appraise the strengths and limitations of these molecules in their potential application to different seizure types. Additionally, this thesis expands the understanding of how seizure burden impacts a novel neurochemical system involved in the response to seizures, that is involving specialized pro-resolving mediators. This research characterizes the specific array of these mediators that are augmented in the brain in a mouse model of Dravet syndrome. This lays an important foundation for the development of treatments in Dravet syndrome that resolve neuroinflammation and brain repair. It is hoped that the results presented in this thesis will help advance the development of novel treatments for refractory epilepsies of which there is the greatest unmet clinical need.

Chapter 6 Reference

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Appendix 1: Supplementary method for Chapter 2

Table S1 Plasma concentrations of compounds in experimental *Scn1a*^{+/-} mice

Compound	Dose (mg/kg)	Plasma Concentration		Brain Concentration	
		µg/mL	µM	µg/mL ^a	µM
Amorfrutin 2	10	54.5 ± 3.5	177.8	1.3 ± 0.3	4.2
	30	108 ± 17.2	353.6	6.6 ± 1.8	21.4
	100	198 ± 12.3	644.6	13.9 ± 7.1	45.2
Honokiol	10	0.9 ± 0.1	3.3	3.4 ± 0.6	12.8
	30	4.9 ± 1.0	18.7	22.3 ± 1.8	88.6
	100	15.7 ± 2.9	58.8	92.3 ± 13.3	346.5
Magnolol	10	0.3 ± 0.0	1	0.9 ± 0.1	3.2
	30	2.1 ± 0.5	7.9	4.7 ± 1.9	17.7
	100	5.2 ± 0.3	19.5	95.3 ± 3.1	347.1

^aBrain concentrations converted from measured concentrations (ng/mg brain) assuming density of 1 g/mL

FLIPR

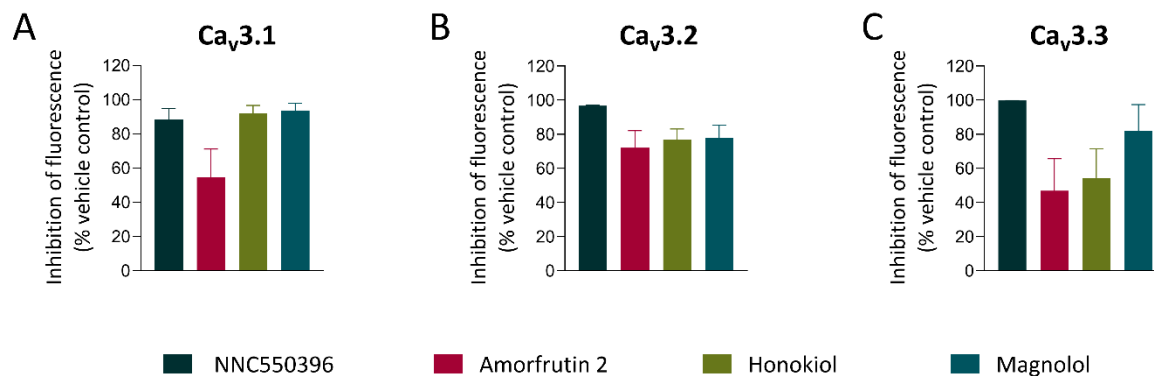


Figure S1. Amorfrutin 2, honokiol and magnolol inhibit T-type calcium channels. Single-point inhibition of 10 mM Ca-induced fluorescence by magnolol, honokiol and amorfrutin 2 (10 μ M) at T-type calcium channels (A) Cav3.1, (B) Cav3.2, and (C) Cav3.3 using FLIPR calcium assay in HEK293 cells. Data presented are mean $\pm$ SEM of % inhibition of fluorescence induced by 10 mM Ca²⁺ (n=5).

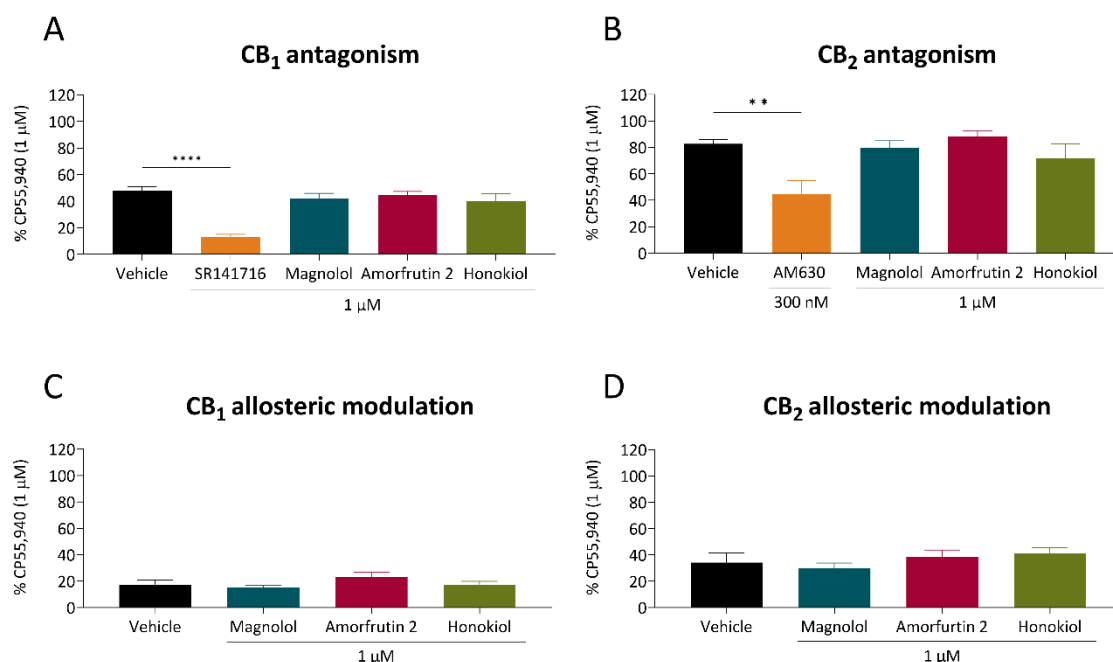


Figure S2. Amorfrutin 2, honokiol and magnolol did not antagonise or potentiate the effects of CP 55,940 on CB₁ and CB₂ receptors. Summary data of the effects of amorfrutin 2, magnolol, and honokiol antagonism of (A) 100 nM CP 55,940 activation of CB₁ (B) 300 nM CP 55,940 activation of CB₂ (C) 30 nM CP 55,940 activation of CB₁ (D) 100 nM CP 55,940 activation of CB₂ using membrane potential assay. After 2 min of baseline reading, AtT20 cells expressing CB₁ and CB₂ receptors were pretreated with 1 μM of amorfrutin 2, magnolol, and honokiol for 5 min followed by the addition of CP 55,940. SR141716 (1 μM) and AM630 (300 nM) are selective CB₁ and CB₂ inverse agonists, respectively. Unless otherwise stated, results are expressed as mean ± SEM after normalization to 1 μM CP 55,940 induced cellular hyperpolarization (n = 3-5)

Appendix 2: Supplementary data for Chapter 2

Materials

Amorfrutin 2, honokiol and magnolol were purchased from LinkChem (Shanghai, China). For cellular assays, drugs were dissolved in DMSO at 10 mM stock, stored at -20°C and diluted with 0.01% bovine serum albumin (BSA, Sigma, Castle Hill, Australia) in HEPES-buffered low potassium Hanks Balanced Salt Solution (HBSS) for experiments. HBSS comprises (mM) NaCl 145, HEPES 22, Na₂HPO₄ 0.338, NaHCO₃ 4.17, KH₂PO₄ 0.441, MgSO₄ 0.407, MgCl₂ 0.493, Glucose 5.56, CaCl₂ 1.26 (pH7.4). Final DMSO concentration was 0.1%. Acetonitrile, methanol and formic acid were obtained from Merck Millipore (Massachusetts, USA). Diazepam was supplied by Sigma-Aldrich (Missouri, USA). NNC550396 was obtained from Tocris (Bristol, United Kingdom). All drugs and chemicals achieved were $\geq 98\%$ pure based on HPLC.

Animals

Scn1a^{+/-} mice were obtained from the Jackson Laboratory (stock 37107-JAX, Bar Harbour, USA) and maintained as a congenic line on the 129S6/SvEvTac background (129.*Scn1a*^{+/-}).³¹⁰ F1 mice were produced by breeding heterozygous 129.*Scn1a*^{+/-} with wild type (WT) C57BL/6J mice (Jackson Laboratory stock 000664, Animal BioResources, Moss Vale, Australia) (19, 20). Genotyping of F1 mice was performed to determine *Scn1a*^{+/-} genotype (19, 20). Male F1 (129 and C57BL/6J) mice with a WT genotype (F1.WT) aged P21-28 were used in pharmacokinetic studies, and male and female P14-16 F1.*Scn1a*^{+/-} were used for hyperthermia-induced seizure studies (20). Adult male C57BL/6J mice, aged between 6 to 7 weeks, were purchased from Australian Bioresources (Mossvale, NSW) for the Maximal electroshock test (MES) tests.

Gabrb3^{+/D120N} knock-in mice were also obtained from the Jackson Laboratory (stock 035437, Bar Harbour, USA).¹²³ *Gabrb3*^{+/D120N} knock-in mice were produced by breeding male *Gabrb3*^{+/D120N} knock-in hemizygous mice with female WT C57BL/6J mice (Jackson Laboratory stock 000664, Animal BioResources, Moss Vale, Australia). Genotyping of offspring mice was performed to determine *Gabrb3*^{+/D120N} genotype as previously described.¹²³ All mice were housed at a maximum of 5 mice per cage in ventilated cages with *ad libitum* access to food and water. They were habituated for one week upon arrival before any experimental procedures. The mouse facility was maintained in 12-hour light-dark cycles in a pathogen free environment.

Pharmacokinetic studies

Mice were randomly allocated to three treatment groups (amorfrutin 2, honokiol or magnolol). They received a single i.p. injection of the test compounds at 10 mg/kg in an injection volume of 10 mL/kg. Blood samples were collected in five selected time points (15 – 120 min, n = 4 each time point) by cardiac puncture under isoflurane anaesthesia.^{221, 237} The blood was then centrifuged at 10,000 g for 10 min at 4°C and plasma was collected. Whole brains were harvested and snap frozen on dry ice. All samples were stored at -80°C until analysed.²³⁷ The samples were then extracted and analysed as described previously.^{216, 221, 237} The linear-long trapezoidal method was used to calculate the area under concentration time curve (AUC) and pharmacokinetic parameters.^{216, 221, 237} All parameters were fitted to noncompartmental analysis. Time to maximum concentration in the plasma (plasma T_{max}) was used to determine the target time point for hyperthermia-induced seizure and the MES test. T_{max} for amorfrutin 2 was 30 min, and magnolol and honokiol were 15 min from our pharmacokinetic studies.

Sample preparation for analytical chemistry

Plasma samples (50 µl) were spiked with 10 µl of 2 µg/ml diazepam as internal standard. 10 µl of methanol and 140 µl of cold acetonitrile were vortex mixed with the plasma for protein denaturation. The organic phase was isolated by centrifugation in 4,000 g for 10 min at 4°C and subjected to blow dried with nitrogen gas (3). The samples were reconstituted in 90 µl of acetonitrile and 300 µl of 0.1% formic acid in water. A single use of Isolute® SLE+ columns from Biotage (Uppsala, Sweden) was used for solid-liquid extraction and samples were eluted in 900 µl methyl tert-butyl ether (MTBE) via robotics machine. The samples were evaporated to dryness with nitrogen gas for 30 min and reconstituted in 50 µl of HPLC mobile phase.

Brain sample extraction was similar to plasma extraction with minor modifications (3). Briefly, brain hemispheres were homogenised in 5x of acetonitrile (w/v) by a Biospec Tissue-Tearor (Bartlesville, USA). Homogenates were centrifuged (20,000 g for 30 min at 4°C) and 100 µl supernatant was spiked with 15 µl of 10 µg/ml diazepam as internal standard. Three times volume of cold acetonitrile was added to the extracts and centrifuged at max speed for 15 min at 4°C. The supernatant was transferred to Ultra-0.5 mL Centrifugal Filter Unit (Missouri, USA) and centrifuged at 14,000 g for 1.5 hours and evaporated to dryness with nitrogen gas. The samples were reconstituted in 90 µl of acetonitrile and 300 µl of 0.1% formic acid in water, followed by solid-liquid extraction as above. The elutes were evaporated to dryness with nitrogen gas for 30 min and reconstituted in 100 µl of HPLC mobile phase.

Analytical chemistry method

Amorfrutin 2, honokiol and magnolol concentrations in brain and plasma samples were assessed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) using a Shimadzu Nexera ultra-high-performance liquid chromatograph coupled with

a Shimadzu 8,030 triple quadrupole mass spectrometer (Kyoto, Japan). 20 µl of reconstituted sample was injected into the system equipped with a Zorbax XDB-C18 (100 × 2.1 mm, 3.5 µm, Agilent) reverse-phase column and with a Zorbax C8 guard column (Santa Clara, USA). Mobile phase of honokiol, magnolol and amorfrutin 2 was 40% acetonitrile and 60% of 0.1% formic acid in water, 45% acetonitrile and 55% of 0.1% formic acid in water and 30% acetonitrile and 70% of 0.1% formic acid in water, respectively. All mobile phases were delivered at a flow rate of 0.6 mL/min. Treatment concentrations in brain and plasma were averaged at each time point. Samples were quantified against an eight-concentration standard curve ($R^2 > 0.98$).

Hyperthermia-induced seizures in $Scn1a^{+/-}$ mice

$Scn1a^{+/-}$ mice were randomly assigned to vehicle or treatment groups (amorfrutin 2, honokiol or magnolol, $n = 12 - 18$ for each group). A single dose of the treatment was delivered by i.p. injection at an injection volume of 10 mL/kg. All drugs were freshly dissolved in a sequence of ethanol, tween 80 and 0.9% saline (1:1:18, ratio). Doses of 10 and 30 mg/kg were fully dissolved in solution, while the 100 mg/kg dose was cloudy and injected as a suspension for all compounds. The detailed experimental protocol is described previously.^{190, 237} Briefly, a RET-3 rectal temperature probe was inserted 15 min prior to the target experimental time point based on the plasma T_{max} of the compounds. Body temperature was measured by a rodent TCAT-2DF temperature regulator (Physitemp Instruments, New Jersey, USA). Mice were acclimated to the temperature probe for 5 min, and the hyperthermia protocol was started. A heat lamp was used to elevate 0.5°C mouse core body temperature every 2 min. The core body temperature was recorded until the onset of the first clonic convulsion with loss of posture or when 42.5°C was reached. Mice were considered seizure-free when their body temperature

reached 42.5°C and held for 3 min without behavioural seizures. Animals were then anaesthetised with isoflurane, followed by whole blood collection via cardiac puncture and brain collection. Plasma was isolated by centrifugation (14,000 g for 10 min, 4°C). These samples were kept in -80°C for drug concentration analysis.

Maximal electroshock test (MES test)

Maximal electroshock seizures were induced using a Ugo Basile electroconvulsive therapy unit (Model 57800, Gemonio, Italy) in C57BL/6J mice aged 7 – 8 weeks randomly assigned to vehicle or treatment groups. The current intensity required to produce tonic hindlimb seizure in 97% of animals (CC₉₇) was determined as 46 mA using previously described probit analysis.^{312, 313} Animals received a dose of vehicle or treatment before seizure induction at the plasma T_{max} of the test compound as determined above (n = 10 each group). 0.5% tetracaine was applied to both corneas 5-15 min prior to testing to provide anaesthesia. A drop of saline solution was applied to both eyes immediately before applying electrodes to ensure adequate electrical conductivity. Corneal electroshocks were delivered at 46 mA, 299 Hz, 0.2 s duration with a 0.9 ms rectangular pulse width. Mice were scored by researchers blinded to treatment for the presence or absence of tonic hindlimb extension occurring within 30 s following the shock delivery. Mice that failed to demonstrate tonic hindlimb extension were scored as protected.

Electroencephalogram recording and analysis

The protocol for EEG implantation is summary in a previous publication.¹²³ Meloxicam (5 mg/kg) was subcutaneously administrated 15 min prior the surgery for analgesia. *Gabrb3*^{+/D120N} mice were placed in an induction chamber with 4% isoflurane. They were then

transferred to a stereotactic frame in prone position with 1-2.5% isoflurane through an anaesthesia unit. A heating pad (Homeothermic Monitoring System, Conductscience, USA) was placed under the mouse to maintain a body temperature of 37 °C. Ophthalmic ointment (Lubrithal eye gel, Pet Chemist Pharmacy, Australia) was applied to the eyes to avoid irritation and a rectal probe inserted to monitor body temperature. Hair was removed from the surgical region by trimmer and then disinfected with 75% ethanol followed by povidone-iodine. A cranial incision was then made from between the eyes to between the occiput, exposing the skull landmarks of lambda and bregma. A prefabricated headmount (Pinnacle Technology, In., USA) was attached to the skull using cyanoacrylate glue. Stainless steel screws (0.10” in anterior and 0.12” in posterior) (Pinnacle Technology, In., USA) were then inserted via bore holes to contact the brain surface. Electromyography (EMG) wires were inserted into the neck muscle and sutured in place. The incision was closed using non-absorbable monofilament sutures. Dental cement was then applied to secure and insulate the headmount. Mice were administered buprenorphine (0.05 mg/kg) subcutaneously. They were housed in a cage of four mice and monitored daily for one week, and meloxicam (5 mg/kg) was given once per day in the 48 hours post-surgery.

On the recording day, mice were transferred to recording chambers for 30 min before the recording, and pre-amplifiers were attached to the exposed headmount, allowing for unencumbered movement within the chamber. Female mice were then randomized to vehicle or 100 mg/kg magnolol treatment according to a crossover Latin-square design with a one week washout period. Treatments were administered (i.p.) according to their T_{max} , prior to dark cycles. EEG and EMG signals were recorded by Sirenia Acquisition software (Pinnacle Technology Inc. Version 2.2.2) for 30 min following treatment administration. The signals were analyzed offline with Somnivore software¹²³ by an experimenter blind to treatment conditions. Recordings were visually scored for atypical absence seizures, which were characterized by

diffuse slow spike waves discharge patterns, that were high-voltage and low frequency (3 -7 Hz) episodes.¹²³ The peak onset of atypical absence seizures *Gabrb3^{+/-D120N}* knock-in mice occurs at the light-to-dark transition and a higher seizure frequency was observed during the dark hours compared to the light hours.¹²³ Therefore, magnolol was administered 15 min ahead of the light-to-dark transition to match its T_{max} with the peak atypical absence seizure activity.

Cell culture

HEK293 Flp-In T-REx cells stably expressing T-type Cav3.1, Cav3.2, or Cav3.3 channels and mouse pituitary tumour AtT20 FlpIn cells stably transfected with haemagglutinin (HA)-tagged human CB₁ (AtT20-CB₁) and human CB₂ (AtT20-CB₂) receptors were cultured in T75 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S; 100 U/ml). The cells were incubated at 37°C in a 5% CO₂ humidified atmosphere. Blasticidin (10 µg/ml) and hygromycin (80 µg/ml) were used to select cells expressing Cav3.1, Cav3.2, or Cav3.3 while Zeocin (100 µg·ml⁻¹, Invivogen, California, USA) and hygromycin (80 µg·ml⁻¹, Invivogen) were used to select wild-type and transfected AtT20 cells respectively. Cells were passaged at 80% confluency and used for assays at 90% confluency within a limit of 15 passages.

FLIPR Calcium flux assay

Calcium 5 dye (Molecular Devices, United States) was utilised to assess T-type calcium channel activities in HEK293 cells overexpressing Cav3.1, Cav 3.2, or Cav 3.3, using a FlexStation III. Cells were resuspended in Leibovitz's L-15 media with 1 % FBS, 1 % P/S, and

15 mM glucose. 120,000 cells/well (90 μ l) were plated in black-walled, 96-well microplates (Corning, NY, United States) and incubated overnight at 37 °C in humidified air. Tetracycline (2 μ g/ml, Sigma-Aldrich) was added at plating to induce channel expression. For assays, 90 μ l of Hanks Balanced Salt Solution (HBSS) with 50% calcium dye and 2.5 mM probenecid (Invitrogen, United States) was added per well and incubated for 1 h at 37°C. Fluorescence changes were measured with FlexStation III ($\lambda_{\text{excitation}}$ = 485 nm, $\lambda_{\text{emission}}$ = 525 nm), recording every 2 s at 37°C. A 2 min baseline was followed by drug addition (20 μ L) and a 5 min read, then 10 mM CaCl_2 (20 μ l) was added, and measurement continued for another 3 min.

Membrane potential assay

AtT20 cells were resuspended in Leibovitz's L-15 media containing 1% FBS, 1% P/S, and 15 mM glucose; 22.5 μ l of the resuspended 50,000 cells were plated in each well of a black-walled, 384-well microplate (Corning) and incubated overnight in humidified air at 37°C for the experiment. Changes in the membrane potential of cells were measured in duplicate, using a FLIPR Membrane Potential Assay Kit (blue #R8034) and a FlexStation 3 Microplate Reader (both from Molecular Devices, Sunnyvale, CA). A reduction in fluorescence is indicative of cellular hyperpolarization. The dye was diluted to 50% of the manufacturers recommended concentration using HBSS. Dye (22.5 μ l) was loaded into each well of the plate and incubated for 1 hr at 37°C prior to testing. The FlexStation 3 recorded fluorescence at 2 s intervals ($\lambda_{\text{excitation}}$ = 530 nm, $\lambda_{\text{emission}}$ = 565 nm), and drugs (5 μ l) were added after an initial 2 min of baseline reading.

For the antagonist assay, after baseline reading cells were pre-treated with 10 μ M of test compounds for 5 min followed by the addition of approximately EC_{50} (CB_1) or EC_{80} (CB_2) concentrations of CP 55,940 and read for 3 min.^{398, 399} We used EC_{50} for CB_1 receptors to obtain

a broader window of receptor modulation because our calculated CP 55,940 EC₈₀ at CB₁ receptors was producing a response substantially near maximal response. A similar paradigm was used to investigate positive allosteric modulation but with EC₂₀ concentrations of CP 55,940 at both receptors.^{398, 399} The cellular response to the drug is presented as a maximal change in fluorescence from baseline after subtraction of the change produced by vehicle addition. The change in fluorescence was then normalized to the change in fluorescence due to 1 μ M CP 55,940 (a high efficacy, non-selective CB₁ and CB₂ receptor agonist) for cannabinoid receptor transfected cells only or 1 μ M somatostatin for both wild type and transfected cells to allow more ready comparison across experiments (AtT20 cells endogenously express somatostatin receptors).⁴⁰⁰ CP 55,940 standard stimuli were obtained in independent experiments in one well of each column of each plate. Concentration-response curves were fitted to a four-parameter sigmoidal dose-response curve in GraphPad Prism 9.0 to derive pEC₅₀ and *E*_{max}.

Automated patch clamp

The automated patch clamp experiments were conducted according to a published protocol.⁴⁰¹ Briefly, the expression of Cav3.1, Cav3.2, or Cav3.3 Flp-In HEK293 cells were induced by adding 200 ng/ μ l doxycycline (Sigma Aldrich) to the flask for 48 hours. On the assay day, cells were digested with Accumax (Thermofisher Scientific) and cold extracellular NMDG 60 recording solution (in mM: NaCl 80, NMDG 60, KCl 4, CaCl₂ 2, MgCl₂ 1, HEPES 10, D-glucose 5; adjusted to pH 7.4 with HCl) was added to detached cells and incubated at 4°C for 15 min. Cells were next transferred into falcon tubes, centrifuged at 200 g for 3 min and resuspended in 2 mL divalent-free extracellular recording solution (in mM: NaCl 140, KCl 4, HEPES 10, D-Glucose 5; adjusted to pH 7.4 with NaOH). Cell suspensions were transferred to

each well on a 12-well trough and rotated at 300 rpm on a cell hotel on the SyncroPatch 384PE (Nanion Technologies, Munich, Germany). Extracellular NMDG 60 with high calcium recording solution (in mM: NaCl 80, NMDG 60, KCl 4, CaCl₂ 10, MgCl₂ 1, HEPES 10, D-glucose 5; adjusted to pH 7.4 with HCl) was used to achieve high-quality seal resistance followed by 2 washing steps using the same extracellular NMDG 60 with high calcium recording solution. A pressure of -200 mbar was applied twice to achieve the whole-cell configuration.

For drug inhibition recording, 100 ms at +20 mV was used to record the peak tail current. At least 20 sweeps at 5 s bins were collected as a baseline before the drug was added, followed by a further 50 sweeps of recording to reach a steady state of drug inhibition. Quality control parameters used to assess cell recordings for analysis include minimum seal resistance of 500 MΩ and capacitance between 1 and 50 pF. Peak tail currents less than 100 pA were also excluded to avoid overestimating drug inhibition.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Pharmacokinetic parameters were calculated by linear regression of the terminal component of the concentration–time curve.²¹⁶ Temperature thresholds of seizure were compared across the treatments using Mantel-Cox log-rank test.^{190, 221, 230} Seizure responses in MES test were compared across groups using the chi-square test.^{190, 219, 237} For the calcium assay, change in fluorescence by 10 mM Ca²⁺ in the presence of drugs was normalised to change in fluorescence caused by 10 mM Ca in the presence of vehicle only to allow for comparison. IC₅₀ values were calculated by fitting electrophysiology data to a four-parameter non-linear dose-response curve.⁴⁰² The total number of atypical absence seizures and the duration of atypical absence seizures in 5 min bins were

analysed in R⁴⁰³ by generalized linear mixed models using the function *glmer* from the package “lmer4”.^{403, 404} The data type (integers or continuous); residual plots; Shapiro–Wilk test of normality, using the function *shapiro.test* from the package “stats”;⁴⁰³ Levene's Test for Homogeneity of Variance (Homocedasticity) using the function *leveneTest* from the package “car”;⁴⁰⁵ and Pearson's dispersion test were used to identify the best distribution (i.e., Gaussian, Gamma, or Negative Binomial) and link (i.e., Identity, Inverse or Log) for each model.⁴⁰⁶ P-values were generated by the type III Wald chi-square test using the function *Anova* from the package “car”.⁴⁰⁵ Pairwise comparisons were performed with Šidák correction through the functions *emmeans* from the package “emmeans”.⁴⁰⁷ Additional statistical analysis and all graphs were generated using GraphPad Prism 9.0 (California, USA). All statistical differences were considered at a 5% level of significance.