An Investigation on the Lipidomic Profile of a Mouse Model of Dravet Syndrome and the Molecular Action of Cannabinoids

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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. I certify that, to the best of my knowledge, the content of this thesis is my own work, and any assistance received in preparing this thesis have been acknowledged. This thesis has not been submitted for any other degree or other purposes.

The research in this thesis was carried out at the Brain and Mind Centre and the Mass Spectrometry facility at the Charles Perkins Centre, both 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.

The work on Chapter 2 was designed by me, together with Dr Timothy Couttas and Professor Jonathon Arnold. I conducted most experimental work, statistical analyses, and interpretation of data. Dr Ka Yip and Dr Dilara Bahceci conducted mice line maintenance and sample collection, while I performed all brain dissections. Sample preparation, LC-MS/MS analysis, data collection, analysis and interpretation were performed by me with the supervision of Dr Timothy Couttas and Beverly Jieu. Dr Miguel Bedoya Perez provided statistical analysis assistance.

The work in Chapter 3 was also designed by me, together with Professor Jonathon Arnold and Dr Timothy Couttas. I conducted most experimental work, statistical analyses, and interpretation of data. LC-MS/MS analysis were performed by me with the assistance of Dr Timothy Couttas and Anna Hoffman. Dr Miguel Bedoya Perez provided statistical analysis assistance.

For the publication in Chapter 4, I was the primary author and designed experiments under the supervision of Dr Lyndsey Anderson and Professor Jonathon Arnold. I performed all cell culture maintenance and assays, statistical analysis, and interpretation of the data. Dr Lyndsey Anderson assisted in the LC-MS/MS data acquisition. Dr Adam Ametovski and Dr Samuel Banister contributed to the synthesis of

cannabichromene. Dr Peter Jones and Dr Anthony George performed the molecular docking experiments at the University of Technology Sydney. Chapter 4 has been published upon the submission of this thesis (Etchart et al., 2022). Prof Arnold is the corresponding author and has granted permission to include the published material.

The student used ChatGPT for the purposes of text enhancement. The use of this generative AI tool includes spelling corrections, minor sentence restructuring, and clarity enhancement. The author confirms that where text was modified by generative AI, the content was reviewed for possible errors, inaccuracies, and bias. The author takes full responsibility for the submitted thesis, confirms the work is their own, and has used generative AI in accordance with University guidelines and policies.

I wrote all chapters with feedback from Prof Jonathon Arnold, Dr Elizabeth Cairns, Dr Richard Kevin, and Dr Timothy Couttas.

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As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

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Publications

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List of Abbreviations

Δ^8-THC	Delta-8-tetrahydrocannabinol
Δ^9-THC	Delta-9-tetrahydrocannabinol
24S-HC	24-S-hydroxycholesterol
2-AG	2-arachidonoylglycerol
5-HT	Serotonin
AA	Arachidonic acid
AAV	Adeno-associated virus
ABC	ATP-binding cassette
ABCB1	ABC subfamily B member 1
ABCG2	ABC subfamily G member 2
AEA	Anandamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ASMase	Acid sphingomyelinase
ASMs	Antiseizure medication
ATP	Adenosine triphosphate
BBB	Blood brain barrier
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBC	Cannabichromene
CBCA	Cannabichromene acid
CBCVA	Cannabichromevarinic acid
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBDVA	Cannabidivarinic acid
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBGVA	Cannabigerovarinic acid

CBN	Cannabinol
cDNA	Complementary DNA
Cer	Ceramide
CerS	Ceramide synthase
ChE	Cholesterol ester
CNS	Central nervous system
CSF	Cerebrospinal fluid
CYP46A1	Cholesterol 24-hydroxylase
ECS	Endocannabinoids system
DES1/2	Dihydroceramide desaturase
DHA	Docosahexaenoic acid
dMePE	Di-methyl-phosphatidylethanolamine
DNA	Deoxyribonucleic acid
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assays
ESI	Electrospray ionisation
GABA	Gamma-aminobutyric acid
GalCer	Galactosylceramide
GlcCer	Glucosylceramide
GPCR	G protein-coupled receptor
HexCer	Hexosyloceramide
ILAE	International League Against Epilepsy
LGS	Lennox-Gastaut syndrome
LPC	Lysophosphatidylcholine
MALDI-MSI	Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging
NMDA	Glutamatergic N-methyl-D-aspartate
$Na_v1.1$	Voltage-gated sodium channel 1 alpha subunit
PA	Phosphatidic acid
PC	Phosphatidylcholine

PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PPARγ	Peroxisome proliferator-activated receptor gamma
PS	Phosphatidylserine
PTZ	Pentylenetetrazole
SM	Sphingomyelin
SUDEP	Sudden unexpected death in epilepsy
TG	Triglyceride
TRPV1	Transient receptor potential vanilloid 1
UGCG	UDP-glucose-ceramide glucosyltransferase
UGT8A	UDP-galactose-ceramide galactosyltransferase

Abstract

Epilepsy is a chronic neurological disorder characterized by a frequent predisposition for seizures, a range of psychosocial-cognitive and motor impacts, and an increased mortality risk. Refractory, or drug-treatment resistant epilepsy is common, affecting about 30% of epileptic individuals. It is thus necessary to better understand the bases of the disease and its sub-classifications through the characterization of novel neurochemical pathways, as well as by unravelling the pharmacology of novel treatment candidates. This may, in turn, improve disease management and treatment. Dravet syndrome is a rare form of developmental and epileptic encephalopathy with onset in infancy. It is characterized by seizures, the development of cognitive-behavioural and social disabilities, sleep difficulties, a high risk of sudden unexpected death, and a very high degree resistance to treatment. Although the causative genetic mutation is known in at least in 80% of cases (the *SCN1A* gene), much remains unclear about the disease, including complete understanding of the pathophysiology, epileptogenesis, and aetiological profile. The association of lipids with Dravet syndrome pathophysiology is of interest given the strong connection between lipid regulation of neuronal excitation as well as growing evidence that suggests that lipid dysregulation may also occur in seizure-related disorders and epilepsy. Such evidence includes the syndrome's association with lipid-linked pathways of neuroinflammation and the endocannabinoid system, the evidence of efficacy of the cholesterol modulator soticlestat, the efficacy of lipid-rich ketogenic diet treatment, and the anti-seizure properties of cannabidiol and other emerging cannabinoids. However, more research is needed to better understand how lipid dysregulation might be involved in the pathophysiology of drug-resistant epilepsies like Dravet syndrome, as well as a need to better understand the mode of action of emerging lipid-targeting therapeutics like cannabinoids. The present thesis aimed to further advance this field of research by obtaining a better understanding of the lipid neurochemistry of Dravet syndrome and the pharmacological action of cannabinoids.

Chapter 1 begins with an introduction to epilepsy, covering the neurological bases of the disease, current treatment, and seizure and epilepsy classifications, before focusing on Dravet syndrome, its hallmarks, treatment options, and the Dravet syndrome

mouse model. It further expands on the emerging role of lipid modulation in the context of Dravet syndrome and the proposal of its further exploration as a key to better understanding the nature of the disease. It then introduces the research area of lipidomics and its establishment as a powerful tool to investigate broad biological lipid profiles. And finally outlines the potential role of cannabis and cannabinoids as novel and emerging anti-seizure therapeutics with unresolved pharmacological characterization, but that may involve lipid modulation.

This thesis encompasses three original studies in chapter 2, 3 and 4 which focused on the advancing the knowledge on Dravet syndrome lipid neurochemistry and pharmacological knowledge of cannabinoids. Chapters 2 and 3 assess the cortical and hippocampal lipidomic profile in the *Scn1a*$^{+/-}$ Dravet syndrome mice model to investigate potential genotype-, strain- and tissue- specific differential modulation of lipids via lipidomic approaches. Chapter 2 performed an explorative untargeted lipidomics analysis to identify potential lipid dysregulations. This examination flagged hexosylceramides as compounds of interests, therefore, chapter 3 performed a follow-up targeted LC-MS/MS analysis of the hexosylceramides family of compounds. Chapter 4 assessed the lipid-like cannabinoids, CBC and CBCA that have anti-seizure effects in the *Scn1a*$^{+/-}$ mice model of Dravet syndrome for their interactions with ABC transporters, efflux pumps found in the blood brain barrier that can limit brain disposition of substrate compounds, including lipids. This was done via an *in vitro* pharmacological screen in a bidirectional transport assay to determine substrate and/or inhibitor activity. Transwell assays with polarized epithelial MDCK cells expressing ABCB1 or ABCG2 were used, and samples were measured using LC-MS/MS.

Chapter 2 performed an untargeted lipidomic analysis of cortical and hippocampal brain tissue of the *Scn1a*$^{+/-}$ mice with differential seizure susceptible backgrounds. This investigation identified unique lipidomic profiles associated with the heterozygous deletion of *Scn1a* and the seizure-susceptible F1 strain. Cortical F1.*Scn1a*$^{+/-}$ versus F1.WT showed alterations in sphingolipid, glycerolipid, and glycerophospholipids families, and, in parallel, differences between the 129 seizure-resistant strain and the F1 seizure-susceptible strain. This suggests we have identified a neurological lipidomic signatures that could be associated with the F1.*Scn1a*$^{+/-}$ seizure phenotype. This

examination flagged hexosylceramides as compounds of interests, therefore, chapter 3 performed confirmatory follow-up targeted LC-MS/MS analysis of the hexosylceramides family of compounds. Region- and strain-specific HexCer modulation with differential structural patterns between the seizure-prone F1 strain and the non-seizure prone 129 strain were observed, namely, a vast hippocampal upregulation of species of a wide structural variety, while the cortex presented minor dual differences in short chain HexCer. Additionally, upregulation of the di-saturated short chain C18 was found in the cortex of *Scn1a*$^{+/-}$ compared to WT. Altogether, the present study reflects the idea that an altered lipid pathway is potentially contributing to the seizure-susceptibility seen in the F1.*Scn1a*$^{+/-}$ mouse, with a potential for additional modulations driven by seizure-presentation.

Chapter 4 sought to advance the pharmacological understanding of cannabinoids CBC and CBCA through characterisation of interaction with ABCB1 and ABCG2 transporters. These cannabinoids are structurally distinct to the approved anti-seizure agent CBD, but also display anti-seizure effects in the *Scn1a*$^{+/-}$ mouse model of Dravet syndrome, through unknown mechanism(s) of action. The key results are: 1) CBCA is an ABCB1 substrate, but not an ABCG2 substrate; 2) CBC was not a substrate of either transporter; 3) Neither CBCA nor CBC inhibited ABCB1 transport of prazosin or ABCG2 transport of digoxin; 4) *In silico* molecular docking suggested CBCA binds ABCB1 in the access tunnel and the central binding pocket. These findings provide important information on basic pharmacological data on these cannabis constituents in their evaluation as next-generation cannabinoid anti-seizure agents as well as advancing on the potential role of ABC transporters on their reported anti-seizure properties.

Finally, chapter 5 provides a general discussion to summarize the finding from all chapter findings and discussed together with a comment on their significance in the context of Dravet syndrome pathophysiology and treatment. This discussion also includes an assessment of study design limitations as well as proposals of future directions of research.

Chapter 1

Introduction

1.1 Epilepsy

Epilepsy is a chronic neurological disorder characterized by a frequent predisposition for seizures, a range of psychosocial-cognitive and motor impacts, and an increased mortality risk (Jones, 1998; Trinka et al., 2023). People with epilepsy have an average of two to three times higher danger of premature death, with those with drug resistant seizures mortality risk (Trinka et al., 2023). Unfortunately, first line anti-seizure medications (ASMs), have a very low success rate for many epilepsy classifications (Devinsky et al., 2018; Pellinen, 2022). It is thus necessary to better understand the bases of the disease and its sub-classifications, and, in turn, improve disease management and treatment. This is essential as epilepsy has no socio-economic boundaries, it affects 50-65 million people worldwide, and it is the 3rd greatest contributor to global disease burden amongst neurological disorders (Chen et al., 2023; Devinsky et al., 2018). This section will introduce the literature regarding seizures and epilepsy, the current state of the art in treatment, and classifications of epilepsy syndromes.

1.1.1 Neurological Bases of Seizures and Epilepsies

The term 'epilepsy' refers to the broad group of neurological conditions of recurrent epileptic seizures (Stafstrom et al., 2015). Epileptic seizures are clinically significant due to their direct impact in adverse health outcomes. Seizures can cause irreparable physiological damage to the brain and other parts of the body (Anwar et al., 2020). Such damage leads to heightened risk of sudden unexpected death in epilepsy (SUDEP), status epilepticus, falls, and injuries (Jones, 1998). Even one seizure can cause neurodevelopmental changes that can lead to cognitive-, behavioural- and motor-associated consequences (Stafstrom et al., 2015). Frequent recurrence is especially detrimental as it leads to extended neuronal disruption which simultaneously exacerbates a feed-forward cycle of dysregulation that reinforces damage and creates further seizure susceptibility, also referred to as kindling development (Dingledine et al., 2014). As a result, the main goal of epilepsy treatment is to reduce seizure frequency as it is the main factor that contributes to the quality of life of epilepsy patients (Jones, 1998).

Seizures are sudden alterations of neurological function driven by abnormal excessive and hyper-synchronous neuronal activity (Ono et al., 2012; Stafstrom et al., 2015). Although sometimes used interchangeably, the terms 'convulsions', 'seizures' and 'epileptic seizures' differ vastly. Convulsions, or involuntary muscle spasms, can often be a seizure symptom, however both can occur in the absence of the other (Delanty et al., 1998). Moreover, epileptic seizures are a sub-category of seizures. They are distinguished by their underlying cause and brain activity profile. While non-epileptic seizures can arise from pharmacological, psychological and/or physiological stress (also called psychogenic nonepileptic seizures), epileptic seizures occur from abnormal underlying electrical activity defined by an epileptogenic and ictogenic context (Devinsky et al., 2011; Larson et al., 2021).

Epileptogenesis is the process of cellular and molecular alterations that results in a patient having an active epileptic condition, the process of transformation from a normal network to a hyperexcitable network. Ictogenesis refers to the process of seizure generation, the transition from an interictal state (between seizures) to a seizure state (ictal) (Bromfield et al., 2006; Devinsky et al., 2018; Pitkanen et al., 2014). Ictogenesis (i.e. neuronal hyperexcitability) is contingent upon the potential distinction between the neuronal membrane's intracellular and extracellular space and the consequent context for action potential generation (Bromfield et al., 2006). A hyperexcitable state can stem from too much excitatory signal, too little inhibitory signal, imbalances in ion concentration, or even small frequent additive signals (Bromfield et al., 2006). The possible epileptogenic mechanisms are also varied; they extend from dysregulation in neuronal and glial function ('intrinsic') and/or issues with network connectivity ('extrinsic'). For example, disturbances at the level of membrane structure, the synapse, ion-channels, other membrane-bound properties, and/or neurotransmitters (glutamate, gamma-aminobutyric acid (GABA), acetylcholine, dopamine, serotonin, noradrenaline, histamine and other neuropeptides and hormones); additionally even if there is only one main factor, all the rest at least have some modulatory effects (Bromfield et al., 2006; McCormick et al., 2001).

Epileptogenic aetiologies are not only complex and diverse, but major determinants of speed of diagnosis, treatment, management and prognosis of the

disorder (Shorvon, 2011). Aetiologies have been divided by the International League Against Epilepsy (ILAE) into seven main categories: structural, genetic (mostly polygenic), infectious, metabolic, immune, neurodegenerative and unknown. Each is further divided into subcategories and take into account that each is not mutually exclusive of the others (Balestrini et al., 2021; Scheffer et al., 2017) (Figure 1.1). Identifying the development of epileptogenesis is challenging because, in most cases, the underlying aetiology doesn't have a clear onset and the condition is only recognised once a seizure has already occurred (Pellinen, 2022). Epileptogenic factors arise from cell loss and/or circuity reorganization, both which can aetiologically develop from developmental malformations of cortical structures, traumatic brain lesions or injury, tumour complexes (e.g., tuberous sclerosis complexes, neoplasms or hypothalamic hamartomas), infections to the central nervous system (CNS) (e.g., meningitis or encephalitis), vascular disease (e.g., cerebral infraction or haemorrhage), autoimmune disorders, other neurodegenerative diseases (e.g., dementia or multiple sclerosis), or various genetic mutations (Ono et al., 2012; Pitkanen et al., 2014). There are more than 950 epilepsy-associated genes that have been identified, with specific gene mutations covering 23 epileptic phenotypes (Wang et al., 2017). Even further, localization and extent of epileptogenic processes within the brain also contribute vastly to the epileptic phenotype.

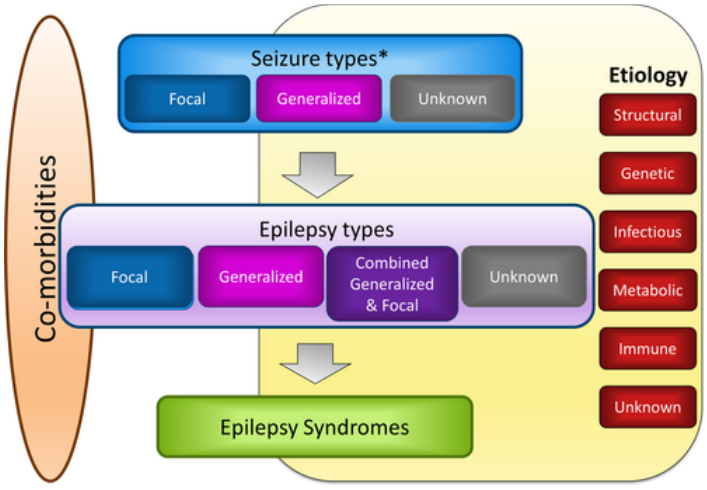

Figure 1.1. Framework for classification of the epilepsies (*Denotes onset of seizure). Image from Scheffer et al. (2017).

In theory, every part of the brain can generate seizures, however, identifying specific anatomical origin and alterations can be integral for syndrome classification and treatment. The most common areas are the hippocampus, amygdala, frontal cortex, temporal cortex, and olfactory cortex (Chauhan et al., 2022). Notwithstanding, researchers have mainly focused on three general structures, the cerebral cortex, the hippocampus and the thalamus (Balestrini et al., 2021; McCormick et al., 2001). For instance, the hippocampus, involved in memory, learning, emotions and spatial navigation, is of particular interest as there is a big link between it and the more common epilepsy syndromes. Seizures are known to cause abnormal neurogenesis in the hippocampus, and features like hippocampal sclerosis are associated with medial temporal lobe epilepsy and is even required for diagnosis (Bromfield et al., 2006; Chauhan et al., 2022). Furthermore, about 75% of epilepsy occurs during childhood, which reflects the heightened susceptibility of the developing brain to generate seizures and form epileptogenesis (Chauhan et al., 2022). All in all, anatomical patterns can vary greatly depending on the type of seizures and the epilepsy syndrome. Understanding the anatomical basis of the specific syndromes can allow a better understanding of the disease, help with diagnosis and tailor treatment options.

1.1.2 Current Treatment Options

The risk of not obtaining proper treatment for subsiding seizure frequency is associated with poor health outcomes, high expenses and healthcare utilization, and the risk of life threatening episodes like status epilepticus and SUDEP (Chen et al., 2023). Consequently, treatment management is essential, with the primary goal being the elimination or reduction of seizure severity and frequency. The specific epilepsy treatment modality depends on seizure type and diagnosis of an epileptic syndrome (Liu et al., 2017). A wide range of treatment options exist, primarily pharmacological and to a minor extent non-pharmacological. Often, management strategies involve the utilization of multiple treatment types contemporaneously. However, even with diagnosis, epilepsy patients often face vast gaps in optimal seizure control with current treatments (Pellinen, 2022).

Pharmacotherapy is the first line of treatment, starting with monotherapy, although combination therapy is readily used (Liu et al., 2017). As of 2024, the US Food and Drug Administration (FDA) had more than 30 drugs approved for treatment of epilepsies (Sánchez et al., 2024). Some of the most widely used ASMs worldwide are: carbamazepine, clobazam, clonazepam, ethosuximide, gabapentin, lamotrigine, levetiracetam, oxcarbezepin, phenobarbital, phenytoin, primidone, valproic acid, vigabatrin, zonisamide, cannabidiol, and ganaxolone (Liu et al., 2017; Sánchez et al., 2024). In Australia, in the period of 2019-2020, valproate, levetiracetam, lamotrigine, topiramate and carbamazepine were the top 5 most prescribed ASMs under the Pharmaceutical Benefits Scheme (Health et al., 2022). The known mechanisms of action of each drug vary, with targets including sodium, potassium and calcium channels, GABA transporters and receptors, glutamatergic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and synaptic vesicle glycoproteins (Sánchez et al., 2024). Each drug is selected for a patient based on the nature of seizure type, age, gender and side effects. Depending on the individual, discontinuing ASMs is considered if seizure-freedom is achieved for a prolonged period of time (Liu et al., 2017).

Sadly, the therapeutic use of many ASMs are hindered by side effects, low tolerability, low selectivity, and treatment resistance (Sánchez et al., 2024). In Australia and globally, ~30% of patients with epilepsy whose seizures are resistant to ASM treatment, also referred to as refractory-epilepsy (Chen et al., 2023; Health et al., 2022; Klein et al., 2024; Stafstrom et al., 2015). This has driven the development of alternative routes of management.

Surgical intervention targeting seizure onset sites is a viable option in a small subset of individuals with epilepsies of refractory-focal nature and well-defined lesions. Although the number of people with refractory epilepsy that are suitable for surgical intervention cannot be adequately determined, it's been hypothesized that it could range from 10% to over 50% (Engel, 2018). Some of the most commonly used surgeries include, but are not limited to: corpus callosotomy, corticectomy, and hemispherectomy (Health et al., 2022). Adequate results have been achieved in many cases of surgical intervention, and many patients do gain seizure freedom; however, the invasive and permanent nature

of the procedure, as well as the current lack of well-defined long-term improvements in cognitive, psychiatric, psychosocial and quality-of-life outcomes, makes this treatment an option of last resort (Engel, 2018; Jobst et al., 2015).

Other non-pharmacological approaches are considered useful adjuncts in intractable patients (Liu et al., 2017; Perucca et al., 2018). One of such methods is the ketogenic diet, a diet comprised of high fat (90% substrate intake), low carbohydrate and low protein consumption. It has been in use for the treatment of epilepsy since the 1920s, where positive outcomes leading to strong clinical support for this treatment modality amongst its exponents. However, more generally there is a low rate of use of this intervention, (Borowicz-Reutt et al., 2024; D'Andrea Meira et al., 2019). One of the biggest challenges is its reported unpalatability, which reduces patient compliance and adherence (Borowicz-Reutt et al., 2024). Regardless, although in the past the ketogenic diet has been considered a last resort for treatment due to its restrictive nature, it is now believed the diet might be better implemented early, especially in paediatric epilepsies (Borowicz-Reutt et al., 2024). The intent of the diet is to transition from using carbohydrates to fats as primary fuel in order to induce catabolism of fatty acids in the liver and produce ketone bodies, which is referred to as ketosis (D'Andrea Meira et al., 2019). Interestingly, the exact mechanism in which the diet offers seizure-relief benefits is still debated. The overall theory centres around diet-induced modification of neuronal metabolism resulting in reduced neuronal hyperexcitability through one or multiple factors associated with to the reduction in glucose concentrations and increase of ketone bodies, including: the modification of mitochondrial function and energy reserves that promote the stabilization of the synapse; diet-related metabolites like ketone bodies inhibiting excitatory receptors and/or enhancement of synaptic GABA concentrations; modulation of sleep patterns, and the remodelling of the gut microbiome and gut-brain axis (Borowicz-Reutt et al., 2024; D'Andrea Meira et al., 2019; Mishra et al., 2024). Nonetheless, research exploring the validity of these proposed mechanisms has been limited, and the true underlying nature of the anti-seizure effects of ketogenic diets remains controversial and unclear (D'Andrea Meira et al., 2019; Mishra et al., 2024).

Other emerging non-pharmacological treatments include neurostimulation procedures that aim to modulate nerve activity and reduce hyperexcitability. Current

options include vagus nerve stimulation, responsive neurostimulation, deep brain stimulation, chronic subthreshold cortical stimulation, transcranial magnetic stimulation, and transcranial direct current stimulation (Liu et al., 2017; Perucca et al., 2018; Schulze-Bonhage, 2019; Skarpaas et al., 2019; Starnes et al., 2019). Each procedure is different, the transcranial stimulations are 'non-invasive', while the rest involve the surgical insertion of stimulation electrodes in different areas of the brain in order to administer electrical impulses into specific or general structures (Starnes et al., 2019; Vonck et al., 2012). These procedures are normally targeted to intractable-focal epilepsies, and have shown strong indications of safety, tolerability and efficacy (Schulze-Bonhage, 2019; Skarpaas et al., 2019). Each treatment has its own strengths and limitations, but many challenges remain for these alternative therapies given they are not yet highly standardized and the their long-term effects and mechanisms of action remain elusive (Schulze-Bonhage, 2019; Vonck et al., 2012). Further research is needed to better understand the underlying processes and create optimal stimulation and patient guidelines (Starnes et al., 2019), but so far, these treatment options remains in their infancy.

Gene therapy is a novel and rapidly emerging treatment in the epilepsy research space. Although no epilepsy gene therapeutic has been approved by any major governmental agency at the time of this thesis being written, we have never been closer due to advancement in gene technologies in vector delivery and discoveries such as CRISPR/Cas9 (Cai et al., 2024). Currently, gene therapy options either aim for symptom attenuation or target the cause, which either involves the modulation and regulation of synaptic receptors, channels, neuropeptides, and/or other neurotrophic factors. These can be done via gene supplementation and modulation, RNA preparations, base editing, gene editing, or epigenetic regulation (Cai et al., 2024; Street et al., 2023). There has been an outstanding amount of preclinical and clinical studies focused on gene therapy for the treatment of monogenic (heavily associated with developmental and epileptic encephalopathies (DEEs)) and refractory-focal epilepsies (Street et al., 2023). A recent notable example for preclinical space is University of Michigan's adeno-associated viral vector (AAV) encoding β1 subunit cDNA (AAV-Navβ1) for treatment of a *Scn1b*-null mice model of DEE52 (associated with a severe variant of Dravet syndrome). Injection of the

vector achieved great success in channel restoration, the intensity and length of spontaneous seizures were significantly reduced, lifespan was extended, hyperthermia-induced seizures were prevented, and cortical neuron excitability was restored (Chen et al., 2025a). In the clinical space, Capsida Biotherapeutics recently (May 2025) received clearance by the FDA for their investigational new drug (IND) CAP-002 for clinical trials, an intravenously administered gene therapy for syntaxin-binding protein 1 developmental and epileptic encephalopathy (STXBP1-DEE) (Capsida-Biotherapeutics, 2025). Given that this space of therapeutic research is just emerging, various limitations still stand for their clinical application. These include challenges in method development, such as the need for therapy individualization due to the vast diversity and genetic complexity of epilepsy types, issues with intervention targeting, timing, and precision, the limited number of available human-relevant models for testing, and the risk of potential side effects like immune reactions and toxicity; as well as ethical considerations, such as the regulation of standardized protocols, generation of ethical frameworks to guide clinical applications, and patient selection prioritization (Cai et al., 2024). Time is still needed before these therapies are introduced alongside traditional pharmacotherapies, but they show great promise.

Whilst there is an extensive list of treatments available and a vast amount of research advancements on the way, epilepsy treatment often remains inadequate. This fact begs the question, “what is contributing to this lack of treatment efficacy?” Although the answer is not crystal clear, a large contributing factor is the extensive complexity, and our limited understanding of this complexity, of the nervous system, and the boundless variety of observed epileptic aetiologies, epilepsy subtypes, and even variability within the subtypes. Better understanding of this complexity will undoubtably allow for the development of more efficacious treatment.

1.1.3 Definitions and Classification of Seizures and Epilepsy Syndromes

While epilepsy can be regarded as a general umbrella term, ‘epilepsy syndromes’ describes the sub-categories of epilepsies defined by a collection of specific characteristic symptoms. i.e. seizure type, age of onset, triggers, structural, metabolic,

immune and genetic profile, electroencephalogram (EEG) patterns, prognosis and response to therapies (Stafstrom et al., 2015; Wirrell et al., 2022a). The most recent ILAE classification framework for epileptic seizures recognizes 21 distinct types (Beniczky et al., 2025), a significant reduction from their 2017 framework which contained 63 (Fisher, 2017). The framework begins with identifying and categorizing 'Classifiers', which identify seizure origin and are divided into four categories: focal (origin limited to one hemisphere or specific location, consistent site of onset), generalized (involves both hemispheres), unknown (some information available, but no clear focal or generalized classification) or unclassified (no information available for classification). Next the level of awareness (or consciousness) during the episode is appraised, defined as the level of responsiveness during the seizure and remembering of the event (i.e. impaired or preserved). Seizures can also manifest with a variety of other signs and symptoms (Ono et al., 2012), and so the next step takes into account additional features, or 'Descriptors' as stated in the framework. Such factors are defined as observable manifestations that are identified by observation, namely, elementary and complex motor manifestations (e.g., atonic, clonic, myoclonic, automatisms, spasms, etc.) and non-motor (aphasic, sensory, autonomic, and emotional) (Beniczky et al., 2025). It is also important to note that the above classification system excludes neonatal seizures due to their possession of unique characteristics, and thus have a separate framework (Pressler et al., 2021).

Epilepsy syndrome diagnosis is an extension of epileptic seizure type identification. The latest ILAE position on epilepsy syndromes classification divides their diagnosis frameworks based on various factors (Wirrell et al., 2022a; Wirrell et al., 2022c). First, age of onset, with its sub-divisions as follows: neonates and infants (up to 2 years), childhood, and variable ages (both paediatric patients and adults), and an additional classification for idiopathic generalized epilepsies (IGEs). Within these, syndromes are further classified based on epileptic seizure type: generalized, focal, focal and generalized, and with a separate category for those considered DEEs or with progressive neurological deterioration (e.g., progressive myoclonic seizures). Presentation of DEEs are often first noted in childhood and associated with strong cognitive impairments, which may result from both underlying aetiology and recurrent epileptic activity (Wirrell et al., 2022a). An additional step in syndrome classification

within the framework has to do with aetiology recognition; i.e., a clearly defined and consistent structure to the syndrome. These include structural anomalies or genetic mutations, such as mesial temporal lobe epilepsy with hippocampal sclerosis, gelastic seizures with hypothalamic hamartoma, and monogenic epilepsies, including *CDKL5*-DEE, *PCDH19* clustering epilepsy, and Dravet syndrome (*SCN1A*) (Wirrell et al., 2022a; Wirrell et al., 2022c).

1.2 Dravet Syndrome

Dravet syndrome is a rare form of DEE with onset in infancy. It is characterized by seizures, the development of cognitive-behavioural and social disabilities, sleep difficulties, and a high risk of SUDEP (Dravet, 2011). Although the causative genetic mutation is known in at least in 80% of cases, the *SCN1A* (Bender et al., 2012), much remains unclear about the disease, including complete understanding of the pathophysiology, epileptogenesis, and aetiological profile. It is no surprise then, that treatment success in Dravet syndrome remains generally low, with patients rarely achieving seizure-freedom through available treatment paradigms (Samanta, 2025), and 73% of patients dying before the age of 10 (Shmuely et al., 2016). Therefore, there is a highlighted need to explore more of the Dravet syndrome pathophysiology, not only for the improvement of disease outcomes, but would also advance the broader field of epilepsy research.

1.2.1 Hallmarks of Dravet Syndrome

Dravet syndrome was first described in 1978 by Dr Charlotte Dravet as a severe myoclonic epilepsy of infancy (Dravet, 2011). It has an estimated incidence of 1 in 16,000 live births (Wu et al., 2015) . The most recent ILAE classification framework categorises Dravet syndrome as a DEE with onset in infants and appearing in both sexes. It presents itself in the first year of life (3-9 months) as prolonged, febrile and afebrile, focal (hemiclonic) tonic-clonic or generalized clonic seizures, with sporadic additional seizure types, like myoclonic and atypical seizures, appearing later in life (Zuberi et al., 2022). Dravet syndrome has a differential diagnosis to myoclonic atonic seizures, Lennox-Gastaut syndrome (LGS), generalized epilepsy with febrile seizures plus, febrile infection-

related syndrome, and hemiconvulsion-hemiplegia-epilepsy syndrome (Specchio et al., 2022). Other notable aspects of the syndrome are its drug-resistance, intellectual disabilities, motor and behavioural impairments, sleeping difficulties, and higher risk of SUDEP than other childhood epilepsies (Dravet, 2011; Lagae, 2021). A review by Shmuely et al. (2016) investigating mortality in Dravet syndrome, reported SUDEP as the major cause of death (almost 50% of cases), followed by status epilepticus (32%), accidental deaths (8%), and infections (5%).

Genetic mutations are common in epilepsy, often being referred to as epileptic aetiologies or epileptogenic factors (Steinlein, 2008). The most common mutations in Dravet syndrome (70-85%) are *de novo* missense mutations in the *SCN1A* gene (Claes et al., 2001; Dravet, 2011; Zuberi et al., 2022), though other less common genetic variants identified include mutations in *SCN1B*, *SCN9A*, *GABRG2*, *GABRA1*, and *STXBP1* (Martins Custodio et al., 2023; Singh et al., 2009; Steel et al., 2017; Zuberi et al., 2022). *SCN1A* encodes for the voltage-gated sodium channel 1 alpha subunit ($Na_v1.1$) and, incidentally, is also associated with other epilepsies, such as, partial epilepsy with antecedent febrile seizures and GEFS+ phenotypes (Ding et al., 2021; Wang et al., 2017). The family of sodium channel proteins comprises of 9 members, $Na_v1.1$ being one of them (Bender et al., 2012). This family is essential for the excitability of neurons via the initiation and propagation of action potentials. $Na_v1.1$ is comprised of four repeated domains (DI-DIX) of six transmembrane segments (S1-S6), each with a different role. The $Na_v1.1$ protein is mainly expressed in the axonal initial segment of GABAergic interneuron and thus, seizure susceptibility is attributed to the channel's loss-of-function impairment of interneuron functionality, and thus disrupted excitatory and inhibitory balance in neurotransmission (Bender et al., 2012; Cheah et al., 2012; Tiraboschi et al., 2020). The onset of the disease in infancy is consistent with the brain developmental expression of $Na_v1.1$, and the functionality of the protein differs depending on the mutation location on *SCN1A* and so does the phenotype severity (Bender et al., 2012).

The diagnosis of Dravet syndrome requires the series of typical clinical features mentioned above and cannot be made due to the *SCN1A* genetic variant alone, and the absence of the variant cannot exclude the possibility of the syndrome diagnosis (Zuberi et al., 2022). Additionally, 10% of patients who are thought to have the *de novo SCN1A*

mutation have a parent who is mosaic for the variant, and 30-50% of them have family history of febrile seizures and other epilepsies (Sun et al., 2010; Zuberi et al., 2022). Additionally, the gene has been identified to undergo various possible mutations, some leading to loss-of-function, but other with apparent gain-of-function (Brunklaus et al., 2022; Clatot et al., 2023); whilst the prior is highly associated with the severe Dravet syndrome phenotype, the latter is mostly associated with familial hemiplegic migraine type 3, but also links to a broader range of early-onset epileptic disorders with distinct features to Dravet syndrome (Brunklaus et al., 2022; Clatot et al., 2023). Altogether, although we know *SCN1A* mutation is often involved in Dravet syndrome's epileptogenesis, a lot of the aetiology and surrounding context of the pathophysiology remain a mystery.

Brain anatomical structures of patients with Dravet syndrome are in most cases considered normal, thus neuroimaging is not usually obtained as it is not required for diagnosis (Zuberi et al., 2022). However, anatomical abnormalities have been reported in older children and adults, i.e., those with an extended record of seizure presentation. These include: hippocampal sclerosis; enlarged ventricles; focal cortical dysplasia; cerebellar and cerebral atrophies; global volume reductions of grey matter and white matter; reduced cortical folding in the right precentral gyrus; and also damage more specifically localized to areas in the brainstem, cerebellum, corpus callosum, corticospinal tracts and association fibres (Pérez et al., 2014; Siegler et al., 2005; Striano et al., 2007), damage commonly observed with atypical myelination (Richards et al., 2021). Notably, one study found that such abnormalities appeared more often in patients absent of the characteristic *SCN1A* mutations (Striano et al., 2007).

Although the role of interneuron malfunction is indisputable in Dravet syndrome, it's been argued that epileptogenesis progression extends beyond it, with evidence for alterations in excitatory neurons (Chopra et al., 2014; Jones et al., 2024). Further, the interneuron dysfunction appears to be transient and is normalized later in development in a mouse model of Dravet syndrome (Favero et al., 2018).

There are currently no reliable biomarkers that can be used to measure epileptogenesis in Dravet syndrome (Pitkanen et al., 2014); however, other epileptogenic

factors have been found in preclinical studies, including abnormally increased mTOR signalling (Tsai et al., 2025) and microglial cell dysfunction (Chen et al., 2024). These findings suggest that much remains to be understood about the underlying biological mechanisms of Dravet syndrome, and it is hoped that the deeper understanding of its biology will uncover new therapeutic avenues.

1.2.2 Current Treatment Strategies for Dravet Syndrome

There is currently no cure for Dravet syndrome. Treatments are available; however, up to 90% of patients still experience inadequate seizure control, evidencing the condition's high resistance to treatment (Samanta, 2025). Managing Dravet syndrome poses several challenges. It involves treating multiple seizure types that can evolve over time, and it needs to account for individual-specific phenotypic variability (Strzelczyk et al., 2022). For the time being, treatment focuses on controlling seizures, but due to the disease's complexity, the clinical goal is often to simply reduce seizure frequency and prevent prolonged seizures or status epilepticus (Wirrell et al., 2022b). For this, polypharmacy is commonly employed, which brings numerous additional complications: increased potential for adverse events, drug-drug interactions, and drug-induced exacerbation of seizures. Pharmacological treatment is further complicated by variable drug availability across countries and the difficulty of optimizing both drug combinations and serum concentrations simultaneously (Strzelczyk et al., 2022).

As previously mentioned, there are currently ~30 ASMs approved to be used for the treatment of epileptic-seizures (Sánchez et al., 2024). However, only a smaller number are recommended for refractory-epilepsies such as Dravet syndrome. According to expert consensus, the hierarchy of medications for Dravet syndrome is as follows:1) First-line medication is valproate, a multimodal agent that inhibits GABA transaminase and voltage-gated sodium channels; 2) Second-line treatments include clobazam, a benzodiazepine that behaves as a positive allosteric modulator (PAM) of the $GABA_A$ receptor, fenfluramine, a serotonergic stimulator, and stiripentol, also a multimodal drug that is also a $GABA_A$ receptor PAM as well as an inhibitor of lactate dehydrogenase and T-type calcium channels; 3) Pharmaceutical grade cannabidiol (CBD) is considered a third-line treatment and is only approved as an add-on therapy under the pharmaceutical form

Epidiolex, though interestingly, the anti-seizure mechanism of action of CBD is not fully understood; 4) Alternative third-line medications include topiramate and bromide. Other minor available pharmacotherapies are levetirecetam, brivaracetam, zonisamide, ethosucimide or perampanel (Cross et al., 2019; Lagae, 2021; Strzelczyk et al., 2022; Wheless et al., 2020; Wirrell et al., 2022b). The ketogenic diet is also recommended as an alternative non-pharmacological therapy, and vagus nerve stimulation is used in rare instances (Strzelczyk et al., 2022).

In contrast, seizure exacerbation in Dravet syndrome has been observed with drugs that block voltage-gated sodium channels, like carbamazepine and lamotrigine (Zuberi et al., 2022). These drugs are therefore contraindicated in the treatment of Dravet syndrome. Additionally, even though some patients have structural brain abnormalities, surgery is currently not often employed due to lack of evidence on its efficacy (Vezyroglou et al., 2020).

Notwithstanding the diversity in available therapies and the vast amount of research presently underway, there is still a lot of unknowns about the disease and current effective therapies are still inadequate to achieve seizure freedom and attenuate comorbidities. Hence, there is a heightened need for the expansion of treatment options, a task that is only possible via a better understanding of the disease. Thankfully, a widely used tool for such explorations exists, namely, mouse models of Dravet syndrome.

1.2.3 Mouse Models of Dravet Syndrome

Several mouse models of Dravet syndrome exist with heterozygous loss-of function mutations in the *Scn1a* gene (Jones et al., 2024). These models include knock-in, knock-out and conditional knock-out models (Jones et al., 2024). These models closely mimic the core clinical features of the syndrome in humans: susceptibility to febrile seizures, spontaneous seizures within the first 2-3 weeks of life, and premature death (Cheah et al., 2012; Higurashi et al., 2013; Jones et al., 2024; Miller et al., 2014; Oakley et al., 2009; Ricobaraza et al., 2019; Uchino et al., 2021; Yu et al., 2006). These models also exhibit neuropsychiatric comorbidities, such as low sociability, learning

deficits, disrupted sleep, hyperactivity and stereotyped behaviours (Bahceci et al., 2020; Han et al., 2012; Ito et al., 2013).

Dravet syndrome mouse models are thus a tool to help support novel insights into the biological basis of Dravet syndrome pathophysiology. For instance, they helped uncover impaired GABAergic signalling in Dravet syndrome, which helped explain the mechanism for excessive neuronal excitability due to disinhibition of GABAergic interneurons in the cortex and hippocampus (Cheah et al., 2012; Jones et al., 2024; Layer et al., 2021; Ogiwara et al., 2007; Yu et al., 2006). Furthermore, the mice emulate the developmental pattern of expression of $Na_v1.1$ seen in humans, where interneuron dysfunction emerges gradually, matches the process of *Scn1a* upregulation, and ultimately converges to seizure expression (Cheah et al., 2013; Favero et al., 2018). In contrast, it has also been found that heterozygous inactivation of *Scn1a* in excitatory pyramidal neurons does not result in an epileptic phenotype, but rather might help explain developmental abnormalities (Dutton et al., 2013). These mouse models of Dravet syndrome also helped explain how loss-of-function of *Scn1a* in different GABAergic neuron populations subserves different behavioural manifestations of the disease. For example, $Na_v1.1$ dysfunction in parvalbumin (PV)-positive interneurons promotes autistic-like symptoms, while deletion in somatostatin-positive interneurons causes motor hyperactivity (Rubinstein et al., 2015).

1.2.3.1 The *Scn1a*$^{+/-}$ Mouse Model of Dravet Syndrome

The *Scn1a*$^{+/-}$ mouse model was developed by A/Prof Jennifer Kearney at Northwestern University, Chicago. This model has been widely used as an effective screening tool for potential ASMs via testing the test intervention's ability to reduce hyperthermia-induced seizures, spontaneous seizures and increase lifespan (Hawkins et al., 2017). The mouse model responds to medications used to treat Dravet syndrome patients. The first-line treatment clobazam has anti-seizure effects in the model, while compounds that are strong sodium channel blockers, e.g. lamotrigine, are pro-convulsant (Hawkins et al., 2017). Similarly, CBD has anticonvulsant effects in the model and even influences the syndrome's comorbidities, such as rescuing autistic-like cognitive symptoms (Anderson et al., 2019a; Kaplan et al., 2017).

This model has also been a great tool to explore the pathophysiological nature of the disease. This comes about via the classical comparison of physiological differences driven by genetics (i.e., comparing the physiology and pharmacology of mice with and without the genetic deletion of *Scn1a*), but also via observing strain-dependent neurochemical variabilities. The latter occurs because, depending on strain, the *Scn1a*$^{+/-}$ mice show distinct phenotypic properties in terms of seizure severity and mortality rate. The *Scn1a*$^{+/-}$ mice on a 129S6/SvEvTac background strain (129.*Scn1a*$^{+/-}$) has no overt phenotype and do not have spontaneous seizures, while the forward crossing of 129.*Scn1a*$^{+/-}$ mice with wildtype C57BL/6 mice results in a F1 (129 x C57BL/6) background strain combination that is susceptible to seizures. The F1.*Scn1a*$^{+/-}$ mice have the severe seizure phenotype, and are sensitive to febrile seizures, develop spontaneous seizures and premature mortality (Hawkins et al., 2016; Miller et al., 2014; Rubinstein et al., 2015; Yu et al., 2006). This observation infers that the resultant modified alleles introduced by the C57BL/6 strain are important to unmasking the epileptic phenotype and the penetrance of the *Scn1a* genetic manipulation. Coincidentally, this quite aligns with human presentation of the disease, as it has been often found that even when two people have the same specific type of *SCN1A* mutation, they can still experience markedly different seizure severity, effectively suggesting the presence of additional genetic modifiers or modulating environmental effects (Hawkins et al., 2012; Hawkins et al., 2016). The application of animal models such as this one have vastly aided on the identification of novel markers and pathways that have had significant impact on understanding disease pathophysiology and guiding new therapeutic approaches (Lai et al., 2022). This mouse model is thus an invaluable tool for the novel exploration of new disease mechanisms and treatment strategies.

1.3 Lipids in the Context of Dravet Syndrome

The association of lipids with Dravet syndrome pathophysiology is worth considering given the strong connection between lipid regulation of neuronal excitation. Fundamentally, the brain is a fatty organ containing an abundance of lipids that help support normal brain functions (Incontro et al., 2025; Tracey et al., 2021). Lipids have been implicated in numerous brain disorders including Alzheimer's disease, Parkinson's

disease, major depression, autism, multiple sclerosis, Huntington disease and Niemann-Pick disease (Adibhatla et al., 2007, 2008; Hussain et al., 2019). Growing evidence suggests that lipid dysregulation may also occur in seizure-related disorders and epilepsy (Wang et al., 2024). However, more research is needed to better understand how lipid dysregulation might be involved in the pathophysiology of drug-resistant epilepsies like Dravet syndrome, as well as a need to better understand the mode of action of emerging lipid targeting therapeutics. This section will begin with defining the concepts of lipids and the lipidome, an introduction to the brain lipidome, the current association between lipid signalling, metabolism and Dravet syndrome, and the case for cannabinoids.

1.3.1 Lipids and the Lipidome: Definitions and Classification

Lipids are hydrophobic or amphipathic biomolecules, often small and soluble in organic solvents. All lipids originate from two distinct building blocks (ketoacyl and isoprene) whose diverse combinations of head groups, fatty acyl chain lengths, and saturation properties contribute to an estimated ~100,000 unique lipid structures (Fahy et al., 2011; Fahy et al., 2005; Yoon et al., 2022). Quantitative and qualitative measurements of lipids are complex due to an abundance of isobaric properties (identical molecular formula and weight but different spatial configurations) and iso-elemental species (compounds with identical elemental compositions, but are different chemical species) (Quehenberger et al., 2010). However, as defined by the LIPID Metabolites and Pathways Strategy (LIPID MAPS), lipids can be classified into eight main categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids and prenol lipids. These categories are structured based on chemical backbones and biosynthetic logic, which is also is tied to function (Fahy et al., 2011; Fahy et al., 2005; Fahy et al., 2009; Liebisch et al., 2020; Liebisch et al., 2013). Table 1.1 presents a summary of the eight lipid categories, together with a basic description of their structure, key variants, and brief summary of biological role.

Table 1.1. Core structure, key variants, and major biological roles of all 8 lipid categories as defined by LIPID MAPS. (Cockcroft, 2021; Fahy et al., 2011; Fahy et al., 2005; Fahy et al., 2009; Liebisch et al., 2020; Liebisch et al., 2013).

Lipid Category	Core Structure	Key Variants	Biological Role
Fatty Acyls	Chains built from acetyl-CoA or malonyl-CoA	Fatty acids, and alcohols, aldehydes, amines, esters functional variants	Central to energy metabolism and structural integrity
Glycerolipids	Glycerol backbone with fatty acid chains	Mono-, di-, and triacylglycerols	Primarily used for energy storage
Glycerophospholipids	Glycerolipids with a phosphate-containing head group	Phosphatidylcholine, phosphatidylethanolamine, etc.	Major components of cell membranes
Sphingolipids	Long-chain sphingoid bases with fatty acid chain	Ceramides (Cer), sphingomyelins (SM), glycosphingolipids	Key structural elements of membranes, involved in cellular signalling (especially in neural tissue)
Saccharolipids	Fatty acyl groups linked directly to a sugar backbone	Lipid A, acylated glucosamines	Found in bacterial membranes
Polyketides	Repeating ketide units derived from acetyl and malonyl building blocks	Rapamycin, doxorubicin, erythromycin and tetracycline	Common in plants and microbes, often bioactive (e.g., antibiotic or toxic properties)
Sterol lipids	Derived from isoprene units; characterised by fused ring system	Cholesterol, steroid hormones	Regulate membrane fluidity, hormone signalling
Prenol lipids	Isoprene-derived chains, without the fused ring structure of sterols	Vitamin K, ubiquinones	Involved in electron transport and vitamin activity

Each main lipid category is further divided into its own subclassification hierarchy, starting with class, then subclass, and in some instances an extra 4th level classification (Fahy et al., 2011; Fahy et al., 2005; Fahy et al., 2009; Liebisch et al., 2020; Liebisch et al., 2013). While classes are classified more broadly by their polar head groups, subclasses vary in rule, but, for example, can be classified based on existence of different chemical bonds or due to differences in their backbone (Han et al., 2022). Consequently, each category can reach thousands of unique molecular species. Currently, LIPID MAPS' database (LIPID MAPS Structure Database (LMSD)) has 49,732 unique structures (LIPID-

MAPS, 2025). Moreover, each class is not stagnant, they transform, interact between each other, and possess their own set of properties (Quehenberger et al., 2010). This totality of lipid structures and dynamic interaction is what is referred to as the lipidome.

The lipidome and its lipids have become increasingly appreciated as a central biological factors affecting human physiology and pathophysiology (Liebisch et al., 2020). They possess multiple functions, including energy source, cell membrane integrity support, and a signalling messenger (Yoon et al., 2021). They have essential roles at every step of the biological hierarchy of size, from micro organelles (Celik et al., 2023), to single cells (Dennis et al., 2010; Ejsing et al., 2009; Klose et al., 2012) and all the way to organs (Ference et al., 2018; Knox et al., 2021), lipids rule physiological homeostatic. Because of this, lipid dysregulation can result in major consequences including the development of disease. Although lipids in pathology are most commonly associated with excessive accumulation in adipose tissue, obesity, and cardiac disease (Yoon et al., 2021), abnormal lipid homeostasis extend beyond that, for instance, they are also linked to disruption in protein misfolding and dysregulation of inflammation pathways (Gleeson et al., 2016; Vendruscolo, 2022). Most notable research spaces in lipid pathophysiology include atherosclerosis (Ekroos et al., 2010), non-alcoholic steatohepatitis (Musso et al., 2023) and various forms of cancers (e.g., lung (Lemay et al., 2019), liver (Chen et al., 2018; Liu et al., 2022), and breast (Bougnoux et al., 2008)). The brain lipidome is of particular importance in the lipid research space, this is because it possesses an exceptionally rich and complex lipid content, and lipids have a pivotal role in neuropathologies (Osetrova et al., 2024; Yoon et al., 2022)).

1.3.2 The Brain Lipidome

The brain is a complex network of lipid-rich systems. Lipids account for 50% of dry brain weight, making it, the most lipid enriched organ in the body aside from adipose tissue (Sastry, 1985). Although brain lipids were once solely thought to be important for membrane structure integrity, we now know their functionality extends to cell signalling, energy metabolism, and inflammatory response (Agranoff et al., 1999; Yoon et al., 2022), and they also play an essential role in neuronal development, neuroplasticity, and action potential propagation (Incontro et al., 2025; Tracey et al., 2021).

The lipid blueprint of the brain is vast and complex. Table 1.2 presents information on major brain lipid categories, common classes, a summary of physiological role, and localization.

Table 1.2. Major brain lipid categories, specific classes, physiological roles, and cellular and region-specific localization. (Bazinet et al., 2014; Bjorkhem et al., 2004; Farooqui et al., 2000; Lauwers et al., 2016; Olsen et al., 2017; Osetrova et al., 2024).

Lipid category	Examples of specific lipid classes	Role	Localization
Glycerophospholipids	Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylglycerol (PG), Phosphatidylserine (PS), plasmalogen analogues of PC and PE	Mediate neuronal signalling; maintain membrane integrity and fluidity	Enriched in neuronal membranes and synapses; plasmalogens also abundant in myelin-rich regions
Sphingolipids	Cer, SMs, Hexosylceramide (HexCer), sulfo-HexCer	Structural support in myelin; formation of lipid-raft signalling domains	Highly concentrated in oligodendrocytes and white-matter tracts; present in lipid rafts
Sterols	Cholesterol	Regulates membrane fluidity, synaptic transmission, myelin function; acts as a signalling molecule via receptor interactions	Predominantly in myelin-rich areas (white matter); also present in neuronal membranes across regions
Fatty acyls	Oleic acid, Docosahexaenoic acid (DHA), and arachidonic acid (AA)	Building blocks for complex lipids; directly participate in cell-signalling pathways	Oleic acid in oligodendrocytes; polyunsaturated fatty acid (DHA, AA) in neocortex and other grey-matter regions
Glycerolipids	Triglycerides (TG), diacylglycerides	Cellular energy reserves	Stored in astrocytic neutral-lipid pools; most abundant in astrocytes

Brain lipids engage in an ever-growing array of roles. In addition to the well-stablished roles in membrane structural maintenance, there is a growing body of evidence that points at an essential role of lipids in brain energy metabolism, modulation of protein complexes, cellular differentiation, inflammation response, and cellular signalling (Adibhatla et al., 2007; Lauwers et al., 2016; Osetrova et al., 2024). Lipid features such as lipid class ratio, charge and size of head groups, as well as number,

length and saturation levels of fatty acid chains define the geometry, fluidity, and compartmentalization of membrane bilayers (Osetrova et al., 2024). The presence of lipids in oligodendrocytes and the overall myelination system is perhaps lipids' most well-known structural localization and functionality. The myelin is the most abundant and complex lipidome composition amongst all cell types and brain regions (Osetrova et al., 2024). Oligodendrocytes wrap neuronal axons with myelin, a modified cell-membrane that is multilayered and deeply lipid-rich (Kister et al., 2023). It is characterized by a 40% cholesterol, 40% phospholipids, and 20% glycolipids composition, in contrast to a ratio closer to 25%:65%:10%, respectively, in most other biological membranes (Poitelon et al., 2020). Additionally, while typical cell membranes have an approximatively equal 50:50 ratio of proteins to lipids, dry myelin sheath is characterized by higher proportion of lipids (70%–85%) over proteins (15%–30%). Altogether, its composition allows for its low ion permeability and ability to be an excellent electrical insulator (Poitelon et al., 2020). Myelin facilitates the saltatory conduction of neuronal action potentials via its reliable insulating property that ensures rapid propagation of nerve impulses that then form the basis of effective neuronal communication (Kister et al., 2023; Montani, 2021). Moreover, the role of lipids in myelin goes beyond a structural one, as they have also been found to regulate oligodendrocyte and myelin development (Montani, 2021).

Lipids also have essential roles in synaptic transmission. They actively engage in membrane-based sorting of neurotransmitters (Borroni et al., 2016; Postila et al., 2020). Lipophilic neurotransmitters (e.g., dopamine, serotonin, and noradrenaline) bind to and travel along the lipid-rich membrane towards their corresponding receptors sites, while lipophobic ones (e.g., glutamate, GABA, and acetylcholine) remain in the water-based medium to reach extracellular receptors (Postila et al., 2020). Further, lipids are tightly linked to protein structural elements in the membrane. Each receptor and transporter has a particularly curated membrane lipid composition that assist in this binding process, a dynamic relationship between neurotransmitters, proteins and lipids that ultimately enhances signal transmission and transduction (Postila et al., 2020). For instance, cholesterol is visible in X-ray crystal structures of various membrane proteins such as serotonin, cannabinoid, μ-opioid, κ-opioid, muscarinic acetylcholine, and adenosine receptors, as well as serotonin and dopamine transporters (Jakubík et al.,

2021; Postila et al., 2020). The link between these functionalities are lipid rafts. Lipid rafts are microdomain clusters of sphingolipids and cholesterol that liaise with membrane proteins; they provide structural support as well as actively modulating signalling complexes (Dart, 2010). Vast neurotransmitter signalling occurs in association with lipids rafts, examples include nicotinic acetylcholine receptors, $GABA_B$ receptors, AMPA and NMDA glutamate receptors, and ion channels (Incontro et al., 2025; Mencarelli et al., 2013). In addition, they play important role in neuronal cell adhesion, axon guidance, and protein localization, and, in oligodendrocytes, rafts mediate the contact between myelin associated glycoprotein and receptive neurons (Mencarelli et al., 2013). Furthermore, at the synapse, membrane lipids undergo dynamic remodelling in response to action potentials via protein-mediated endocytic and exocytic mechanism, generating lipid-rich synaptic vesicles that both release and retrieve neurotransmitters amongst other signalling molecules (Ahrends et al., 2025; Lauwers et al., 2016). Lipids associated with such signalling role include poly-unsaturated fatty acids, glycerophospholipids (phosphatidylinositol (PI) and phosphatidic acid (PA)), ceramide (Cer), cholesterol, oleic acid and arachidonic acid (AA) (Lauwers et al., 2016; Postila et al., 2020; Tracey et al., 2021).

Additionally, lipids' role in signalling dynamics go beyond the traditional protein-centric narrative of neurotransmission, as they themselves actively participate as signalling agents (Postila et al., 2020). Cholesterol stands out in this context: it is an allosteric modulator of G protein-coupled receptors (GPCRs) such as chemokine receptors (van Aalst et al., 2021), and its major metabolite 24(S)-hydroxycholesterol (24S-HC) is a potent allosteric modulator of NMDA (Nebert et al., 2013). Tropomyosin receptor kinase B, essential for cell development and survival, is tightly regulated by cholesterol levels (Postila et al., 2020), it possesses a cholesterol recognition sequence suggesting it can act as a cholesterol sensor (Biojone et al., 2023). Additionally, endocannabinoids, 2-arachidonoylglycerol (2-AG), N-arachidonoyl-ethanolamine (AEA) and are known to inhibit excitatory synaptic transmission via agonism of CB1 receptors (Castillo et al., 2012).

Taken together, the multifaceted lipid expression and functionality role of lipids underscore their involvement to virtually every feature of the brain structure and function,

which positions them as indispensable regulators of brain homeostasis (Yoon et al., 2022). Unfortunately, their high abundance and prevalent influence opens the door to system dysregulation. For instance, while there is a known entanglement between lipids and learning (Kotti et al., 2008; Lei et al., 2017) and neuroprotection (Niemoller et al., 2010; Tran et al., 2020), they are also highly associated with neurodegeneration (Couttas et al., 2018; Shin et al., 2024) and various neuropathologies, including, Alzheimer's disease (Couttas et al., 2014; Couttas et al., 2016; Hejazi et al., 2011; Lei et al., 2019), alcohol-related brain damage (Smith et al., 2022), amyotrophic lateral sclerosis (Tracey et al., 2021), multiple system atrophy (Don et al., 2014) schizophrenia (Couttas et al., 2022), and of most interest, epilepsies.

1.3.3 Lipid Alterations in Seizures and Epilepsies

Epilepsy presents a connection between lipid dysregulation, seizure presentation and epileptogenesis. Most evidence says that seizure activity causes and synergizes with changes in lipid pathways (Johnson et al., 2020; Sun et al., 2024); however, a growing number of investigations suggest that lipid dysregulation itself can also create a seizure prone-state (Almannai et al., 2021).

Various epilepsy animal models display seizure-related lipidomic dysregulation cascades that get aggravated by frequency and duration. For instance, status epilepticus causes disturbances to the metabolism of cholesterol, its major metabolite 24S-HC, and other analogue structures (Hanin et al., 2021). In their paper, Hanin et al., (2021) found that in both, mouse and humans, status epilepticus induced the reduction of 24S-HC levels and was followed by an abnormal increase of cholesterol synthesis (Hanin et al., 2021). Further, an acute kainic acid epileptic mouse model found brain-regions specific seizure-induced lipid alterations in phospholipids, SM, endocannabinoids and fatty acids (Lerner et al., 2017). In parallel, Johnson et al. (2020) found that seizure-prone voltage-gated potassium channel $K_v1.1$ knockout mice displayed distinct metabolic profiles particular to lipid biosynthesis pathway compared to wild-type, with significant upregulation of species, such as cardiolipin, in the hippocampus compared to the cortex (Johnson et al., 2020). In conjunction, Chali et al. (2015) showed that the inhibition of

cholesterol degradation via genetic alterations of CYP46A1 in mouse induced neuronal sclerosis and seizure activity in the hippocampus (Chali et al., 2015).

Moreover, pharmacological connections between lipids and epilepsy exist. For instance, statins, drugs that lower cholesterol levels, have been suggested to possess neuroprotective and anti-epileptic effects. Older adults who are prescribed statins are less likely to be hospitalized for seizures or epilepsy (Scicchitano et al., 2015). Additionally, a systematic review by Quintana-Pájaro et al. (2018) found potential anti-epileptic effects in statin consumption in both humans and non-humans. In animals, they found a reduction in epileptiform, mostly alluded to statins effect in inflammation reduction driven by the suppression of reactive astrogliosis, the stimulation of GABAergic activity and the inhibition of glutamatergic activity (Quintana-Pájaro et al., 2018). These results were only retrospective and there is limited evidence for different types of epilepsies. Consequently, the authors emphasise that although the findings are exciting, more research in this area is needed.

Altogether, there is both physiological and pharmacological evidence that connects lipid dysregulation with seizure presentation and a potential epileptogenic development. Given the vast breath and complexity of lipid involvement in normal brain functioning, it is no surprise that disruptions in its pathway are commonly implicated in disease. Various pathologies with genetic or metabolic dysfunction display seizure presentation as well as lipid dysregulation. Some inherited metabolic disorders include lysosomal and glycosphingolipid storage diseases such as Niemann-Pick type C (Vanier, 1999), GM3 synthase deficiency, Gaucher disease, and Krabbe's disease (Dodge, 2017), all defined by abnormal accumulation of specific lipids due to deficiencies their metabolic pathways. Amongst epilepsies, a syndrome that has picked up lipid-associated momentum is Dravet syndrome; however, although various connections have been made, its lipid pathways remain an underexplored area.

1.3.3.1 Lipid Signalling and Metabolism Dysregulation in Dravet Syndrome

Metabolic lipid dysregulation has been association with Dravet syndrome in both physiological and pharmacological avenues. To begin with, a viable adjunct treatment for

the syndrome is the keto diet, a lipid-rich eating regiment that actively modulates the body's lipid profile. It has shown effectivity in seizure frequency reduction (Caraballo et al., 2005), safety, and minor adverse effects (Wang et al., 2020). It is estimated that 47-60% of Dravet syndrome patients achieve more than 50% seizure reduction under the diet's treatment (Wang et al., 2020). Although it's mechanism of action remains partially unresolved, its effectivity hints at an involvement of lipid metabolism in the disease's blueprint. Miljanovic et al. (2021) explored the keto diet-driven metabolic changes in a of genetic *Scn1a*-A1783V Dravet syndrome mouse. They found that hippocampal metabolic alterations in energy metabolism were driven by both, the *Scn1a* deficiencies and the ketogenic diet, which, conversely, were tightly coupled with the neuronal hyperexcitability and seizure susceptibility of the mice. In particular, changes in glucose, glycolysis and tricarboxylic acid cycle intermediates, noradrenaline, corticosterone, GABA, and two bile acids suggested genotype-and treatment driven modulations of catabolic processes, for instance, there was a marked increased GABA-to-glutamate ratio that was suggested to serve as an endogenous compensatory mechanism for seizure-presentation (Miljanovic et al., 2021). Additionally, Figueroa et al. (2025) further evidenced a link between Dravet syndrome and lipid-associated energy metabolism. They identified metabolic defects driven by mitochondrial dysfunction in lymphoblast cells derived from Dravet syndrome patients with ketogenic diet responsiveness. The cells presented impaired bioenergetics via a reduced mitochondrial respiratory capacity, and showed a metabolic shift to fatty acid utilization, suggested to counter cellular stress (Figueroa et al., 2025). Altogether, the connection between the ketogenic diet and Dravet not only highlights lipid modulation as a viable and effective pharmacotherapy, but also evidences a link between the disease and an altered lipid-driven energy and metabolism pathway.

Furthermore, Dravet syndrome's characteristic hyperexcitability is modulated by lipids regulation. A novel lipid-associated DEE pharmacotherapy, Soticlestat, is currently on clinical trials for the treatment of Dravet syndrome and LGS. It has demonstrated therapeutic potential in various epilepsy model as well as in humans. Clinical phase 1b/2a trials found exploratory efficacy evaluation showed a reduction in seizure frequency over time (Halford et al., 2021). In animal models, it effectively reduced acute

seizure burden and chronic epilepsy-related behavioural deficits in mice following Theiler's virus infection (Barker-Haliski et al., 2023); inhibited kindling development, and significantly reduced seizure development in the absence of sedation in a PTZ mouse model (Nishi et al., 2018); and also reduced seizures and premature death in *Scn1a*$^{+/-}$ Dravet syndrome mice (Hawkins et al., 2021). Soticlestat acts as a cholesterol 24-hydroxylase (CYP46A1) inhibitor, the enzyme in charge of breaking down of cholesterol into 24S-HC, an essential process in the maintenance of cholesterol-homeostasis in the brain (Koike et al., 2021). This is essential as cholesterol is one of the most abundant lipids in the human body, the brain contains ~25% of the total body's cholesterol concentration, and it account for ~20-25% of lipid molecules in plasma membranes of most cells (Dietschy et al., 2004). Its vast expression and extensive interaction with various physiological processes means its homeostatic balance is of crucial importance, including that of its major metabolite, 24S-HC. Coincidently, to an important but minor extent, 24S-HC has also been reported as a modulator of neuronal signalling and cell survival and signal transduction, in particular, it has been reported as a positive allosteric modulator of NMDA receptors (Sun et al., 2016). Both molecules have been implicated in neuronal hyperexcitability and its dysregulation (Wheless et al., 2025), hence pharmacotherapy for their stabilization has shown great promise. Altogether, Soticlestat evidences once again that the modulation of essential homeostatic lipid pathways not only modulates neural hyperexcitation in mice (Nishi et al., 2020), but that lipid-modulation is an effective pharmacological strategy to treat epilepsies such as Dravet syndrome (Nishi et al., 2018; Salamone et al., 2022), further suggesting an underlying lipid-dysregulation in Dravet's epileptogenic events.

A crucial lipid-linked pathway also associated with Dravet syndrome is neuroinflammation. Epileptogenesis is known to involve inflammation, immune responses and disrupted brain circuits (Sumadewi et al., 2023). Seizures induce inflammatory chain reactions in the brain which often involve lipid signalling molecules. Lipids are heavily involved in neuroinflammation processes, microglial cell activation during neuroinflammation lead to the breakdown of membrane lipids and the production of AA and DHA, both which lead to the production of pro-and anti-inflammatory mediators (Farooqui et al., 2007). This mechanism has been identified in Dravet

syndrome. For instance, studies using *Scn1a*$^{+/-}$ mouse model have reported that high seizure burden increase levels of such bioactive brain lipid compounds, namely, prostaglandin and specialized pro-resolving mediators (Yip et al., 2025; Zhou et al., 2024). Another lipid- and Dravet syndrome- associated pathway is the endocannabinoid system (ECS). The ECS is an endogenous network of lipid-like molecules called endocannabinoids, most notably AEA and 2-AG, enzymes involved in their production and degradation, as well as cannabinoid receptors CB1 and CB2 (Crocq, 2020). The function of the ECS has been linked to a wide variety of physiological processes including mood, sleep, appetite, pain and immune response (Crocq, 2020), through mechanisms such as modulation of synaptic signalling and function (Castillo et al., 2012). There has been a strong connection between these networks of bioactive lipids and seizure burden in Dravet syndrome. For instance, *Scn1a*$^{+/-}$ mice with severe seizure burden showed reduced levels of the CB1 receptor, suggesting a link between the ECS and seizure susceptibility; additionally, pharmacological intervention with GAT229 (a CB1 receptor modulator) and ABX-1431 (a 2-AG degradation inhibitor) decreased the mice's susceptibility to heat induced seizures (Anderson et al., 2022).

As mentioned earlier, CBD, a plant-derived cannabinoid, is approved in several jurisdictions as an add-on therapy for the treatment of Dravet syndrome, LGS and tuberous sclerosis complex (Urits et al., 2019). It displays efficacy at seizure reduction and is often chosen due to its low adverse effects profile. Moreover, many other cannabis constituents have also shown anti-seizure properties in mouse models of the disease (Anderson et al., 2021a; Anderson et al., 2021c; Anderson et al., 2022b). And while pharmacological research on CBD as well as other cannabinoids is growing, their therapeutic mechanism of action in Dravet as well as their overall pharmacology remain underexplored (ElSohy et al., 2015), though could involve lipid modulating mechanisms. The relationship of cannabis and cannabinoids with lipid systems and Dravet syndrome, is further explored in section 3.5. For now, there is evidence that endogenous pathways such as inflammation and ECS pathways are involved in Dravet syndrome, once again stablishing the link between lipid-pathways and the disease.

As highlighted, many connections exist between lipids pathways, brain homeostasis, seizure presentation, epilepsy and Dravet syndrome. Although Dravet has

traditionally been seen as a neurogenic disease, both past and emerging research suggests that dysregulations in metabolic lipid pathways exists and may potentially be contributing to the disorder's pathophysiology. Much remains unknown about the extent of the full scale of lipid involvement in disease presentation. Whilst here I've commented on a small portion of connections, particularly to cholesterol, a broad and tightly linked lipid-network exists. To date, such system remains to be underexplored in the Dravet syndrome context, highlighting a clear need for further research in this area.

1.4 Lipidomics: The Study of the Lipidome

Due to the joint complexity of lipid biology and the disease itself, advancing our knowledge and addressing the current research gap requires novel wide-ranging approaches. A lot has been discovered in terms of the prominence of involvement and impact of lipids in health and disease. However, much of this exploration has been within a narrow scope and much is yet to be uncovered regarding the alterations in the lipidome, that is, approaches not only concerned with individual lipids, but also the interactions between themselves, other molecules, living systems as a whole and in pathophysiology of disease such as Dravet syndrome (Mutch et al., 2006).

Most recently, the application of 'omic' methodologies, particularly lipidomics, have offered a viable path for further exploration. Lipidomics has gained vast momentum since the early 2000s. This is mostly due to the earlier 'omics' revolution, a wave of investigations, most notably in genomics and proteomics, facilitated by exponential advancements in bioinformatics and analytical chemistry qualitative and quantitative methods (Voit, 2020). Such wave revolutionized and advanced the concept of profiling biological systems and allowed for the recognition of importance of other molecules within other areas, such as the metabolome (Seppänen-Laakso et al., 2009; Subramaniam et al., 2011). The metabolome is the global term used to define the profile of small low-weight molecules (also known as metabolites). It is regarded as a prominent link between genotype and phenotype (Sobti et al., 2022; Tripathi et al., 2019), as it stands in line with the genome, the transcriptome, and proteome, all which ultimately connect to phenotypical expression. It comprises of sugars, nucleotides, amino acids, and the focus of this section, lipids. The term lipidome falls under the umbrella term

metabolome, and although the area of lipidomics falls under the term metabolomics, it is considered its own distinct discipline. This is not only because they appear in significantly higher relative distribution (Quehenberger et al., 2011), but they also possess particular chemical and physical properties in comparison to the other water-soluble compounds in the metabolome (Han et al., 2022). Figure 1.2 displays the full schema of the 'omes.'

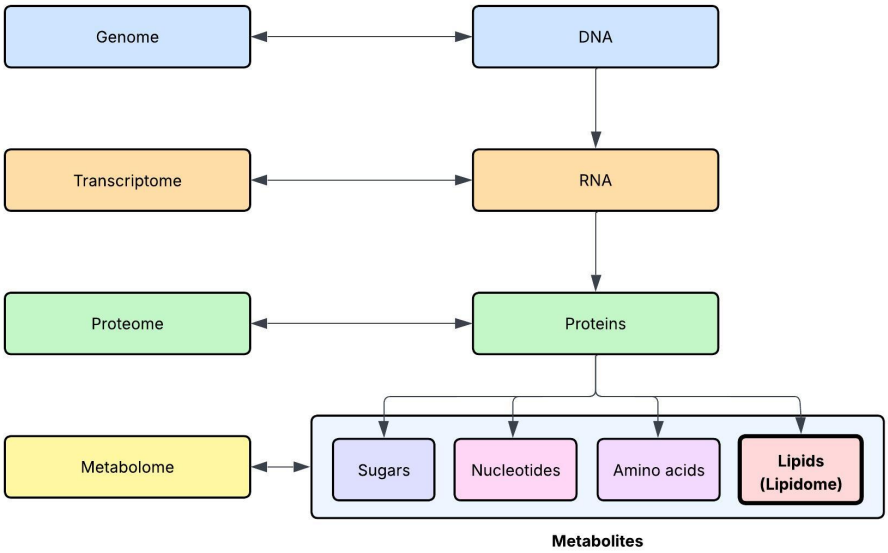

Figure 1.2. Schema of the 'omes' displaying the hierarchical connection between the genome, transcriptome, proteome and the metabolome with their corresponding molecules of interest. Figure generated with Lucid Chart.

The field of lipidomics uses complex analytical chemistry machinery, broad molecular databases, and intricate mathematical software in order to identify and quantify lipid profiles across biological samples (Kyle et al., 2021; Mutch et al., 2006). The lipidomics workflow conventionally begins with sample preparation and total lipid extraction, is then followed by mass spectrometry (MS) techniques, and wraps up with advanced bioinformatics tools to perform computational identification of compounds, data enrichment and statistical analysis (Seppänen-Laakso et al., 2009).

The breakthrough analytical technology that created the bases of the field of lipidomics were brought to life between the 1980s to 90s. These include advances in MS soft ionization methods (like electrospray ionization (ESI) and matrix-assisted laser

desorption/ionization (MALDI)), multistage fragmentation, enhancement of resolution and mass accuracy, and multilevel sample analysis (Han et al., 2022). The eventual incorporation of even more advanced strategies in direct infusion, chromatographic separation, and charge-switch derivatization allowed access to a huge pool of lipids of low abundance (Han et al., 2022). It was also in the early 2000s when the previously mentioned LIPID MAPS consortium originated. It began in 2003 by researchers of the University of California, San Diego, and funded by the National Institute of Health with the goal of advancing the state of lipidomics (Conroy et al., 2023; O'Donnell et al., 2020). Over the years, the consortium has created a standardized global system of lipid classification, nomenclature and structural drawing system as well as a leading resource of databases, tools, protocols and standards which accelerated the grow of the area of lipidomics and are now considered essential in the field (Conroy et al., 2023; Han et al., 2022).

Nowadays, the field of lipidomics utilizes the above technologies and mostly follows two strategies: untargeted and targeted. Untargeted lipidomics is used for hypothesis generation via the identification of global alterations in abundance of known and unknown molecular species. In contrast, targeted lipidomics aims to identify and quantify the abundance of specific metabolites (Han et al., 2022). MS based technologies are the primary analytical tool for both approaches. During MS analysis, samples are either analysed via shotgun-lipidomics or chromatography-based techniques, such as liquid or gas chromatography (LC-MS or GC-MS). The most widely used MS ionization techniques include ESI and MALDI as stated above, but other minorly used options are atmospheric pressure chemical ionization, atmospheric pressure photoionization, secondary ion mass spectrometry and desorption ESI. Alternatively, another relevant technique is nuclear magnetic resonance (NMR). Additionally, tandem mass spectrometric techniques are also commonly used to add additional dimensions to the data (i.e. MS/MS analysis), these include: product ion scan, precursor ion scan, neutral loss scan, and selected/multiple reaction monitoring (SRM/MRM) (Checa et al., 2015; Yang et al., 2016).

Untargeted lipidomic scans will often focus on a wide range of molecular masses and thus result in a large amount of MS or MS/MS spectra. Such data present vast

complexity in diversity of lipids and other species, consequently, it demands the use of complex bioinformatic tools to perform identification and quantification (Conroy et al., 2023; Kyle et al., 2021) such as LIPID MAPS, but the range of databases and software for lipidomic analysis expands beyond it. Other notable examples, some stemming from the LIPID MAPS family, include LipidXplorer, LipidFinder, Lipid Data Analyser, LION/web, XCMS, and LipidSeach (Breitkopf et al., 2017; Ni et al., 2023). When deciding which approach to use, researchers will consider a variety of factors, such as their underlying data structure, the research question being asked, but also what is available in their facilities.

The resulting outputs of lipidomic analysis can vary from profiling of system lipidomes, underpinning mechanisms of disease, identifying biomarkers, and even assess the efficacy of therapeutics (Ni et al., 2023; Yang et al., 2016). This wide range of lipidomic-based outcomes, together with small-scope lipid-driven investigations, has led to an immense expansion in the collective knowledge of the state of the lipidome in health and disease, as well as treatment mechanisms.

Changes in the lipidome have yet to be explored in Dravet Syndrome. Consequently, the present thesis aimed to evaluate potential changes using both untargeted and targeted lipidomic techniques on the *Scn1a*$^{+/-}$ mouse model. Chapter 3 and 4 provide a detailed account of these investigations.

1.5 Cannabis, Cannabinoids, and Epilepsy

The endocannabinoid system, as mentioned above, relies on lipid-based signalling to function. Interestingly, this system can also be modulated by a range of compounds found in the cannabis plant, and several such compounds have been investigated as novel ASMs. Thought to have originated in the north-eastern Tibetan Plateau (McPartland, 2018), cannabis has evolved and travelled together with humans amongst various cultures and timepoints in history. Although its purposes have vastly varied from being used as a textile, food, oil, consumed in religious ceremonies, and for leisure, its use as a medicine greatly stands out for its impact in society. Such medicinal uses include treatment for pain, nausea, anxiety, improvement of appetite, sleep, and

epilepsy (Crocq, 2020). Currently, the plant and its components are treated as restricted in many parts of the world; however, a growing number of jurisdictions have legalized it for recreational and/or medicinal use (Carliner et al., 2017; MacPhail et al., 2022).

Several bioactive compounds have been isolated from cannabis, including the phytocannabinoids, a group of structurally related highly lipophilic molecules produced in the trichomes of the plant. Several approved therapeutic drugs either include or are based on phytocannabinoids. For example, a synthetic form of (-)-Δ^9-trans-tetrahydrocannabinol (Δ^9-THC), the primary intoxicating constituent of cannabis, was first approved by the US FDA in 1980s in the form of dronabinol for treatment of nausea and vomiting during chemotherapy (Crocq, 2020). Nabixiols, an oromucosal spray that contains a 1:1 combination of plant-derived CBD and Δ^9-THC, was first approved in the UK in 2010 for spasticity and pain associated with multiple sclerosis. Most recently Epidiolex (or "Epidyolex" in the UK and Australia), a purified preparation of CBD, was first approved by the US FDA in 2018 for the treatment of refractory LGS and Dravet syndrome, and later for tuberous sclerosis complex (Urits et al., 2019). Interestingly, although anti-seizure activity of CBD is thought to be significantly different from other ASMs, its mechanism of action has not been fully elucidated. For example, coadministration of CBD with other ASMs such as clobazam have shown pharmacokinetic interactions, although administration of CBD in Dravet mouse models shows anti-seizure activity when administered alone, suggesting that this pharmacokinetic effect alone does not explain this activity (Anderson et al., 2019a).

Some of the difficulty in understanding possible mechanism(s) of action of CBD's anti-seizure effects may be attributed to its complex pharmacology (Schouten et al., 2023). For instance, some key known interactions include activation of channels such as transient receptor potential vanilloid 1 (TRPV1), transcription factors like peroxisome proliferator-activated receptor gamma (PPARγ), and different GPCRs such as CB1, GPR55, serotonin 1A receptor (5-HT1A), adenosine A2a receptor, $GABA_A$ receptors, and a range of cytochrome P450 enzymes (Bakas et al., 2017; Doohan et al., 2021; Peng et al., 2022; Schouten et al., 2023). Many of these targets operate within lipid-membrane environments and even have strong associations with lipid signalling. For example, CBD interacts with cholesterol binding sites on many of these targets (Martin et al., 2021a),

and has been shown disrupt cholesterol homeostasis and overall membrane fluidity of human cell lines. Guard et al. (2022) found that CBD incorporates into membranes, causes an increase in cytosolic calcium, AMPK activity, cholesterol biosynthesis, and cholesterol storage, and also a decrease in cholesterol accessibility and lateral diffusion in the membrane (Guard et al., 2022).

To date, the majority of scientific investigations on cannabis and cannabinoids have focused on CBD and Δ^9-THC. However, the reported efficacy of many 'full-spectrum' cannabis extracts used in childhood epilepsies suggest cannabinoid activity may extend beyond CBD, as anti-seizure effects were even reported in extracts with low CBD content (Suraev et al., 2018). This highlights the potential involvement of other components of the cannabis plant. The cannabis plant has a highly complex phytochemistry, with 500 different compounds identified thus far (ElSohly et al., 2017). The phytocannabinoids can generally be grouped into broad sub-classes: THC, CBD, cannabigerol (CBG), cannabichromene (CBC), cannabinodiol (CBNL), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), cannabitriol (CBT), and miscellaneous type (ElSohly et al., 2017). In the plant, phytocannabinoids are generated from cannabigerolic acid (CBGA) (Figure 1.3), with the amount of each varying dramatically by chemovar and growing conditions.

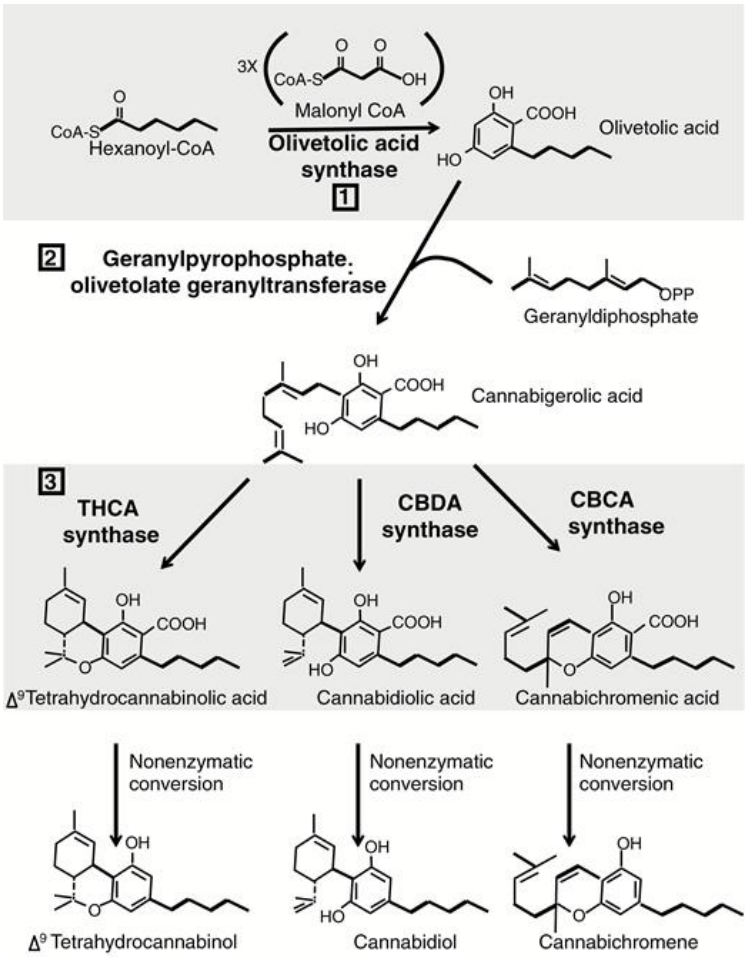

Figure 1.3. Cannabinoid synthesis pathway. The biosynthesis of phytocannabinoids begins with (1) olivetolic acid synthase produces olivetolic acid, (2) olivetolic acid and geranyl diphosphate, yield cannabigerolic acid (CBGA) via olivetolate geranyltransferase, also referred to as the mother of cannabinoids. (3) CBGA is further oxidised by different synthases to produce Δ^9-THC acid (Δ^9-THCA), cannabidiolic acid (CBDA) and cannabichromenic acid (CBCA). From these 4 compounds stem Δ^9-THC, CBD, CBC, and derivatives which total 120 constituents (ElSohly et al., 2017; Vergara et al., 2019). Original image from Vergara et al. (2019).

Various cannabis constituents display anti-seizure properties in preclinical investigations using the *Scn1a*$^{+/-}$ Dravet syndrome mouse model. These include low dose Δ^9-THC, CBC, cannabichromene acid (CBCA), cannabichromevarinic acid (CBCVA), CBDA, cannabidivarinic acid (CBDVA), CBGA, cannabigerovarinic acid (CBGVA), and olivetolic acid (Anderson et al., 2021a; Anderson et al., 2021c; Anderson et al., 2019b; Anderson et al., 2020b; Anderson et al., 2022b). However, the mechanism(s) of action for these effects are also unknown. Given the effects of CBD and the structural similarity of many of these compounds it is possible that these cannabinoids may also be involved in

lipid pathway modulation, and that through studying these effects we may be able to elucidate common targets that may help to explain mechanism(s) of action.

1.5.1 Cannabinoids, ABC Transporters and Dravet Syndrome

A target of CBD that may help to explain its anti-seizure effects are transporters. Adenosine triphosphate (ATP)-binding cassette (ABC) transporters are a superfamily of proteins that mediate the movement of compounds across physiological membranes, using energy from ATP hydrolysis. They are widely distributed in apical membranes of tissues with greatest expression in the epithelial cells of intestinal epithelium, kidneys, placenta, liver, and blood-brain barrier (BBB) (ter Beek et al., 2014). ABC subfamily G member 2 (ABCG2, also known as 'breast cancer resistance protein') and ABC subfamily B member 1 (ABCB1, also known as "P-glycoprotein 1" or "multidrug resistance protein 1") are the most studied ABC transporters. They are involved in a vast variety of physiological processes, including maintenance of membrane lipid composition, and xenobiotic defence (Glavinas et al., 2004).

Of great importance is that ABC transporters interact with endogenous pathways, including lipids. Their function extends from regulation of membranes and lipid rafts composition, to modulation of membrane protein functionality, as well as involvement in phagocytosis and immune system response (Kotlyarov et al., 2022). Almost half of the human ABC transporter proteins are thought to facilitate lipid movement (Tarling et al., 2013), including movement of cholesterol, glycerophospholipids (e.g. PC, PE, and PS), sphingolipids (e.g., HexCer), and lipopolysaccharides (Kotlyarov et al., 2022; Neumann et al., 2017). Plant cannabinoids are themselves lipids and have been suggested to share binding sites and modulate the metabolism of lipids like cholesterol (Guard et al., 2022; Martin et al., 2021a). That's why it's not surprising that various cannabinoids have also been shown to interact with ABC transporters. For instance, CBD has been characterized as an inhibitor, but not substrate, of the ABC transporters ABCB1 and ABCG2, while Δ^9-THC has been characterized as a substrate and inhibitor of both (Anderson et al., 2021b; Anderson et al., 2020a; Arnold et al., 2012; Bonhomme-Faivre et al., 2008; Brzozowska et al., 2017; Feinshtein et al., 2013; Holland et al., 2007; Holland et al., 2006; Spiro et al., 2012; Tournier et al., 2010; Zhu et al., 2006). Nonetheless, CBD and Δ^9-THC are not alone,

as various other cannabinoids have also been shown to interact with ABC transporters. A complete summary of published cannabinoid interactions with ABC transporters can be found in Table 1.3. This extended relationship between cannabinoids and ABC transporters, as well as their associated connections to epilepsy, may suggest their interaction and potential disruption of physiological processes could be tied to their yet unresolved mechanism(s) of action.

Table 1.3. Summary of published information cannabinoid interaction with ABC transporters ABCB1 and ABCG2. (Anderson et al., 2021b; Anderson et al., 2020a; Arnold et al., 2012; Bonhomme-Faivre et al., 2008; Brzozowska et al., 2017; Feinshtein et al., 2013; Holland et al., 2007; Holland et al., 2006; Spiro et al., 2012; Tournier et al., 2010; Zhu et al., 2006).

Cannabinoid	Transporter interaction profile	ABC transporters implicated
CBD	Competitive inhibitor of ABCB1 and ABCG2 ATPase/efflux; weak ABCG2 substrate; not an ABCB1 substrate in knock-out mice	ABCB1 inhibitor, ABCG2 inhibitor + weak substrate
CBDA	Robust substrate whose efflux is strongly inhibited by THC and CBG; no ABCB1 transport	ABCG2 substrate
CBDVA	Substrate behaviour mirrors CBDA, but weaker; not transported by ABCB1	ABCG2 substrate
Δ^9-THC	Dual substrate and inhibitor; active efflux lowers brain levels, while high doses block both pumps	ABCB1 and ABCG2 substrate + inhibitor
Δ^9-THCA	Minimal transport; not classed as substrate for either pump at tested concentrations	No significant ABCB1 or ABCG2 interaction
CBG	Potent inhibitor of BCRP-mediated CBDA efflux; very weak BCRP substrate; no ABCB1 substrate activity	ABCG2 inhibitor (weak substrate)
CBGA	No detectable substrate or inhibitory action under the same assay conditions	None detected

Several other drugs interact with ABC transporters. Some of the most well-known substrates of ABCB1 include digoxin, dabigatran, and fexofenadine, while known inhibitors include verapamil and carvedilol (Crawford et al., 2018). Moreover, some drugs

can indirectly modulate ABC transporter function, for instance through modulation of expression (statins, metformin) (Ye et al., 2020). Interestingly, seizure presentation has been shown to upregulate transporter expression, and therefore drugs that interact and/or modulate ABC transporter functionality could convey a direct or indirect mechanisms of action via this interaction.

Cannabinoid-ABC transporter interaction has also been studied in the context of 'entourage effect' assessment, the concept of cannabis constituents interacting pharmacologically to create a greater effect that the individual parts, often employed in the context of treatment for epilepsy. This ties back to the fact that even though CBD has anti-seizure properties on its own, there is also reported efficacy of 'full-spectrum' cannabis extracts that possess a mix of cannabinoids and where CBD content doesn't match with the reported symptom relief (Suraev et al., 2018). In the ABC transporter context, Anderson et al. (2021b) found that oral administration of CBDA to mice lead to a substantially lower bioavailability in plasma when given alone than when co-administered with other cannabinoids. ABC transporter interaction assessment discovered that CBDA is a ABCG2 substrate, while cannabinoids Δ^9-THC and CBG are inhibitors (Anderson et al., 2021b). This discovery evidenced the pharmacokinetic interactions between cannabis constituents can substantially alter systemic cannabinoid concentrations, which in some cases may relate to their therapeutic effects (Anderson et al., 2021b). This also highlights a potential additional interaction that may also alter pharmacokinetics of other co-administered drugs.

Indeed, interaction with ABC transporters has been a source of drug-drug interactions that may result in modulation of efficacy of therapeutic agents and/or their safety profiles (Chen et al., 2016; Glavinas et al., 2004; Liu, 2019; Marquez et al., 2011). Anti-seizure drugs that are ABC transporter substrates (e.g., clobazam (Nakanishi et al., 2013)), are thus vulnerable to such interactions which may lead to reduced treatment efficacy. Further, the over-expression of ABC transporters due to the pathophysiology of epilepsy may also itself contribute to drug-resistance in epilepsy treatment through such a pharmacokinetic interactions (Lazarowski et al., 2007), and thus screening of novel therapeutic drugs against ABC transporters should be a priority in order to understand these potential liabilities in drug efficacy.

Altogether, using tools such as cannabinoids to further understand the role of ABC transporters in intractable epilepsies such as Dravet syndrome, enables the further elucidation of underlying pathophysiology, and aids in the advancement of identification of novel therapeutic targets as well as the development of more efficacious therapies.

1.5.2 The Case for CBC and CBCA

The effects of two sometimes prominent cannabinoids CBC and CBCA (Andre et al., 2016; Izzo et al., 2009) both show anti-seizure effects when administered alone in Dravet mouse models (Anderson et al., 2021a), yet their interactions at ABC transporters are unknown. As with other phytocannabinoids, CBCA non-enzymatically decarboxylates into CBC (Morimoto et al., 1998). In addition, CBC and CBCA have demonstrated therapeutic potential in various other preclinical models. CBC exhibits anti-inflammatory properties in mouse models of oedema and colitis, anti-cancer properties in *in vitro* cancer models, and antidepressant activity in mouse models (Anis et al., 2021; De Petrocellis et al., 2012; DeLong et al., 2010; El-Alfy et al., 2010; Izzo et al., 2012; Ligresti et al., 2006b; Romano et al., 2013). CBCA has also been reported to have antibacterial properties against methicillin-resistant *Staphylococcus aureus* (MRSA) (Galletta et al., 2020). Altogether, it is of interest to progress the pharmacological characteristics of these drugs due to their potentially high therapeutic value.

Characterisation of the molecular actions of CBC is ongoing; however, several therapeutically relevant molecular targets have already been identified. CBC has been shown to activate cannabinoid CB2 receptors and transient receptor potential vanilloid (TRPV) channels (De Petrocellis et al., 2008; Udoh et al., 2019). CBC also inhibits lactate dehydrogenase and anandamide uptake (Ligresti et al., 2006a; Martin et al., 2021b). It is noteworthy that unlike CBD, CBC had negligible to weak inhibitory effects on an array of the major drug metabolising cytochrome P450 (CYP450) enzymes (Doohan et al., 2021). The pharmacological targets of CBCA are currently unknown.

In conclusion, investigating CBC and CBCA may provide opportunity to better understand the pharmacological properties cannabis and cannabinoids, while leveraging this knowledge to better understand the pathophysiology of intractable

epilepsies, as well as advance their development as therapeutic drugs. One area of grave interest to this advancement is the unravelling of their interaction with ABC transporters. This is explored in chapter 4, where we aimed to characterize CBC and CBCA against ABC transporters ABCB1 and ABCG2.

1.6 Aims

The global objective of this thesis was to better understand the neurochemistry of Dravet syndrome and leverage the lipid-like structure and anti-seizure activity of cannabinoids as tools to advance the development of effective treatments. The thesis had two major aims:

(1) Identify the lipid signatures in the brain of seizure-susceptible *Scn1a*$^{+/-}$ Dravet syndrome mouse model.
(2) Assess the interaction of cannabinoids CBC and CBCA with ABC transporters ABCB1 and ABCG2.

1.6.1 Specific Aims

Aim 1: Assess the cortical and hippocampal lipidomic profile in the *Scn1a*$^{+/-}$ Dravet syndrome mice model to investigate potential genotype-, strain- and tissue- specific differential modulation of lipids (chapter 2 and 3).

Better understanding of Dravet syndrome neurochemistry may unlock novel characteristics of the disease and inform novel targets for therapeutic development. Lipid pathways are believed to be associated with the Dravet syndrome pathophysiology, nonetheless, characterisation of the brain lipid profile remains underexplored. The area of lipidomics has rapidly gained momentum in the last decade due to great advances in analytical chemistry and bioinformatic tools, thus providing a novel avenue of exploration. Chapter 2 aimed to perform an explorative untargeted lipidomics analysis of cortical and hippocampal tissue of the *Scn1a*$^{+/-}$ Dravet syndrome mouse model to identify potential lipid dysregulation associated with genotype- or strain-driven modulation of seizure-susceptibility. This examination flagged hexosylceramides as

compounds of interests, therefore, chapter 3 aimed to perform confirmatory targeted LC-MS/MS analysis of the hexosylceramides family of compounds.

Aim 2: Assess the interaction of cannabis constituents CBC and CBCA with ABCG2 and ABCB1 transporters

Cannabinoids CBC and CBCA show promising anticonvulsant therapeutic potential in preclinical models, so advancing their pharmacological profiling is of great interest. Previous literature has identified various cannabinoids as substrates and/or inhibitors of ABC transporters ABCB1 and ABCG2, yet CBC and CBCA had not been characterized. ABC transporter characterization is important because these transporters play a major role in maintaining neurological homeostasis, are involved in endogenous signalling modulation, are especially involved in epileptogenic processes, they modulate drug pharmacokinetics, and are known drivers of drug-drug interactions. Chapter 4 therefore aimed to perform an *in vitro* pharmacological screen of CBC and CBCA in a bidirectional transport assay using epithelial MDCK cells expressing ABCB1 or ABCG2 to determine substrate and/or inhibitor activity.

Chapter 2

Untargeted Lipidomics of Mouse Brain in the *Scn1a*$^{+/-}$ Model of Dravet Syndrome

2.1 Introduction

Dravet syndrome is a rare form of developmental and epileptic encephalopathy with onset in infancy. It is characterized by seizures, the development of cognitive-behavioural and social disabilities, sleep difficulties, and a high risk of SUDEP (Dravet, 2011). Despite a clear aetiology driven by a genetic mutation in the *SCN1A* gene in 80% of cases, much remains unknown about the disease. This lack of knowledge is clearly outlined by the fact that Dravet syndrome is highly refractory, such that patients rarely achieve seizure-freedom with the currently available medication (Samanta, 2025). Consequently, there is a need to obtain a better understanding of disease profile and create more efficacious therapeutics. One of the most viable investigational approaches is the characterization of Dravet syndrome's underlying neurobiology. The application of 'omic' methodologies may offer a viable research path for further exploration of the disease's neurobiology, as they use advanced high-throughput technologies to analyse broad and specific molecular families (Hasin et al., 2017).

Several Dravet syndrome mouse models exist that closely mimic the core clinical features of the syndrome in humans, namely, susceptibility to febrile seizures, spontaneous seizures within the first 2-3 weeks of life, and premature death (Cheah et al., 2012; Higurashi et al., 2013; Jones et al., 2024; Miller et al., 2014; Oakley et al., 2009; Ricobaraza et al., 2019; Uchino et al., 2021; Yu et al., 2006). These models commonly have heterozygous loss-of function mutations in the *Scn1a* gene; however, they often require a specific genetic background for the mutation to be penetrant and induce the characteristic Dravet syndrome phenotype. That is, in *Scn1a*$^{+/-}$ mice on a 129 background strain (129.*Scn1a*$^{+/-}$) do not exhibit a seizure-phenotype, whereas when they are forward crossed onto the C57BL6/J strain, the resulting F1 strain (i.e. F1.*Scn1a*$^{+/-}$ mice) manifests the severe seizure phenotype. In this way, the model can be exploited to observe the impact of: 1) heterozygous deletion of *Scn1a*; or 2) background strain and strain-related modifier genes; and 3) the interaction of *Scn1a* genotype and strain.

A number of studies have investigated the genomic and transcriptomic alterations in Dravet syndrome mouse models. One important study (Hawkins et al., 2016) conducted low resolution global genetic mapping and RNA sequencing (RNA-seq) in the

same *Scn1a*$^{+/-}$ mouse model utilised in the current study to discern the impact of genotype and strain. By comparing the 129 and F1 strains in *Scn1a*$^{+/-}$ mice, they uncovered various candidate modifier genes that might influence the severity of the disease phenotype. Differences emerged in ion channels (Na^{2+}, Ca^{2+}, K^{+}, Cl^{-}), membrane potential regulation (*Cacna2d1, Kcnj11, Clcn3*); related to neurodevelopment and synaptic structure (*Reln, Lgi2*); membrane transport and homeostasis (*Atp1a3, Slc7a10*); intracellular signalling (*Mapk10*); and most importantly GABAergic signalling (e.g., *Gabrb3, Gabrg3, Gabra2*). Notably, *Gabra2* stood out as a high priority putative modifier gene found to influence the *Scn1a*$^{+/-}$ Dravet syndrome mice survival (Hawkins et al., 2016; Miller et al., 2014). Most recently, Hawkins et al. (2019) extended on this study by performing a global RNA-seq analysis in hippocampal tissue of WT and *Scn1a*$^{+/-}$ mice on both F1 and 129 strains. Heterozygous deletion of *Scn1a* had a minimal effect on gene expression, whether of the seizure-resistant 129 strain or on the seizure susceptible F1 strain. However, a large difference on overall gene expression (>1.5-fold) was observed between the 129 and F1 strains, irrespective of *Scn1a* genotype. The largest difference was again in the *Gabra2* gene which encodes the alpha2 subunit of the inhibitor $GABA_A$ receptor, where the F1 strain had significantly lower expression compared to 129, again suggesting *Gabra2* as a candidate genetic modifier.

The above investigations underscore the utility of omic approaches to uncover novel pathophysiological dysregulation pathways and infer disease modifiers in a mouse model of Dravet syndrome. Nonetheless, omic approaches extend beyond genomics, with comparable techniques available for metabolomics, proteomics, and lipidomics. Lipidomics is the most recent omic approach (since early 2000s), which investigates the lipid profile of cells, tissues, or more complex systems. Technological advancements in analytical chemistry and informatics tools have enabled improved lipid profiling in recent years (Conroy et al., 2023; Kyle et al., 2021). The lipidomics workflow conventionally begins with sample preparation and total lipid extraction, followed by mass spectrometry (MS) analysis, followed by advanced bioinformatics tools to perform computational identification of compounds, data enrichment and statistical analysis (Seppänen-Laakso et al., 2009). Lipidomics, broadly, includes two approaches: untargeted and targeted analyses. While untargeted is used for hypothesis generation via the

identification of global alterations, targeted is hypothesis driven, and it aims to identify and quantify the abundance of specific lipids and related compounds (Han et al., 2022).

Many connections exist between Dravet syndrome and lipids pathways. Dravet has traditionally been seen as a neurogenic disease, largely underpinned by SCN1A mutations, however, both past and emerging research suggests that dysregulations in metabolic lipid pathways exist and may potentially be contributing to the disorder's pathophysiology, including associations with lipid-linked systems like immune response (Yip et al., 2025; Zhou et al., 2024) and the endocannabinoid system (Anderson et al., 2022a), as well as lipid-based therapeutics such as the keto diet, cannabinoids, and the novel drug Soticlestat (Caraballo et al., 2005; Cross et al., 2019; Hahn et al., 2022). Nonetheless, the vast biological lipid-network system is underexplored in the Dravet syndrome context to date. Due to the joint complexity of lipids biology and the disease itself, the application of lipidomics offers a viable path for further exploration of Dravet syndrome.

The current chapter aimed to conduct an exploratory, untargeted lipidomics analysis of brain tissue using the *Scn1a*$^{+/-}$ mouse model of Dravet syndrome. Analysis focused on the effects of genotype (i.e., *Scn1a*$^{+/-}$ versus WT mice) and strain (seizure susceptible F1 versus seizure-resistant 129 strains). Lipidomics was applied in an effort to advance our understanding of the pathophysiology of Dravet syndrome and potentially uncover novel therapeutic targets.

2.2 Methods

2.2.1 Animals

All animal care and research procedures were 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. The animal protocol outlined below has been employed in various other publications (Anderson et al., 2022a; Anderson et al., 2023; Yip et al., 2025; Zhou et al., 2024). Mice with 129S6/SvEvTac (129) background strain with heterozygous deletion for *Scn1a* (129.*Scn1a*$^{+/-}$) and wild-type (129.WT) were obtained from the Australian BioResources (New South Wales, Australia) and The

Jackson Laboratory (stock 37107-JAX; Bar Harbor, USA) respectively and were maintained by continuous backcrossing. C57BL/6J (B6) mice were obtained from Animal Resources Centre (Perth, Australia). The 129.*Scn1a*$^{+/-}$ were crossed with B6 to generate 129xB6.*Scn1a*+/- (F1.*Scn1a*$^{+/-}$) mice (with Dravet syndrome phenotypic combination), as well as wild-type (F1.WT) mice. Genotyping procedures were conducted as previously described by Miller et al. (2014). The facilities in which the animals were pathogen-free and held operated on a 12 h light/dark cycle. All animals were group housed in individually ventilated cages and had ad libitum access to food (Irradiated Rat and Mouse Cubes (SF00-100 Irr) (Speciality Feeds, WA, Australia)) and water.

2.2.2 Sample Preparation

Brain samples from mice were collected from the following four groups: 129.WT, 129.*Scn1a*$^{+/-}$, F1.WT and F1. *Scn1a*$^{+/-}$ at postnatal day 24 (P24) (n = 3-5 per group). P24 was chosen because it is a seizure-prone developmental age, and it is commonly used as the culling age for these specific mice strain and genotype combination. The sex of the animals was balanced across experimental groups, with an equal number of males and females included in each. Animals were euthanized via cervical dislocation. Brains were rapidly collected and immediately snap-frozen in liquid nitrogen. At the time of analysis, brains were thawed on ice and hippocampal and cortex sections dissected (Zhou et al., 2024). Once separated, tissues were stored individually in 1.5 mL Eppendorf tubes and snap-frozen again. Samples were stored in -80 °C (Zhou et al., 2024).

Tissue homogenization and lipid extraction was performed under a modified Matayash approach (Matyash et al., 2008). Each tissue was weighed, submerged in 200 µL of methanol (MeOH) and spiked with 50 µL of an internal standard mixture composed of: 5 nmol of PC 16:0-d9/16:0, 2 nmol of D-erythro/L-threo SM(18:1/17:0), galactosylceramide d18:1/12:0 (all Cayman Cayman Chemical, MI, USA), 1 nmol of PE 17:0 and C17 ceramide (Cer) (both Avanti Polar Lipids, AL, USA). Samples were then sonicated for 30 min with 850 µL of methyl tert-butyl ether (MTBE). To ensure samples remained cold, sonication occurred in a cold room and with the bath filled with ice. Tissues were then homogenised using a metal spatula and sonicated again for an hour. To induce phase separation, samples were vortexed and centrifuged for 5 min at 2000 g

with an additional 212 µL of MilliQ water. The upper organic layer was extracted into a glass collection tube. The same extraction process was done twice, with the remaining sample starting with an additional 500 µL of MTBE and 150 µL of MeOH and finishing with the collection of the upper MTBE layer into the same collection glass tube. The final extracts were dried using a Speedvac on its medium heat setting, reconstituted in 400 µL MeOH, vortexed, and sonicated. The collection tubes were centrifuged at 2000 g for 10 min to pellet insoluble material. The final lipid extract was transferred to a glass HPLC vial and stored at -80 °C until LC/MS/MS analysis. Handling of samples accounted for freeze-thaw stability with 3 aliquots prepared to minimise repeated freeze-thaw procedures.

2.2.3 Untargeted LC-MS/MS Analysis of Lipids

All untargeted LC-MS/MS lipid analysis of samples was done as previously described (Couttas et al., 2020; Jieu et al., 2024). Briefly, extracts were analysed on a QExactive-HFX mass spectrometer coupled to a Vanquish UHPLC system (Thermo Scientific, Bremen, Germany). Lipid extracts were resolved on a 2.1 x 100 mm Waters Acquity C18 UHPLC column (1.7 µm pore size), using a 25 min binary gradient at 0.28 mL/min flow rate. Solvent A was 10 mM ammonium formate, 0.1% formic acid in acetonitrile:water (60:40) and solvent B was 10 mM ammonium formate, 0.1% formic acid in isopropanol:acetonitrile (90:10). Each run was as follows: 0 min, 80:20 solution A/B; 3 min, 80:20 solution A/B; 5.5 min, 55:45 solution A/B; 8 min, 36:65 solution A/B; 13 min, 15:85 solution A/B; 14 min, 0:100 solution A/B; 20 min, 0:100 solution A/B; 20.2 min, 70:30 solution A/B; 27 min, 70:30 solution A/B. Each sample was analysed in both positive and negative mode under a full scan/data-dependent MS2 mode (resolution 70,000 FWHM, scan range 200–1200 *m/z*). The ten most abundant lipid ions in each cycle were subjected to fragmentation (MS2), with an isolation window of 1.4 *m/z*, collision energy 30 eV, resolution 17,500 FWHM, maximum integration time 110 ms and a dynamic exclusion window of 10 s. Blank sample injections were run every five samples.

2.2.4 Data Processing and Refinement

LC/MS/MS raw files were run through LipidSearch software (version 4.2, Thermo Fisher, CA, USA) for peak integration, chromatogram alignment and lipid annotation.

Lipid annotation was based on precursor (mass tolerance 4 ppm) and product ions (mass tolerance 8 ppm) for both positive and negative ion modes. The selected adducts were +H, $+NH_4$, $+H-H_2O$ for positive mode search and –H and CH_3COO^- for negative mode given that ammonium acetate was used in the mobile phase.

The resulting dataset was further cleaned and refined with the R studio software (version 2024.9.0.375) using the tidyverse, padr, lubridate, base, readr, and readxl packages. Unreliable lipid annotation and abundance of false positives are known issues with LipidSearch (Xu et al., 2018); therefore, to further maximize data accuracy and reliability, individual lipids counts were removed if their 'Rating' values were absent and/or their 'M-score' was below 5; these two measurements are indicative of LipidSearch's scoring algorithms and reveal confidence in lipid identification (Xu et al., 2018). Finally, lipids that were confidently identified in more than 80% of samples were included for the final data analysis. Remaining relative lipid abundances were further normalised to the internal standard mix and tissue weight (Jieu et al., 2024).

2.2.5 Statistical Analysis

Analysis aimed to evaluate strain and genotype effects in the lipid profiles of the mice in two different brain regions, the cortex and hippocampus. Specifically, the focus was first on the potential differences in the lipidome between the seizure susceptible F1.*Scn1a*$^{+/-}$ mice in comparison to the F1.WT, with a prioritisation of those that were absent in 129.*Scn1a*$^{+/-}$ versus 129.WT comparison. And second, the differences between the seizure susceptible F1.*Scn1a*$^{+/-}$ mice in comparison to the 129.S*cn1a*$^{+/-}$, in contrast to those that were absent in F1.WT versus 129.WT comparison. Conducting power analysis for untargeted methods is recognized as complex given that the list of measured metabolites and effect size is not known before hand; for animal tissue, a minimum of 5-10 samples per group is usually recommended (Rakusanova et al., 2024). Thus, sex was collapsed for the following analysis (n = 7-10 per group).

The following data evaluation workflow was performed as previously described (Jieu et al., 2024; Marian et al., 2023) with some modifications to suit the present study's data structure. A multivariate analysis of the resulting untargeted lipidomic data was

performed using MetaboAnalyst (version 6.0)'s 'Statistical Analysis [One Factor]' module. Missing values were assigned a value of 1/5 of the minimum observed value for that lipid. Lipid levels were filtered based on interquartile range (25%) and log_{10}-transformed to achieve normal distribution. Supervised partial least squares discriminant analyses (PLS-DA) were applied to the samples per tissue to obtain an overview of the data, the general clustering trend and to identify outliers that laid significantly outside the cluster. Further, supervised orthogonal partial least squares discriminant analyses (OPLS-DA) were performed together with their variable importance in the projection (VIP) scores to also identify the most important differential lipids occurring between F1.*Scn1a*$^{+/-}$ and F1.WT and how they compare to 129.*Scn1a*$^{+/-}$ and 129.WT to assess the genotype effect, and also by comparing F1.*Scn1a*$^{+/-}$ and 129.*Scn1a*$^{+/-}$ and how they compare to F1.WT and 129.WT to assess strain effects. Lipids with VIP scores > 2.0 were considered significantly different and manually verified to their total ion chromatogram, parent ion, and fragmentation spectra observed in LipidSearch; ambiguous lipids were removed from further analysis, these were defined by visual inspection -a lipid was identified as 'ambiguous' if, for example, it did not present a clean chromatogram or possessed relevant fragmentations profile. Lipid candidates from the OPLS-DA models were subjected to classical univariate receiver operating characteristics (ROC) curve analysis to report model performance; an area under the curve (AUC) >0.8 was considered significant. The OPLS-DA models were validated using MetaboAnalysts's built-in cross-validation and permutation tests while ROC curves were also tested using internal cross-validation to assess model performance.

All lipids appearing in the resulting list of hits were additionally assessed in the previous group pairings using a pairwise analysis Mann-Whitney (MW) test (multiple t-test) for unpaired nonparametric comparisons with false discovery rate (FDR) adjustments using the Benjamini-Hochberg correction with statistical significance set at Q = 5% (GraphPad Prism version 10.2.3, MA, USA).

2.3 Results

2.3.1 PLS-DA Analysis to Identify Separations Between the 4 Experimental Groups

Untargeted lipidomic profile analysis was performed on cortex and hippocampus samples of F1.*Scn1a*$^{+/-}$, F1.WT, 129.*Scn1a*$^{+/-}$ and 129.WT mice. LipidSearch identified a total of 8562 lipid ions in both positive and negative mode across 61 different lipid classes. After data cleaning and filtering, a total of 1406 lipid ions in cortex and 907 in the hippocampus remained for analysis. PLS-DA plots compared all groups and suggested greater group differences in the cortex than in the hippocampus (Figure 2.1A&B). There was clear separation of the F1.*Scn1a*$^{+/-}$ and F1.WT groups in the cortex, and a separation between them and their 129 counterparts, but no separation between the 129.*Scn1a*$^{+/-}$ and 129.WT groups (Figure 2.1A). There was no as distinct separation of the 4 groups in the hippocampus, however a small strain driven effect can be observed (Figure 2.1B).

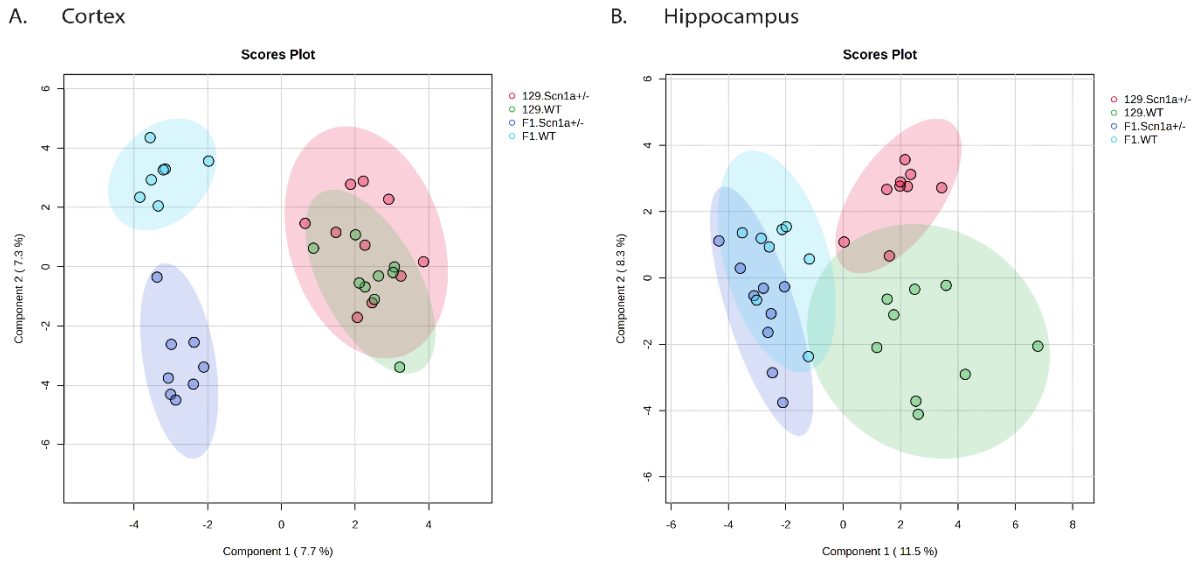

Figure 2.1. Untargeted lipidomic analysis of the cortex and hippocampus in a mouse model of Dravet syndrome. The figure shows PLS-DA score plots for cortex (A) and hippocampus (B) for F1.*Scn1a*$^{+/-}$, F1.WT, 129.*Scn1a*$^{+/-}$ and 129.WT mice. All images generated by Metaboanalyst 6.0.

2.3.2 OPLS-DA Analysis to Compare 2 Groups of Interest

The comparison between F1.*Scn1a*$^{+/-}$ and F1.WT, as well as F1.*Scn1a*$^{+/-}$ and 129.*Scn1a*$^{+/-}$ are of most pathophysiological interest as they hold the potential to reveal

genotype- and strain-dependent differences in lipid profiles associated with the epileptic phenotype of the F1.*Scn1a*$^{+/-}$ mice. Accordingly, these comparisons were furthered examined in both tissues using an OPLS-DA approach to identify such potential lipids.

All OPLS-DAs were successful in creating distinct separations. For the genotype-effect analysis driven by the F1.*Scn1a*$^{+/-}$ comparison with F1.WT, the key lipids driving the separation in the cortex were HexCer(d42:2), TG(54:6), PC(33:1), PC(35:1), PC(37:1), PC(38:3), and PC(40:4e); and in the hippocampus are PE(40:6p), PG(38:5), PS(36:1) and PS(36:2) (Figure 2.2).

To appraise the strain-dependent impact of *Scn1a* deletion, F1.*Scn1a*$^{+/-}$ and 129.*Scn1a*$^{+/-}$ groups were compared. The key lipids driving the separation in the cortex were Cer(34:1), Cer(36:2), Cer(38:2), di-methyl-PE-(dMePE)-(30:0), lyso-PC-(LPC)-(18:0), PC(28:0), PC(29:0), PC(30:0), PC(42:2), PS(38:4) and PS(44:10); and in the hippocampus are dMePe(30:0), PC(28:0), PC(30:0), PC(31:2), PC(32:1), PC(32:2e), PC(36:6), PC(38:2e) and PI(28:4) (Figure 2.3).

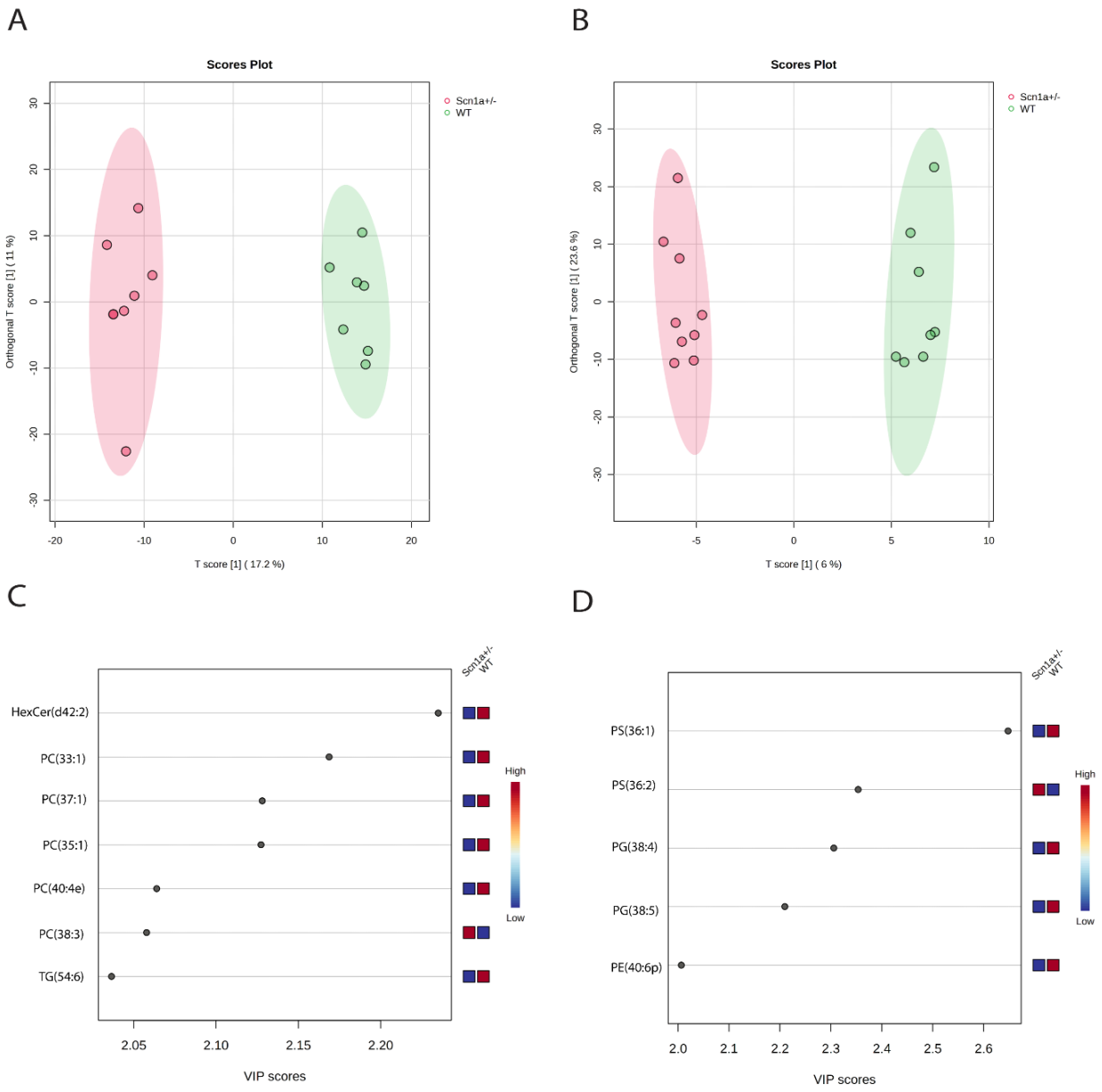

Figure 2.2. OPLS-DA results for the analysis of genotype-effect between F1.WT and F1.*Scn1a*$^{+/-}$ in cortex (A & C) and hippocampus (B & D). (A & B) OPLS-DA plot of orthogonal T score vs T score. (C & D) VIP plots displaying the lipids contributing to the OPLS-DA separation in A & B respectively, lipids displayed are those where VIP score > 2.0. Both images were generated by Metaboanalyst 6.0.

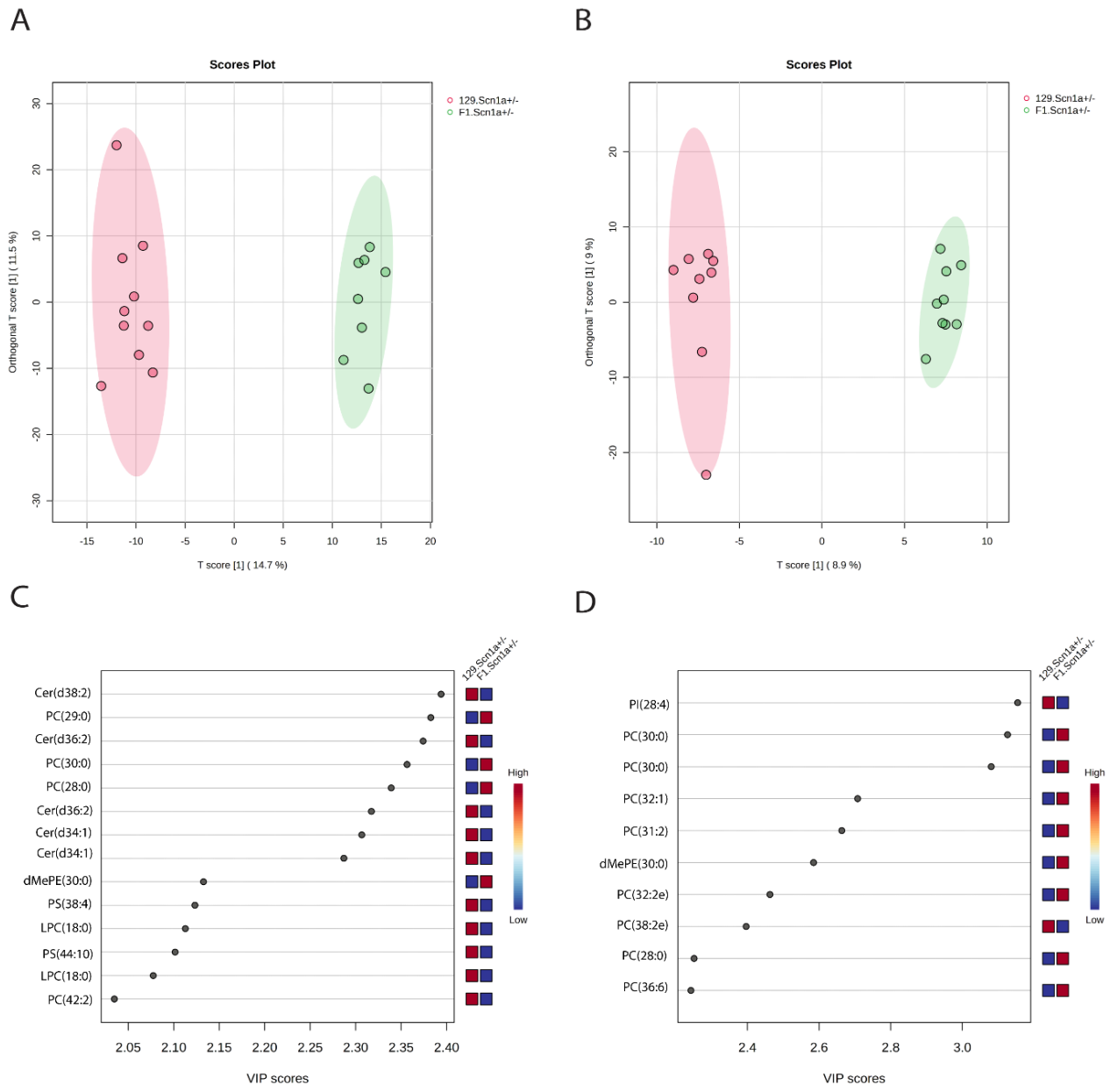

Figure 2.3. OPLS-DA results for the analysis of strain-effect between F1.*Scn1a*$^{+/-}$ and WT.*Scn1a*$^{+/-}$ in cortex (A & C) and hippocampus (B & D). (A & B) OPLS-DA plot of orthogonal T score vs T score. (C & D) VIP plots displaying the lipids contributing to the OPLS-DA separation in A & B respectively, lipids displayed are those where VIP score > 2.0. Both images were generated by Metaboanalyst 6.0.

2.3.3 Pairwise MW Tests and ROC Analyses of Genotype and Strain

Given the PLS-DA analyses showed good separations between F1.*Scn1a*$^{+/-}$ versus F1.WT and F1.*Scn1a*$^{+/-}$ versus 129.*Scn1a*$^{+/-}$ groups in the cortex, it was decided to further characterize the differential lipids identified in the above OPLS-DAs between these groups. The subsequent analysis was conducted in two stages, first focusing on the genotype effects, and second, strain effects.

2.3.3.1 Genotype Effects

First, the lipid profiles identified in F1.*Scn1a*$^{+/-}$ and F1.WT groups were compared (Figure 2.2C). To provide a point of comparison to other groups and brain regions, analyses were also conducted of the differential lipids in: (1) cortical 129.*Scn1a*$^{+/-}$ versus 129.WT groups (the 129 strain does not have a seizure phenotype following partial *Scn1a* deletion, and (2) hippocampal F1.*Scn1a*$^{+/-}$ versus F1.WT groups -the hippocampus showed not marked lipid effects based on PLS-DA analysis (Figure 2.1B). The complete list of putative lipid biomarkers with the corresponding statistical analysis is presented in Table 2.1.

To reiterate, the differential lipids distinguished in the cortex of F1.*Scn1a*$^{+/-}$ versus F1.WT OPLS-DA (Figure 2.2C) were: HexCer(d42:2), TG(54:6), PC(33:1), PC(35:1), PC(37:1), PC(38:3), and PC(40:4e). Pairwise MW tests confirmed these lipids displayed significantly decreased concentrations, except for PC(38:3) which showed upregulation, in F1.*Scn1a*$^{+/-}$ mice compared to the F1.WT. Multivariate analysis results were mirrored by those in the MW tests, which followed an identical lipid differentiation pattern ($q < 0.05$). Additionally, ROC analysis indicated great discrimination ability of the model as all lipids identified by the OPLS-DA were also identified as significantly different between groups (AUC >0.8). In contrast, none of the identified lipids appeared in the parallel cortical 129.*Scn1a*$^{+/-}$ versus 129.WT comparison. Further none of the lipids were found, except for PC(38:3), in hippocampal F1.*Scn1a*$^{+/-}$ versus F1.WT comparison.

Table 2.1. Putative differential lipids identified in the OPLS-DA between F1.*Scn1a*$^{+/-}$ and F1.WT in the cortex, their chemical details, and differential appearance in the OPLS-DAs of 129.*Scn1a*$^{+/-}$ vs 129.WT in cortex and F1.*Scn1a*$^{+/-}$ vs F1.WT in hippocampus, as well as their corresponding MW test results (q-value and mean rank difference), and ROC AUC scores.

Lipid name	Lipid Category	Ion	m/z Ion	Ion Formula	F1.*Scn1a*$^{+/-}$ vs F1.WT in Cortex				129.*Scn1a*$^{+/-}$ vs 129.WT in Cortex				F1.*Scn1a*$^{+/-}$ vs F1.WT in Hippocampus			
					Lipid appears in OPLS-DA?	q-value	Mean rank difference	AUC	Lipid appears in OPLS-DA?	q-value	Mean rank difference	AUC	Lipid appears in OPLS-DA?	q-value	Mean rank difference	AUC
HexCer(d42:2)	Sphingolipid	+H	810.6817	C48 H92 O8 N1	**Y**	**0.01**	-7.5	**1**	N	0.52	-4.86	0.76	N	0.93	-0.83	0.55
PC(33:1)	Glycerophospholipid	+H	746.5694	C41 H81 O8 N1 P1	**Y**	**0.01**	-7.5	**1**	N	0.68	2.96	0.66	N	0.92	-5.79	**0.85**
PC(35:1)	Glycerophospholipid	+H	774.6007	C43 H85 O8 N1 P1	**Y**	**0.01**	-7.5	**1**	N	0.66	3.27	0.67	N	0.92	-2.83	0.67
PC(37:1)	Glycerophospholipid	+H	802.6320	C45 H89 O8 N1 P1	**Y**	**0.02**	-7.1	**0.96**	N	0.59	-4.01	0.71	N	0.92	-2.48	0.65
PC(38:3)	Glycerophospholipid	+H	812.6164	C46 H87 O8 N1 P1	**Y**	**0.01**	7.5	**1**	N	0.53	4.64	0.74	Y	0.92	5.9	**0.85**
PC(40:4e)	Glycerophospholipid	+H	824.6528	C48 H91 O7 N1 P1	**Y**	**0.01**	-7.5	**1**	N	0.88	1.37	0.58	N	0.94	-1.89	0.61
TG(54:6)	Glycerolipid	+NH4	896.7702	C57 H102 O6 N1	**Y**	**0.01**	-7.5	**1**	N	0.67	-3.17	0.62	N	1	-0.71	0.54

2.3.3.2 Strain Effects

To further explore strain dependent effects, we found there was a good separation between F1.*Scn1a*$^{+/-}$ versus 129.*Scn1a*$^{+/-}$ groups in the cortex (Figure 2.3C). Again to further evaluate the uniqueness of these effects we conducted additional analyses comparing: (1) F1.WT versus 129.WT in the cortex (i.e., in the absence of the genotype modification); and (2) F1.*Scn1a*$^{+/-}$ versus 129.*Scn1a*$^{+/-}$ in the hippocampus (again, the brain region that didn't display as marked separation in PLS-DA analyses (Figure 2.1B). The complete list of putative lipid biomarkers with the corresponding statistical analysis is presented in Table 2.2.

The differential lipids distinguished in cortical F1.*Scn1a*$^{+/-}$ versus 129.*Scn1a*$^{+/-}$ groups OPLS-DA analysis (Figure 2.3C) were: Cer(34:1), Cer(36:2), Cer(38:2), dMePE(30:0), LPC(18:0), PC(28:0), PC(29:0), PC(30:0), PC(42:2), PS(38:4), and PS(44:10). Pairwise MW tests showed that all Cer species were significantly decreased in concentration in the F1.*Scn1a*$^{+/-}$ compared to the 129.*Scn1a*$^{+/-}$ group comparisons. The dMePE had significantly higher concentrations, while LPC significantly lower concentrations in the F1.*Scn1a*$^{+/-}$ compared to 129.*Scn1a*$^{+/-}$ groups. The PC species showed mixed patterns: the shorter chain PCs (PC(28:0), PC(29:0), and PC(30:0)), had higher concentrations in the F1.*Scn1a*$^{+/-}$ group, whereas the PC(42:2) had lower concentrations compared to the 129.*Scn1a*$^{+/-}$ group. Finally, both PSs appeared have lower concentrations in the F1.*Scn1a*$^{+/-}$ group compared to 129.*Scn1a*$^{+/-}$ group. Again, the multivariate analysis results were closely mirrored by those in the MW tests, and the ROC analysis indicated great discrimination ability of the model.

Interestingly, many of the identified lipids also appeared significantly, or near-significantly, differentially expressed in the parallel cortical F1.WT versus 129.WT comparison, particularly the Cer species, the LPCs and PCs, but not the dMePE nor the PSs. To a smaller extent, some overlap was also seen in the hippocampus F1. *Scn1a*$^{+/-}$ vs 129. *Scn1a*$^{+/-}$ comparison, namely the dMePE, PC(29:0) and PC(30:0).

Table 2.2. Putative differential lipids identified in the OPLS-DA between F1.*Scn1a*$^{+/-}$ and 129.*Scn1a*$^{+/-}$ in the cortex, their chemical details, and differential appearance in the OPLS-DAs of 129.WT vs F1.WT in cortex and F1.*Scn1a*$^{+/-}$ and 129.*Scn1a*$^{+/-}$ in hippocampus, as well as their corresponding MW test results (q-value and mean rank difference), and ROC AUC scores.

					F1.*Scn1a*$^{+/-}$ vs 129.*Scn1a*$^{+/-}$ in Cortex				F1.WT vs 129.WT in Cortex				F1.*Scn1a*$^{+/-}$ vs 129.*Scn1a*$^{+/-}$ in Hippocampus			
Lipid name	Lipid Category	Ion	m/z Ion	IonFormula	Lipid appears in OPLS-DA?	q-value	Mean rank difference	AUC	Lipid appears in OPLS-DA?	q-value	Mean rank difference	AUC	Lipid appears in OPLS-DA?	q-value	Mean rank difference	AUC
Cer(34:1)	Sphingolipid	+HCOO	582.5100	C35 H68 O5 N1	**Y**	**0.01**	-8.78	**0.99**	N	0.06	-6.22	**0.89**	N	0.46	4.11	0.73
Cer(34:1)	Sphingolipid	-H	536.5100	C34 H66 O3 N1	**Y**	**0.01**	-8.78	**0.99**	N	0.07	-5.97	**0.87**	N	0.46	4.11	0.73
Cer(36:2)	Sphingolipid	+HCOO	608.5259	C37 H70 O5 N1	**Y**	**0.01**	-9.00	**1.00**	**Y**	**0.03**	-6.97	**0.92**	N	0.94	0.94	0.75
Cer(36:2)	Sphingolipid	-H	562.5205	C36 H68 O3 N1	**Y**	**0.01**	-9.00	**1.00**	**Y**	**0.04**	-6.73	**0.84**	N	0.37	4.56	**0.85**
Cer(38:2)	Sphingolipid	-H	590.5518	C38 H72 O3 N1	**Y**	**0.01**	-9.00	**1.00**	N	0.11	-5.46	**0.94**	N	0.18	6.33	0.51
dMePE(30:0)	Glycerophospholipid	-H	690.5079	C37 H73 O8 N1 P1	**Y**	**0.01**	8.55	**0.98**	N	0.65	1.91	0.62	**Y**	**0.02**	8.33	**0.96**
LPC(18:0)	Glycerophospholipid	+HCOO	568.3620	C27 H55 O9 N1 P1	**Y**	**0.01**	-8.10	**0.95**	N	0.06	6.22	**0.89**	N	0.94	1.00	0.56
LPC(18:0)	Glycerophospholipid	+H	524.3711	C26 H55 O7 N1 P1	**Y**	**0.01**	-8.78	**0.99**	**Y**	**0.01**	-8.00	**1.00**	N	0.81	1.89	0.60
PC(28:0)	Glycerophospholipid	+H	678.5068	C36 H73 O8 N1 P1	**Y**	**0.01**	8.78	**0.99**	**Y**	**0.05**	6.48	**0.90**	N	0.13	6.78	**0.88**
PC(29:0)	Glycerophospholipid	+H	692.5225	C37 H75 O8 N1 P1	**Y**	**0.01**	9.00	**1.00**	N	0.06	6.22	**0.89**	**Y**	**0.01**	9.00	**1.00**
PC(30:0)	Glycerophospholipid	+H	706.5381	C38 H77 O8 N1 P1	**Y**	**0.01**	9.00	**1.00**	N	0.06	6.22	**0.89**	**Y**	**0.01**	9.00	**1.00**
PC(42:2)	Glycerophospholipid	+H	870.6946	C50 H97 O8 N1 P1	**Y**	**0.01**	-8.33	**0.88**	N	0.07	-5.97	**0.87**	N	0.68	-2.89	0.65
PS(38:4)	Glycerophospholipid	-H	810.5291	C44 H77 O10 N1 P1	**Y**	**0.01**	-8.33	**0.96**	N	>0.99	0.123	0.51	N	0.98	0.56	0.53
PS(44:10)	Glycerophospholipid	-H	882.5291	C50 H77 O10 N1 P1	**Y**	**0.01**	-8.78	**0.99**	N	>0.99	0.13	0.59	N	0.37	-4.56	0.75

2.4 Discussion

Dravet syndrome is a highly complex and detrimental epilepsy. It is generally characterised by a heterozygous mutation in the *SCN1A* gene, a gene that codes for a subunit of voltage-gated sodium channels ($Na_v1.1$) (Claes et al., 2001). However, much remains unknown about the disease pathophysiology, and its treatment remains inadequate (Samanta, 2025). Aiming to uncover novel pathways of dysregulation, this chapter aimed to investigate a previously underexplored area of Dravet syndrome, alterations in the lipidome. Thus, the present study sought to characterize the brain lipidome of Dravet syndrome via an untargeted lipidomic analysis of the *Scn1a*$^{+/-}$ mouse model, also utilising differing background strains lacking the seizure phenotype (Hawkins et al., 2016; Miller et al., 2014) to aid in identifying related changes.

Results observed unique lipidomic changes driven by the heterozygous deletion of *Scn1a* and related to genetic background. Specifically, this investigation identified distinct lipidomic profile between cortical F1.*Scn1a*$^{+/-}$ and F1.WT mice and between F1s and 129 mouse strain counterparts. As well as a minor hippocampal distinction of F1 versus 129 strain and no cortical segregation was observed between cortical 129.*Scn1a*$^{+/-}$ and 129.WT mice. Prior research has identified lipid dysregulation in epilepsy and seizure-related disorders; however, this is the first study to examine and characterize the distinct lipidomic profile found in the brains of a preclinical mouse model of Dravet syndrome.

The large separation between F1.*Scn1a*$^{+/-}$ versus F1.WT mice suggested distinct lipidomic profiles in the cortex that may be unique to the impact of partial deletion of *Scn1a* on the seizure-susceptible F1 background strain. These lipidomic differences appeared to be driven by multifaceted changes in sphingolipids, glycerolipids, and various glycerophospholipids. Additionally, there was also a large difference in cortical lipidomic profiles in F1.*Scn1a*$^{+/-}$ versus 129.*Scn1a*$^{+/-}$ mice. These differences appeared to be driven by changes in various sphingolipids and glycerophospholipids.

Sphingolipids are characterized by sphingoid bases with a fatty acid chain (Fahy et al., 2011; Liebisch et al., 2020). They are key structural elements of membranes and

are also thoroughly involved in cellular signalling, particularly in neurological tissue (Pant et al., 2020). HexCer(d42:2) was decreased in the cortices of F1.*Scn1a*$^{+/-}$ mice compared to F1.WT mice. Additionally, the concentration of Cer species Cer(34:1), Cer(36:2), and Cer(38:2) were decreased in cortical F1 compared to 129. Cer species are bioactive molecules that are essential structural components of membranes and are considered a major building block of sphingolipid (Pant et al., 2020). More specifically, HexCer is the umbrella term for Cer metabolites galactosylceramide and glucosylceramide, both metabolised from the Cer addition of glucose or galactose via UDP-glucose-ceramide glucosyltransferase (UGCG) or UDP-galactose-ceramide galactosyltransferase (UGT8A), respectively (Reza et al., 2021). Both molecules are expressed in the CNS; however, the galactose version is the most predominant species in the brain, and is particularly enriched in myelin (Reza et al., 2021). The decrease in cortical HexCer (d42:2) concentrations, and presumably GalCer, in the F1.*Scn1a*$^{+/-}$ mice suggests that there might be reduced myelination in these mice. Demyelination has been identified in both Dravet syndrome patients and mouse models, including in this mouse model as previously identified by Richards et al. (2021), who recognized abnormal axonal growth and reduced myelination before the induction of seizures in the same *Scn1a*$^{+/-}$ Dravet syndrome mouse model utilized in our experiment. Consistent with this, Mito et al. (2024) identified widespread disruption in white matter and fibre density and cross-section in one Dravet syndrome patient (Mito et al., 2024). Neither study looked into the specific expression of lipid species, however the present study suggests that the poverty of myelination in Dravet syndrome might be related to reductions in HexCer, a critical component of myelin.

In parallel, the observed reduction of Cer species in cortical F1 compared to 129 suggests an alteration of lipid raft composition between these strains. Lipid rafts are cholesterol and sphingolipid-rich specialized microdomain of cell membrane that organise and stabilise signalling complexes (Gielen et al., 2006; Jana et al., 2009; Olsen et al., 2017; Viljetić et al., 2024). Their variable lipid composition, principally constituted by cholesterol, SM and Cer, creates an environment that may alter the localization and gating behaviour of numerous receptors and channels (D'Avanzo, 2016; Mencarelli et al., 2013). Although $Na_v1.1$ has not yet been explicitly localized to lipid rafts, cholesterol

depletion is known to mislocalize the sodium channel β2 subunit and impair its mobility (Cortada et al., 2021). In pathological states, depletion of sphingolipids and cholesterol disrupt raft architecture, destabilizing AMPA receptors, and leads to gradual loss of synapses and dendritic spines, and ultimately impaired nerve conduction (Hering et al., 2003; Jana et al., 2009; Mencarelli et al., 2013). Interestingly, in normal physiology, the ratio of cholesterol to ceramide has important implication in signalling: ceramide embedding into lipid raft selectively displaces cholesterol, and is associated with protein signalling amplification (Megha et al., 2004; Yu et al., 2005; Zhang et al., 2009a). Altogether, reduced Cer species in the cortex of seizure-susceptible F1 compared to 129 mice may suggest a possible baseline shift in lipid raft composition that exists in both presence and absence of the *Scn1a* haploinsufficiency and seizures. This may suggest that the baseline difference in this background strain could prime F1 animals to have an altered lipid raft composition that then synergizes with the aberrant sodium-channel behaviour, potentially lowering the threshold for hyperexcitability.

Moreover, both Cer and HexCer have been linked to modulation of neuronal excitability and other epilepsies. For instance, whole-cell patch-clamp recordings of sensory neurons showed the application of Cer increased action potential frequency, attributed to the activation of Na^+ current and suppression of delayed-rectifier K^+ currents (Zhang et al., 2002). Cer was proposed to engender this effect as a secondary messenger of nerve growth factor (Zhang et al., 2002). In humans, myoclonic epilepsies have been linked to mutations in the genes *CERS1* and *CERS2*, that encode ceramide synthases (CerS), which produce different sphingolipid of varying chain lengths (C18- and C24-26 respectively). These mutations result in a reduction of long-chain Cer and HexCer sphingolipids, including HexCer(d42:2), and disruptions of myelin sheets integrity (Mosbech et al., 2014; Vanni et al., 2014). Conversely, some lysosomal storage disorders (LSDs) that give rise to drug-resistant seizures display increased concentrations of Cer and HexCer species (Abed Rabbo et al., 2021; Dodge, 2017; Mencarelli et al., 2013). Moreover, increased brain concentrations of ceramide have been observed in kainic acid and pilocarpine rat models of status epilepticus (Mikati et al., 2003; Mikati et al., 2008). Furthermore, a multi-omic analysis of rats that underwent

chronic treatment with the chemoconvulsant PTZ found an upregulation of Cer and HexCer species in the hippocampus (Chen et al., 2025b).

Altogether, the conflicting evidence at the very least suggests that Cer and HexCer are dysregulated in epilepsy models, likely reflecting functionality differences driven by their underlying molecular architecture, such as saturation levels of sphingoid backbone, length of amide-linked to fatty-acyl chains, and hydroxylation state. Each sphingolipid structural feature have a profound impact on a lipid's biophysical properties, functionality and localization, and thus tend to have differential roles in pathophysiology (Hannun et al., 2018; Quinville et al., 2021). To resolve these ambiguities, future studies could employ targeted lipidomic analysis that covers the broad spectrum of Cer and HexCer structures. This application on the present *Scn1a*$^{+/-}$ mouse model would allow a clearer correlation of specific sphingolipid species with the epileptic phenotype.

The *Scn1a* mutation on the F1 seizure-susceptible background strain affected glycerophospholipids across various subclasses and chain lengths particularly, PC, LPC, PS and dMePE. This suggests a broad modulation of lipid species in the glycerophospholipid pathway. In line with this Koh et al. (2024) recently examined lipidomic profiles in *SCN1A* knockout cerebral organoids and also found dynamic modulation of PC and LPC analogues of varying chain length. Glycophospholipid are characterized by a glycerol backbone with various types of fatty acid chains, and different phosphate-containing head groups (Vance, 2015). The characteristic head group determines the lipid subclass, which include most commonly choline (PC), ethanolamine (PE), serine (PS), inositol (PI), glycerol (PG), no head group (i.e., PA), and may even include derivative molecules including lyso (e.g. LPC), and dimethyl (e.g. dMePE) analogues, each with differential biophysical properties and functionalities (Fahy et al., 2011; Liebisch et al., 2020). Glycerophospholipids are the major component of lipid bilayers, and play a crucial role in maintaining membrane structure as well as in signalling (Alberts et al., 2002). Glycerophospholipids are known to influence neuronal excitability. For instance, PS plays a role in exocytosis, via Ca^{2+} triggered vesicle fusions of neurotransmitters, and facilitates synaptic plasticity (Zhang et al., 2009b). Interestingly, LPC, a PC metabolite formed by phospholipase A2-mediated PC hydrolysis, modulates NMDA activity of the excitatory glutamatergic NMDA receptor (Ikeuchi et al.,

1995). Whole-cell patch clamp studies on rat hippocampal glial cells revealed that LPC reversibly inhibited NMDA-gated currents, with the suggestion that LPC exerting its effect via modulation of channel-gating kinetics or interaction with components surrounding the channel's membrane space (Ikeuchi et al., 1995). Schilling et al. (2004) identified that LPC promoted microglial cell de-ramification via non-selective activation of cation currents in patch-clamp experiments of mice. PCs are also important for neuronal axon development, as reduction of PC synthesis reduces axonal branching (Strakova et al., 2011). Altogether, the existing literature agrees with the role of glycosphingolipids as an essential structural component in neurons and glia, as well exerting an influence over neuronal excitation and signalling. Hence, is logically follows that dysregulation of these mediators could contribute to the pathophysiology of brain disorders like epilepsy including Dravet syndrome.

In rats, the seizure-inducing agents soman and bicuculline increased PC metabolism as measured by the increased concentrations of its metabolites in the brain(Flynn et al., 1987). Further, seizures altered glycerophospholipids amongst other lipid classes in a brain-region specific manner in an acute kainic acid-induced mouse model of epilepsy. Mirroring this study, there was a reduction in glycerophospholipids in the cortex, but not hippocampus, including PC, PE, PG, PS, and LPC(18:0) which occurred in first hour of seizure onset (Lerner et al., 2017). In humans, dysfunction of biosynthetic enzymes for PC and PE production are involved in autosomal recessive rare diseases such as a neurodevelopmental disorder with muscular dystrophy (*CHKB*), bone abnormalities and cone-rod dystrophy (*PCYT1A*) and spastic paraplegia (*PCYT2*, *SELENOI*) that, like Dravet syndrome, display cognitive disability and seizures (Klöckner et al., 2022; Vaz et al., 2019). Mondal et al. (2024) found decreased hippocampal concentrations of positively charged glycerophospholipids, e.g., PC, and PE, with a complementary decrease in negatively charged glycerophospholipids, particularly PS and PG in brain tissue resections from temporal lobe epilepsy patients. In contrast, Johnson et al. (2020) found that seizure-prone voltage-gated potassium channel $K_v1.1$ knockout mice displayed distinct lipid biosynthesis pathways compared to wild-type mice, with increase concentrations of various lipid species including PC, and decreased concentrations of PS and PI in the hippocampus compared to the cortex (Johnson et al.,

2020). The ketogenic diet elevated levels of HexCer, TG, PC and PS in patients with super-refractory status epilepticus (Dickens et al., 2023). This suggests that the ketogenic diet help reverse the epileptic-induced reductions in these lipid species, which could also translate to the lipids observed here in the seizure-prone F1.*Scn1a*$^{+/-}$ mice, a hypothesis that might be explored in future studies. Future research is needed to provide a more detailed characterisation of the identified glycosphingolipids in the present study, to better understand their role in Dravet syndrome.

Reduced TG(54:6) in the seizure-susceptible F1 strain was also identified. TGs are neutral lipids that fall under the glycerolipid family. Their structure is characterized by a glycerol backbone with three fatty acid chains (Fahy et al., 2011). Physiologically, they are the main constituents of body fat and have an essential function in energy storage and metabolism (Viecili et al., 2017). TGs are commonly stored in cell lipid droplets with metabolites of excess lipids, namely, cholesteryl acyl ceramides and sterol esters (Yang et al., 2022). In the brain, lipid droplets are considered metabolic hubs that are tightly regulated and contribute to various physiological processes including energy and signalling precursor storage, but also protection from lipotoxicity (Yang et al., 2022). Lipid droplets have been linked to Alzheimer's disease and Parkinson's disease as are sites of α-synuclein aggregation (Yang et al., 2022). Research into the involvement of TGs in seizures and epilepsy has been more limited, though. overexpression of triglycerides (hypertriglyceridemia) decreased seizures compared to controls in a hamster neonatal seizure model (Shen et al., 2024). The authors proposed that the protective property of TGs might be explained by the stabilization of potassium channels, via increased palmitic acid levels from triglyceride breakdown (Shen et al., 2024). However perhaps the strongest connections between glycolipids and Dravet syndrome is the utilisation of the ketogenic diet to treat the condition (Borowicz-Reutt et al., 2024; Caraballo et al., 2005; D'Andrea Meira et al., 2019; Ikeda et al., 2023; Jancovski et al., 2021). Under a ketogenic diet, the body transitions from using carbohydrates to fats, including TGs, as primary fuel that produces ketone bodies (ketosis) (D'Andrea Meira et al., 2019). Interestingly, the exact mechanism in which the diet offers seizure-relief benefits is still debated and theories for its efficacy are controversial (D'Andrea Meira et al., 2019; Mishra et al., 2024). However, my findings might suggest that the upregulation of an underlying

pathophysiological lower TG concentration could be tied to this mechanism of action. In the present study, the concentration of TG(54:6) is decreased in the cortex of F1.*Scn1a*$^{+/-}$ compared to F1.WT, an effect that was absent in the hippocampus nor different between 129.*Scn1a*$^{+/-}$ and 129.WT mice in the cortex. Thus, as F1.*Scn1a*$^{+/-}$ mice are the only group to present seizures, the observed reduction in TG may reflect the unique impact of seizures in this group of mice. Considering that lipid-rich diet such as the ketogenic diet enriches the body in TG and fatty acids content, nutrients that cross the BBB and are able to reach the brain (Banks et al., 2018), here we hypothesise the possibility that a factor behind the ketogenic diet's therapeutic efficacy lies the restoration of TG levels in the epileptic brain. Such restoration could further aid the seizure-driven energy depletion and potentially contributing to seizure mitigation and the alleviation of the epileptic phenotype. Due to the inherent limitations of untargeted lipidomics, the specific fatty acyl chain lengths of the identified TG cannot be fully elucidated (Contrepois et al., 2018; Smirnov et al., 2021); thus, future studies could perform targeted analysis to broaden the TG quantification and resolve the identity of the detected changes, as well as expand on the lipid family. Additionally, it would be interesting to assess the impact of the ketogenic diet on the lipidome in the *Scn1a* mouse model of Dravet syndrome.

There are a number of limitations to this study that point to crucial areas for further investigation. Untargeted lipidomic studies aim for a broad range of measurement by comparing all possible measurable lipids, capturing thousands of features simultaneously in a single sample, often at the cost of sensitivity and/or specificity (Smirnov et al., 2021). These characteristics make it a powerful discovery tool; however, this approach comes with numerous challenges and limitations regardless of specific workflows (Contrepois et al., 2018). These includes: 1) only relative abundances can be reported due to its semiquantitative nature; 2) signal suppression is common due to the broad range of masses being captured simultaneously; 3) prevalence of isomer ambiguity due to it only being able to report sum-composition level of compounds and consequently failing to identify important structural differences; 4) challenges in data processing and annotation commonly leading to false positives; and 5) low levels of standardization and reproducibility in the field (Contrepois et al., 2018; Forest et al., 2018; Smirnov et al., 2021; Xu et al., 2018). Because of these limitations, untargeted

approaches are often required to be validated by hypothesis driven targeted analysis. The most popular approach is identification and quantification of a specific group of metabolites via LC-MS/MS with multiple-reaction monitoring (Cajka et al., 2016; Contrepois et al., 2018). Therefore, future work could focus on the targeted validation of the present results studies, including the glycosphingolipid TG family, glycerophospholipid pathways, as well as sphingolipids Cer and/or HexCer, an investigational approach that will be applied in the next chapter.

2.5 Conclusion

This investigation identified unique lipidomic profiles associated with the heterozygous deletion of *Scn1a* and the seizure-susceptible F1 strain. Cortical F1.*Scn1a*$^{+/-}$ versus F1.WT showed alterations in sphingolipid, glycerolipid, and glycerophospholipids families, and, in parallel, differences between the 129 seizure-resistant strain and the F1 seizure-susceptible strain. This suggests we have identified a neurological lipidomic signatures that could be associated with the F1.*Scn1a*$^{+/-}$ seizure phenotype. Futures studies will focus on further validating these results via targeted lipidomic analysis (Chapter 3).

Chapter 3

Targeted LC-MS/MS Analysis of Hexosylceramides in the *Scn1a*$^{+/-}$ Mouse Model of Dravet Syndrome

3.1 Introduction

Chapter 3 provided an untargeted LC-MS/MS analysis of the effect of heterozygous deletion of *Scn1a* on the brain lipidome of a seizure susceptible F1 strain versus a seizure-resistant 129 strain. This analysis suggested that the HexCer family of lipids may be of particular interest, given that one structure, HexCer(d42:2), appeared to be the most significantly differentially expressed lipid between F1.*Scn1a*$^{+/-}$ and F1.WT mice in the cortex. Untargeted approaches are used in lipidomics for hypothesis generation via the identification of global alterations in the abundance of lipids, followed by targeted methods that aim to identify and quantify differences in specific metabolites (Han et al., 2022). Accordingly, the current chapter aimed to extend on the untargeted approach used in Chapter 3 with the objective of verifying and expanding on the quantification of various HexCer species the *Scn1a*$^{+/-}$ mouse model of Dravet syndrome.

HexCer species are part of the sphingolipid class of lipids, which are a diverse class of amphiphilic molecules. Their defining structural characteristics are a sphingoid base backbone that is amide-linked to fatty-acyl chains of varying lengths and saturation levels, and O-linked to a one of various head group possibilities (Hannun et al., 2018). Sphingolipid biosynthesis originates from the fatty acid palmitate and serine and follows a multi-enzymatic chain-reaction that culminates in the creation of Cer, a process that occurs via the CerS and the dihydroceramide desaturase (DES1/2) family of enzymes. Cer, a main sphingolipid subclass, is the precursor of various other sub-classes, specifically, sphingosine (via ceramidase), SM (via sphingomyelin synthase), and cerebrosides, also referred as glycoceramides (Hannun et al., 2018; Quinville et al., 2021; Shing et al., 2023). The latter species are metabolised in the golgi-apparatus, where a Cer is linked to a hexose sugar, either a glucose or galactose depending on the metabolising enzyme (UGCG and UGT8A respectively); the resulting species is then referred to as one of two epimers, glucosylceramide (GlcCer) or galactosylceramide (GalCer) (Figure 3.1). Given GlcCer and GalCer are mass isomers with indistinguishable fragmentation patterns, conventional LC-MS lipidomic analyses are unable to distinguish between them, and so they are collectively referred to as HexCer species (Hannun et al., 2018; Reza et al., 2021) (Figure 3.2).

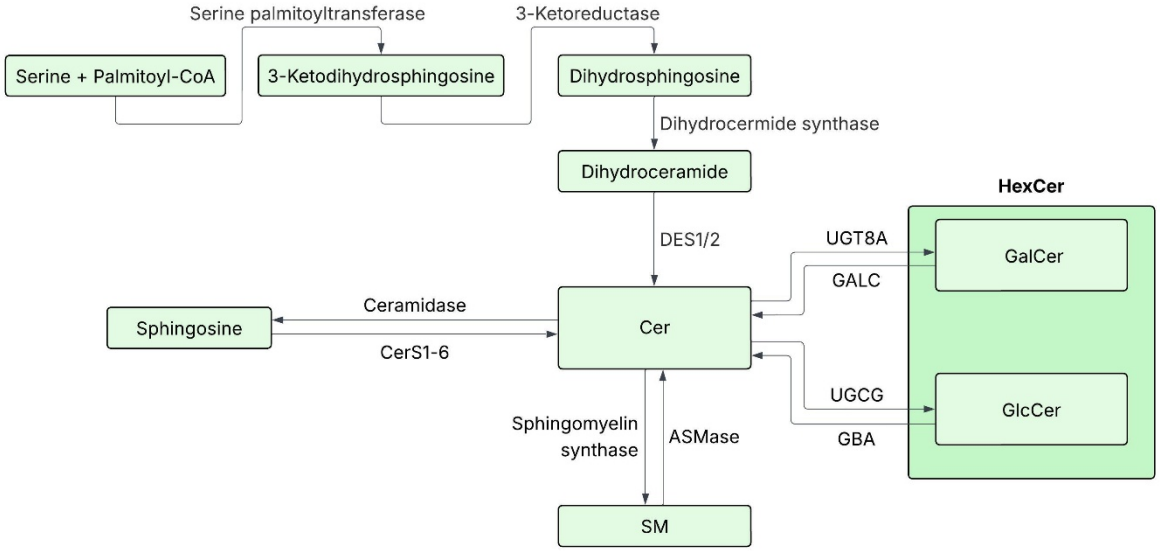

Figure 3.1. Overview of sphingolipid metabolism. Image made using Lucid Chart and inspired by Hannun et al. (2018) and Shing et al. (2023).

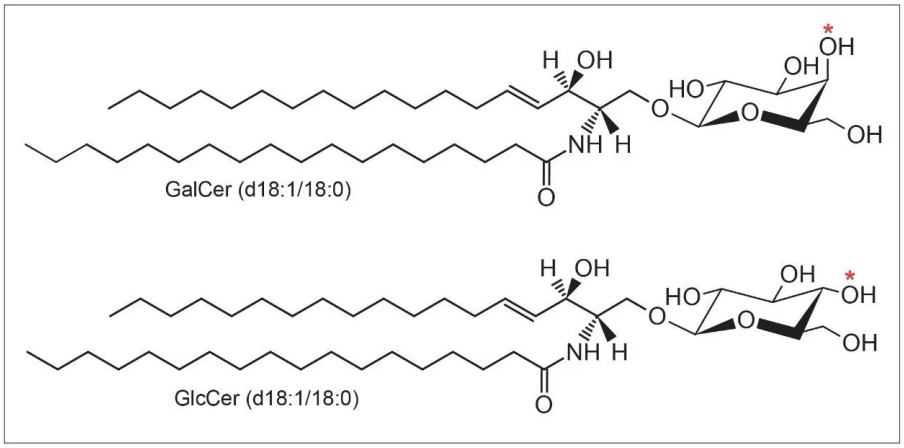

Figure 3.2. Chemical structure of GalCer and GlcCer isomers GalCer(d18:1/18:0) and GlcCer(d18:1/18:0). Red asterisk indicates the difference in the position of the hydroxyl group at the C-4 position. Image from Isaac et al. (2022).

Sphingolipids, and particularly HexCer, are highly abundant in the nervous system. Their function extends from maintenance of structural order and integrity, to being heavily involved in signalling pathways, and as precursors of more complex glycosphingolipids (Olsen et al., 2017; Yu et al., 2009). They reside in the plasma membrane, both in the lipid bilayer but particularly in cell surface lipid rafts. Lipid rafts

are clusters of sphingolipids and cholesterol that form microdomains that liaise with membrane proteins, in turn, they provide structural support as well as actively participation in the signalling complexes (Dart, 2010). GlcCer is predominantly expressed in all cell types in peripheral organs including gastrointestinal tracts, liver, and adrenal medulla, and only appears in trace amounts in the nervous system. In contrast, GalCer is highly abundant in the brain and only in trace amounts in the peripheral nervous system, namely, in Schwann cells (Reza et al., 2021). Although GalCer species appear in small quantities in neurons (Van Zyl et al., 2010), they are generally identified as oligodendrocyte myelin-specific lipids, being abundantly expressed in myelin sheaths (Norton et al., 1966). Here they actively participate in the stabilization and maintenance of myelin (Schmitt et al., 2015), but are also involved in oligodendrocyte differentiation and maturation during development (Marcus et al., 2002). Altogether, HexCer species are abundant in the brain and play an important role in homeostatic balance of the nervous system, particularly in neuronal excitability, given the important role for myelin in the conduction of action potentials.

Given the importance of HexCer species in nervous system function, it is perhaps not surprising that these lipids have been linked to several neurological diseases including Parkinson's disease and Alzheimer's disease (Olsen et al., 2017; Quinville et al., 2021). Relevant to this thesis, the broad sphingolipid biosynthesis and degradation pathways are linked to epilepsy. Dysregulation of ganglioside biosynthesis, a major type of glycosphingolipid, has been associated to epilepsy syndromes such as West syndrome (Izumi et al., 1993), temporal lobe epilepsy (Yu et al., 1987), and an autosomal recessive infantile-onset symptomatic epilepsy syndrome (Simpson et al., 2004). In addition, sphingolipids have a well-established connection to a wide range of lysosomal storage diseases (LSD) and which are often accompanied by refractory seizures (Pastores et al., 2013; Yu et al., 2009). Mutations in the CerS enzyme's corresponding genes, CERS1 and CERS2, leading to the development of progressive myoclonic epilepsies (Ferlazzo et al., 2016; Mosbech et al., 2014; Vanni et al., 2014). Similarly, mutations in acid ceramidase, an essential enzyme for sphingolipid degradation, showed increased risk of spinal muscular atrophy with progressive myoclonic epilepsy (Zhou et al., 2012). In animal models, the link between sphingolipids, seizures and epilepsy has

been further emphasized. For instance, transgenic mice designed to over-accumulate GalCer and its sulfatide version in neurons, showed vast neuronal hyperexcitability and had increased sensitivity to lethal audiogenic seizures, and a decreased lifespan (Van Zyl et al., 2010). Additionally, bioinformatic analysis of hippocampal tissue damage from rats treated with the chemoconvulsant agent pentylenetetrazole (PTZ) yielded changes in sphingolipid pathways, including SM, ceramides, and HexCer; in the study the acute PTZ model yielded decreased hippocampal HexCer levels while the chronic administration displayed upregulation compared to controls, thus suggesting a direct modulation of HexCer species based on intensity and duration of neuronal excitability, synaptic dysfunction and hippocampal tissue damage (Chen et al., 2025b).

Thus far, the connections to specific sphingolipid perturbations to Dravet syndrome have been limited. The ketogenic diet, a diet high in lipids known to modulate physiological lipid levels in the body, is commonly used and can be effective in treating Dravet syndrome, however it is a latter-line therapy that is utilised when pharmaceutical treatments fail (Borowicz-Reutt et al., 2024). In adult patients with super-refractory status epilepticus, adjunctive treatment with ketogenic diet was positively correlated with improved clinical outcomes, including seizure frequency reduction, and the modulation of lipid sub-species, including HexCer (Dickens et al., 2023). In relation to Dravet syndrome, metabolic profiling of genetic Scn1a-A1783V Dravet syndrome mouse revealed hippocampal metabolic alterations in energy metabolism driven by both the *Scn1a* deficiencies and the ketogenic diet. These were in turn highly associated with neuronal hyperexcitability and seizure susceptibility modulation of the mice. In particular, changes in glucose, glycolysis and tricarboxylic acid (TCA) cycle intermediates, noradrenaline, corticosterone, and GABA, suggest genotype-and treatment driven modulations of catabolic processes (Miljanovic et al., 2021). Given the tight connection within the lipidome and the broader metabolome, as well as the ketogenic diet association, it is plausible that the observed catabolic modulation in Dravet syndrome could also extend to the lipidomic profile associated with the disease. In Chapter 3, the use of an untargeted lipidomic approach suggested that the specific HexCer lipid sub-class may be influenced by partial genetic deletion of *Scn1a* in the seizure-susceptible strain. Here we aimed to extend on this research by conducting a

targeted quantitative analysis across the wide array of structural variants in the HexCer class. We therefore assessed whether *Scn1a* genotype or background influence the concentrations of HexCer variants across two brain regions, the cortex and hippocampus.

3.2 Method

3.2.1 LC-MS/MS Analysis of HexCer

Analyses of HexCer were adapted from Jieu et al. (2024). Samples measured under our untargeted LC-MS/MS (Chapter 2, section 2.2) from mice cortex and hippocampal tissue of 129.WT, 129.*Scn1a*$^{+/-}$, F1.WT and F1. *Scn1a*$^{+/-}$ strain and genotype combinations, balanced by sex and collected at postnatal day 24 (P24) (n = 7-10 per group) were quantified for absolute values using a TSQ Altis triple quadrupole mass spectrometer, coupled to a Vanquish HPLC System (Thermo Fisher, CA, USA). Separation of HexCer was performed on an Agilent XDB-C8 column (5 µM pore size, 3 × 150 mm) using a modified binary gradient (Hejazi et al., 2011) set at 30°C and a flow rate of 0.2 mL/min. Solvent A was 0.2 % formic acid, 2 mM ammonium formate in water and solvent B was 1 % formic acid, 1 mM ammonium formate in methanol. Sample injection was 2 µL and the following chromatographic parameters were used: initial conditions were 80% B for 2 minutes, ramped up to 87% B for 5 minutes, then 100% B for 7 minutes and held for 6.5 minutes, lowered to 80% B for 0.5 minutes and held for 3 minutes; overall run time was 24 minutes per sample. MS was operated in positive ion mode (MRM) with a heated electrospray ionization (H-ESI) source; additional MS/MS conditions were as follows: 4500 V, 8.5 Arb sheath gas, 10 Arb aux gas, 10 Arb sweep gas, 275°C ion transfer tube temperature and 220°C vaporiser temperature. MS conditions for each analyte of interest are provided in Table S3.1. Each HexCer of interest's corresponding precursor ion was scanned against known products for sphingoid backbones sphingosine (*m/z* 264, d18:1) and sphingadiene (*m/z* 262, d18:2).

3.2.2 Data Processing

Peak visualization and quantification were carried out using XCalibur software (version 4.2.28.14, Thermo Fisher, CA, USA). ReTimeML (Allwright et al., 2024) was employed to assign retention times using reference spectra for GalcCer(d18:1/12:0), HexCer(d18:1/16:0), HexCer(d18:1/18:0), HexCer(d18:1/18:1), and HexCer(d18:/24:1) (all Cayman Chemical, MI, USA). Results were cross validated manually in XCalibur to ensure peak assignment could be consistently distinguished from background noise (S/N < 3). The resulting dataset was further refined and processed with R studio software using dplyr and base R packages. A HexCer was excluded from analysis if it wasn't confidently quantified in ≥80% of the samples. A total of 29 individual HexCer were quantified. Relative lipid abundances were further normalized to the internal GalCer (d18:1/12:0) and further normalized to tissue weight. The final quantified HexCer were expressed in pmol/mg of tissue. In addition to analysing individual HexCer concentrations, total HexCer levels were also assessed statistically.

3.2.3 Statistical Analysis

All statistical analyses were performed in GraphPad Prism (version 10.4.1, MA, USA). Outliers were removed using ROUT tests (Q = 5%) (Jieu et al., 2024). HexCer concentrations were evaluated for normality using Shapiro-Wilk test (alpha=0.05), non-normally distributed data were $\log_{10}$-transformed to improve normality. Statistical comparisons of HexCer concentrations were conducted using two-way ANOVA with fixed effects for strain, genotype and strain by genotype interactions. Sidak's post hoc test was applied to correct for multiple comparisons. Significant differences were established at $p < 0.05$.

3.3 Results

HexCer structures vary in various structural points. Specifically, variability can be found in the sphingoid base saturation, most commonly found in the mono (d18:1) and di-saturated (18:2) versions, and fatty acyl chain length and saturation, extending from C16 to C24 (e.g. C16:0, C18:1, C24:1, etc...) (Reza et al., 2021). The resulting HexCer

nomenclature, e.g. HexCer(d18:1/18:0), one of the most abundant species, is therefore implying it has a mono-unsaturated sphingoid backbone, together with a saturated fatty acyl chain of 18-carbon length. Each structural characteristic possesses its own differential functionality, localization, and mode of trafficking (Reza et al., 2021), thus quantification of a wide variety of structural motifs is of great importance if the aim is to identify overarching patterns of dysfunction. Previously in Chapter 3, HexCer(d42:2) was identified as the lipid with the highest VIP score in the cortex F1.WT vs F1. *Scn1a*$^{+/-}$ OPLS-DA. It showed that HexCer(d42:2) was significantly decreased in the cortex of *Scn1a*$^{+/-}$ mice compared to WT controls, an effect that occurred exclusively in the F1 seizure-susceptible background strain and in the cortex. This suggested this HexCer as a lipid of interest, but given that untargeted LC-MS/MS analysis restricts the identification of a compound to its sum-composition, a targeted analysis was conducted to examine HexCer structures in more detail (Porta Siegel et al., 2019).

First, the differences in total HexCer concentrations across experimental groups in the hippocampus and cortex were examined (Figure 3.3). Consistent with prior observations, hippocampal tissue contained higher HexCer concentrations than in the cortex of mice ($p < 0.0001$). However, two-factor ANOVA showed no main effects of *Scn1a* genotype, strain, or genotype by strain interactions. This suggested that any effects of genotype or strain may be more confined to specific HexCer species.

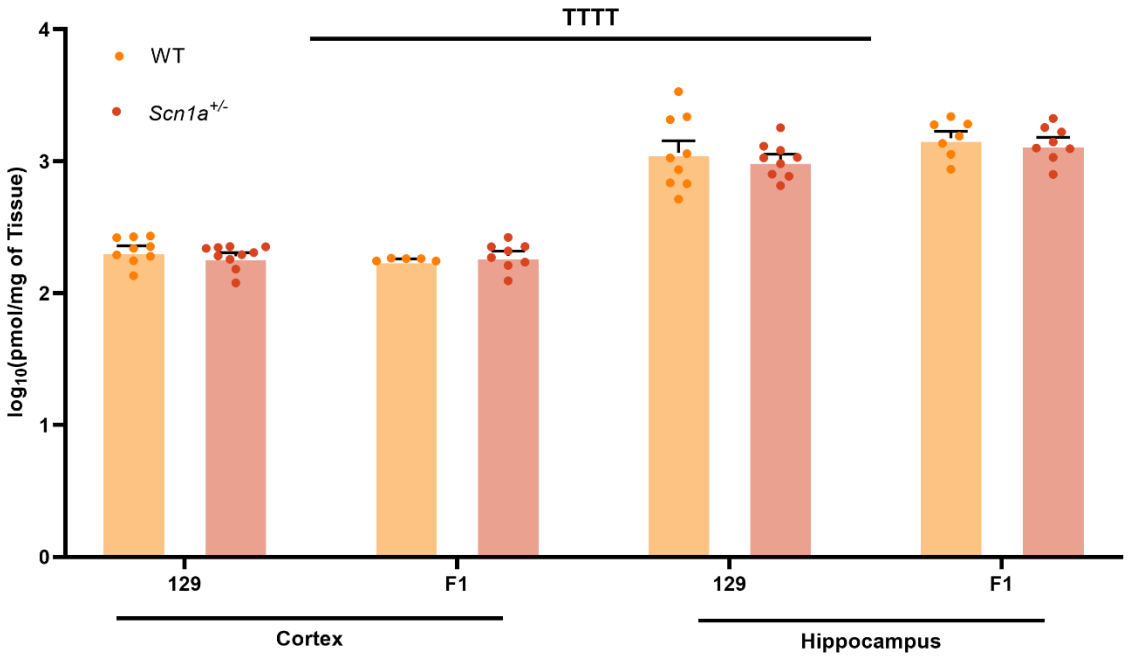

Figure 3.3. Total HexCer mean concentrations ± SEM per strain (129 vs F1) and genotype (WT vs *Scn1a*$^{+/-}$) in two regions of mouse brain, the cortex and hippocampus.TTTT indicates overall increased total HexCer concentrations in the hippocampus compared to the cortex ($p<0.0001$). Graph is in log scale for consistency and clarity of exposition.

3.3.1 Targeted Analyses in the Cortex

Differences were then examined for the specific HexCer species identified in Chapter 3, i.e., HexCer(d42:2). The distribution of saturation of the potential HexCer is ambiguous, with the possibility of more specific species such as d18:2/24:0 or d18:1/24:1. Targeted analysis of cortex tissue showed that there was no effect of genotype, strain or a genotype by strain interaction on either HexCer(d18:1/24:1) or HexCer(d18:2/24:0) in the cortex (2-way ANOVA) (Figure 3.4).

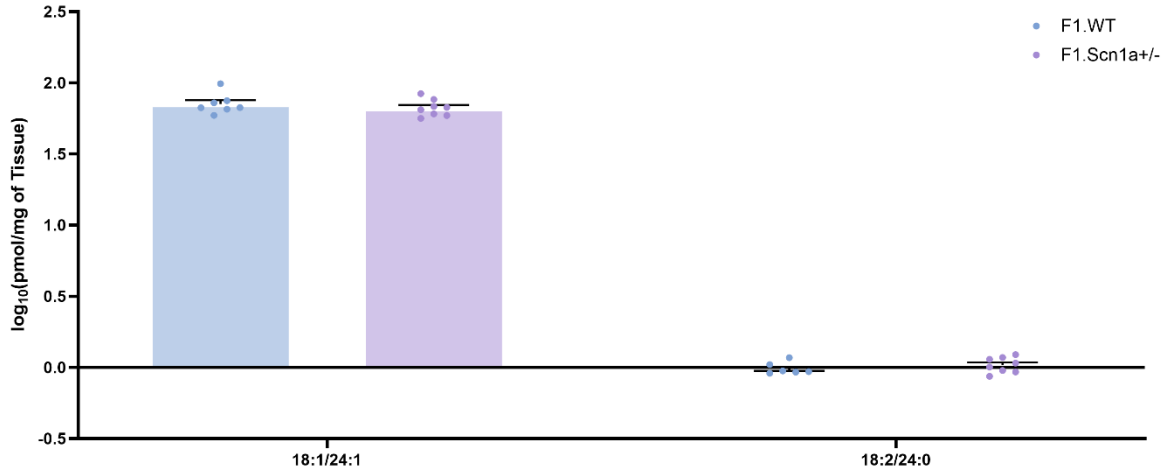

Figure 3.4. HexCer mean concentration ± SEM for HexCer d18:2/24:0 and d18:1/24:1 in the F1 strain for the WT and *Scn1a*$^{+/-}$ genotypes in the cortex of the mouse brain. No significant differences were found. Graph is in log scale for consistency and clarity of exposition.

A more comprehensive analysis of a total of 29 HexCer species with varying sphingolipid backbone saturation (d18:2 and d18:1), fatty acyl chain length and saturation, and level of hydroxylation was then conducted. Mean concentrations of total and individual HexCer species per tissue and strain x genotype group can be found in Table S3.2, and further statistical results can be found in Table S3.3.

Two-way ANOVA showed significant strain and genotype effects on specific HexCer, but no strain and genotype interactions in the cortex (Figure 3.5). Strain-driven effects were seen amongst short chain mono-saturated (d18:1) sphingoid base HexCer, specifically, C16:0 ($p = 0.026$), C18:0 ($p = 0.037$), and C18:1 ($p = 0.002$); while the C16:0 and C18:1 showed increased concentration in the F1 strain compared to 129, C18:0 showed reduced concentrations in the F1 versus 129 strain (Figure 3.5A). Only one di-unsaturated (d18:2) HexCer, C20:1 had significantly higher concentrations in the F1 versus 129 strain ($p = 0.007$); consistent with this was that in the WT mice specifically this species was significantly increased in F1 compared to 129 strain mice ($p = 0.040$). Amid the d18:2 sphingoid backbone, *Scn1a*$^{+/-}$ mice, irrespective of strain, had higher concentrations of this C18:0 species compared to WT mice ((effect of genotype: $p = 0.025$)) (Figure 3.5B). Finally, no significant differences were seen in any individual HexCer with hydroxyl groups (Table S3.3).

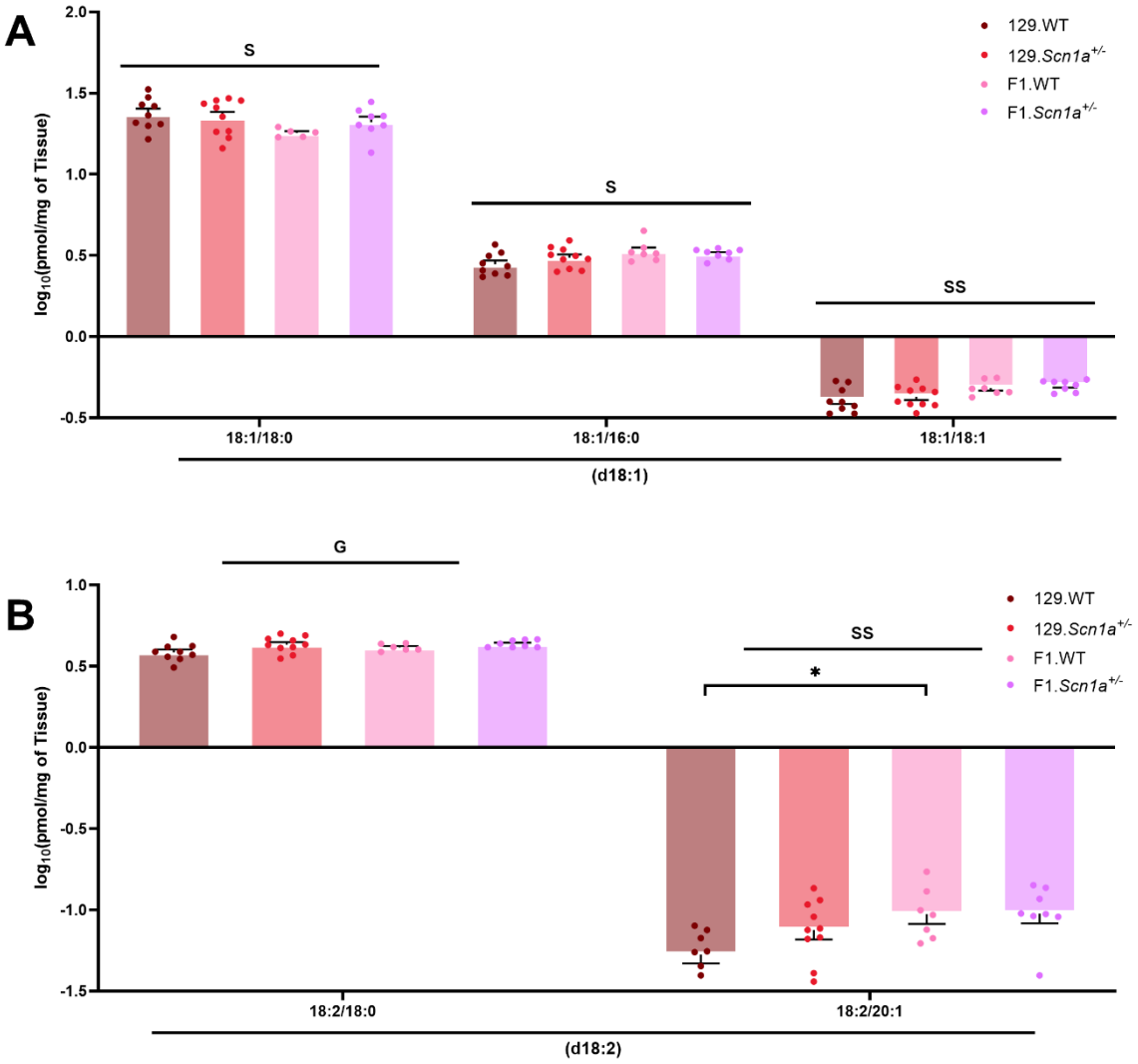

Figure 3.5. HexCer content per strain (129 vs F1) and genotype (WT vs *Scn1a*$^{+/-}$) in the cortex (n= 7-10). Figure displays only structures that displayed significant statistical differences (A) Shows non-hydroxylated d18:1 structures, (B) shows non-hydroxylated d18:2. HexCer are expressed as $\log_{10}$ (pmol/mg of tissue). Samples were analysed by two-way ANOVA with fixed effects corrected for multiple comparisons using Sidak's statistical hypothesis testing. Significant differences were established at *p < 0.05, **p < 0.01 (S indicates strain effects and G for genotype effect). Graphs are in log scale for consistency and clarity of exposition.

3.3.2 Targeted Analyses in the Hippocampus

Notably, almost 40% of individual HexCer tested were significantly different between strains in the hippocampus, with F1 strain mice having significantly higher concentrations than 129 in all cases (Figure 3.6 & Figure 3.7). Further, 18 additional HexCer species trended towards significance ($p<0.1$) (Table S3.3). In contrast, no HexCer species were influenced by the *Scn1a*$^{+/-}$ genotype, nor were there interactions between strain and genotype identified.

Structures containing mono-saturated sphingoid bases (d18:1) showed the most prominent strain effects, namely, C22:1 ($p = 0.013$), C20:1 ($p = 0.044$), C18:1 ($p = 0.029$), and C16:0 ($p = 0.001$); post hoc analysis revealed significantly higher C16:0 levels in the F1 strains across both genotypes *Scn1a*$^{+/-}$ ($p = 0.038$) and WT ($p = 0.044$) compared to the 129 strain (Figure 3.6A). Similarly, various hydroxylated counterparts also displayed this pattern, specifically, C22:0-OH ($p = 0.026$), C22:1-OH ($p = 0.005$), and 24:0-OH ($p = 0.001$), with the latter showing significantly elevated concentrations in the F1.*Scn1a*$^{+/-}$ compared to 129.*Scn1a*$^{+/-}$ ($p = 0.004$) as confirmed by post hoc test (Figure 3.7Figure 3.7A).

Within the di-unsaturated d18:2 HexCer, the same strain differences were seen for C16:0 ($p = 0.027$) and C24:1($p = 0.007$) (Figure 3.6B), as well as hydroxylated counterparts C18:0-OH ($p = 0.006$) and C24:1-OH ($p = 0.033$); post hoc analysis again revealed significantly higher C18:0-OH concentrations in the F1.*Scn1a*$^{+/-}$ compared to 129.*Scn1a*$^{+/-}$ ($p = 0.020$) (Figure 3.7B).

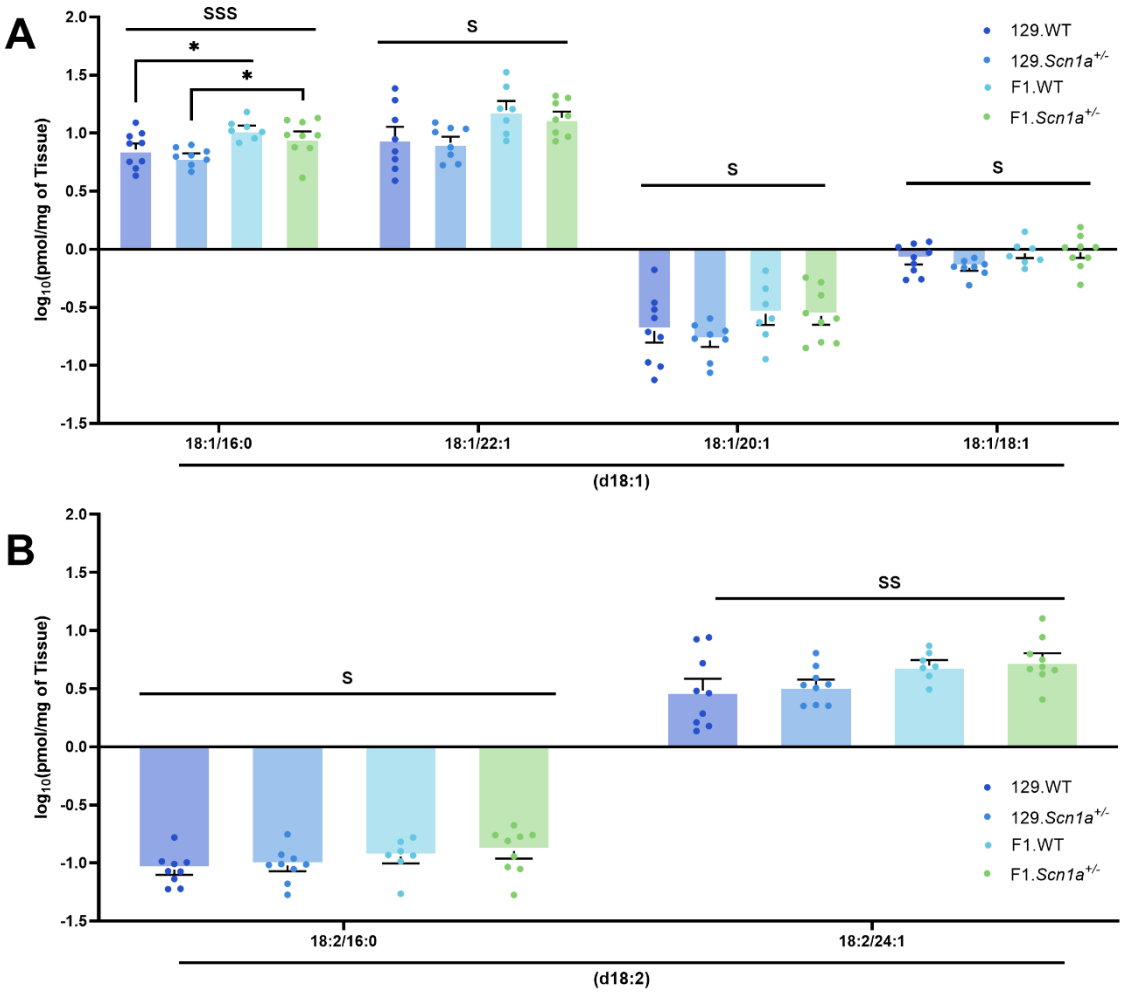

Figure 3.6. HexCer content per strain (129 vs F1) and genotype (WT vs *Scn1a*$^{+/-}$) in the hippocampus (n= 7-10). Figure displays only structures that displayed significant statistical differences (A) Shows non-hydroxylated d18:1 structures, (B) shows non-hydroxylated d18:2. HexCer are expressed as $\log_{10}$(pmol/mg of tissue). Samples were analysed by two-way ANOVA with fixed effects corrected for multiple comparisons using Sidak's statistical hypothesis testing. Significant differences were established at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (S indicates strain effects). Graphs are in log scale for consistency and clarity of exposition.

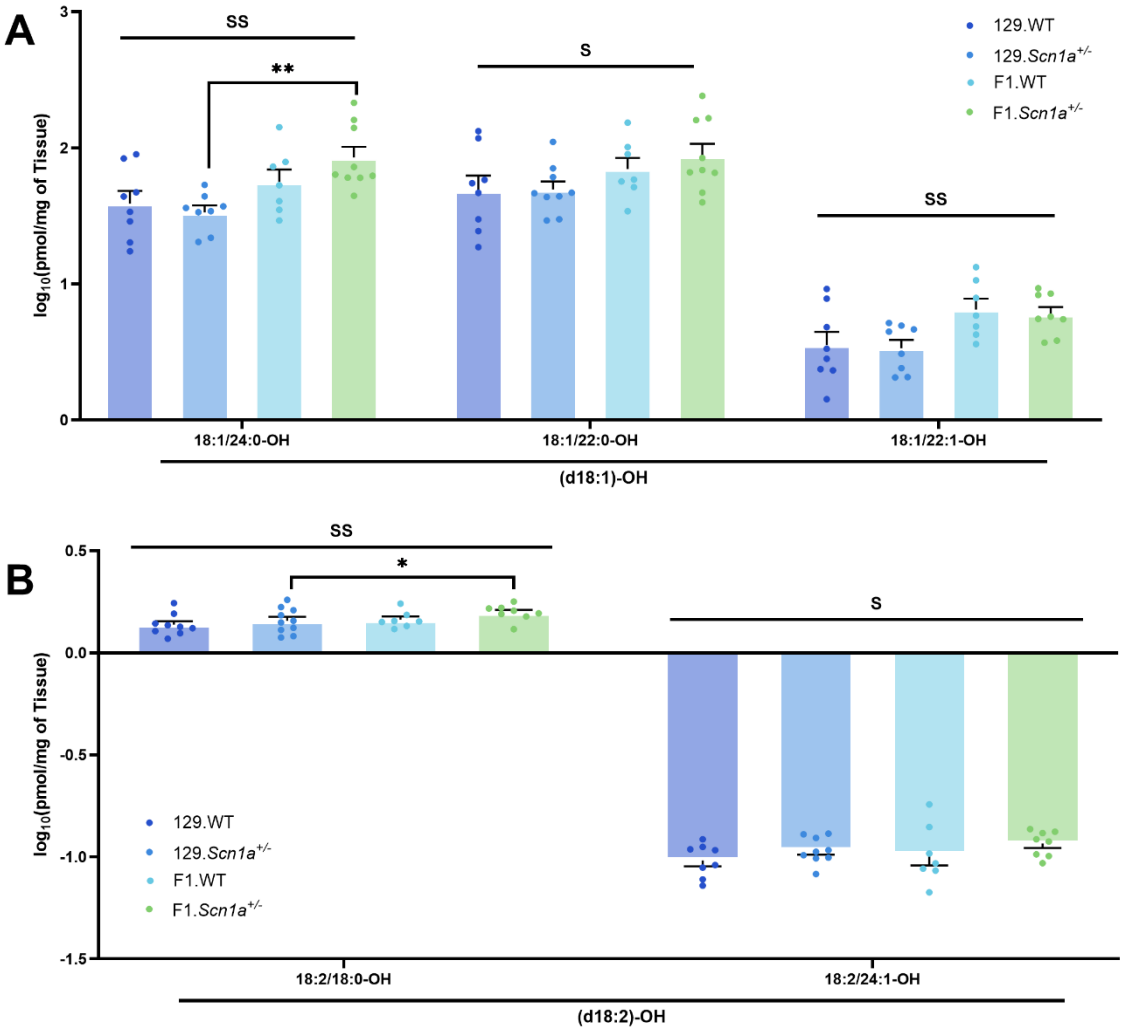

Figure 3.7. HexCer content per strain (129 vs F1) and genotype (WT vs *Scn1a*$^{+/-}$) in the hippocampus (n= 7-10). Figure displays only structures that displayed significant statistical differences (A) Shows hydroxylated d18:1 structures, (B) shows hydroxylated d18:2. HexCer are expressed as $\log_{10}$(pmol/mg of tissue). Samples were analysed by two-way ANOVA with fixed effects corrected for multiple comparisons using Sidak's statistical hypothesis testing. Significant differences were established at *$p < 0.05$ and **$p < 0.01$ (S indicates strain effects). Graphs are in log scale for consistency and clarity of exposition.

3.4 Discussion

Dravet syndrome is a highly complex and detrimental epilepsy with onset in infancy. It is characterised by a heterozygous mutation in the *SCN1A* gene, a gene that codes for a subunit of voltage-gated sodium channels ($Na_v1.1$) (Claes et al., 2001). However, much remains unknown about the disease pathophysiology, and its treatment remains inadequate (Samanta, 2025). This has encouraged the continuous investigation of previously underexplored aspects of the disease, aiming to uncover novel pathways of dysregulation that can lead to the creation of more efficient therapeutics. Seizures and epilepsy syndromes have associations with lipid dysregulation; however, studies examining the brain lipidome of Dravet syndrome are limited. The work presented here aimed to identify both *Scn1a* genotype- or background strain-associated changes in the brain lipidome using targeted LC-MS/MS, by looking at various species in the HexCer class of lipids including specifically HexCer(d42:2). To achieve this, we analysed a series of HexCer species in two epilepsy-relevant brain tissues, the cortex and hippocampus, of 129.WT, 129.*Scn1a*$^{+/-}$, F1.WT and F1. *Scn1a*$^{+/-}$ mice.

The first focus was on HexCer(d42:2), according to the two possibilities of d18:2/24:0 or d18:1/24:1, as the saturation distribution of the potential HexCer as defined by the untargeted approach is ambiguous (Lee et al., 2018; Porta Siegel et al., 2019). Both lipids were quantified, however, neither followed the pattern identified in the untargeted analysis, with neither of these species being influenced by *Scn1a* genotype, background strain or their interaction. The reason behind this discrepancy is unclear, but it could be attributed to inaccuracies in untargeted LC-MS/MS analysis processes. Untargeted lipidomic studies aim for a broad range of measurement by comparing all possible measurable lipids, capturing thousands of features simultaneously in a single sample, often at the cost of sensitivity and/or specificity (Smirnov et al., 2021). These characteristics make it a powerful discovery tool, however, this approach is often regarded as a semiquantitative technique, with numerous challenges and limitations regardless of specific workflows (Contrepois et al., 2018). The most challenging step is data processing and annotation, where thousands of peaks are identified, aligned, quantified, and then annotated against data libraries. In the present study, this process

was allocated to the available software LipidSearch, which has been reported to have a library of ~1,500,000 theoretical MS/MS fragment ions from 18 major lipid species (Xu et al., 2018). In our workflow, LipidSearch originally identified ten different HexCer(d42:2) species in the 'supposed' form of d18:1/24:1 (repeated identifications of a single lipid type is common in this type of analysis), out of which it itself rejected seven, leaving three species for manual identification. Based on the lipid visualizations that the LipidSearch offers, all three lipids were deemed 'real', as: 1) each were quantified in sufficient amount of samples; 2) each showed structurally relevant fragments (i.e. SPH(d18:1) and NL[G1]; 3) all had a valid grade and m-score assigned to them, and 4) even two of them had two different ion keys identified (+H and +H-H_2O). Out of the three, two showed the significant difference reported in this study, while the third did not, which begs the question as to whether this last compound was correctly identified as HexCer(d42:2). Furthermore, because of the broad and complex nature of lipid structures as well as limitations in analytical chemistry instruments, numerous challenges and limitations exist in untargeted lipidomic approaches that lead to the common identification of false positive, these include: 1) signal suppression is common due to the broad range of masses being captured simultaneously, 2) there is vast prevalence of mass isomer ambiguity due to it only being able to report sum-composition level of compounds and consequently failing to identify important structural differences, and 3) challenges in data processing and annotation (Contrepois et al., 2018; Forest et al., 2018; Smirnov et al., 2021; Xu et al., 2018). Consequently, there is a possibility that the identified m/z value actually corresponded to, overlapped with, and/or were co-eluted with different lipid/s. As a result, while LipidSearch annotated the HexCer(d42:2)+H at 810.6825, it may in fact have corresponded to a different lipid, or an accumulation of numerous species (Koelmel et al., 2017). Using LipidMAPS' structure search function with the indicated mass under positive mode returns 15 possible matches which include other HexCer species, PEs, PCs and TGs, some lipid classes which also appeared in the same OPLD-SA profile. Future studies might then conduct targeted analyses of these predicted analytes which could not be done in the present thesis due to time limitations. While it is clear that the untargeted metabolomic and lipidomic to targeted analysis approach has led to reputable scientific publications (e.g. (Becktel et al., 2024; Couttas et al., 2020; Feng et al., 2024; Gong et al., 2020; Jieu et al., 2024; Tang et al., 2025)), this area of

research is still considered to be in its infancy, and it is likely that the unsuccessful untargeted-to-targeted validations often remain unpublished in the “file drawer” of science. Nonetheless, as exemplified by this investigation, this reality only highlights once more the necessity for targeted analysis. And, notably, our expanded targeted investigation of HexCer species revealed additional exciting findings.

Before moving to a discussion of additional HexCer species, it is important to gauge whether our calculated concentrations of the HexCer species more generally reflects reported literature values. Here we observed an enrichment of HexCer in the hippocampus compared to the cortex, which matches previous findings on mice where overall HexCer content (Muralidharan et al., 2021) and particularly GalCer (Nakajima et al., 2016) followed the same pattern. Although, the overall HexCer concentrations observed in our study appeared slightly lower in comparison, but within the same pmol/mg range previously reported and inside a ~three-fold difference to those reported specifically in C57BL/6 mice (Muralidharan et al., 2021; Nakajima et al., 2016). This might be simply explained by a difference in the background strains analysed here (i.e. a 129 and a mixed 129/C57BL/6 strain). Another difference that might explain the slightly discrepant results is reflected in different lipid extraction methods. Muralidharan et al. (2021) used a single phase butanol:methanol method together with a bead mill tissue homogenizer, while Nakajima et al. (2016) used a Bligh and Dyer method. It has been reported that different lipid extraction processes influence lipid recovery, especially in regard to the solvent used. Here we used the MTBE solvent, which was reported to reduce the recovery of specific lipid classes, including SMs and sphingosines (Omar et al., 2023) which could the slightly lower concentrations of HexCer measured in this study.

Here we report for the first time that an array of HexCer species are modulated and mostly upregulated in brain tissue of the seizure-prone F1 strain. Although strain itself did not appear to affect total HexCer concentration, both the hippocampus and cortex displayed strain differences in an array of specific HexCer lipids. Around ~40% were of HexCer were significantly increased in the F1 compared to 129 strain in the hippocampus. In the cortex there were minor biphasic changes between F1 and 129 strain mice dependent on the specific HerCer being measured.

Although all strain-effects appeared as the collective aggregation of both genotypes and no gene and strain interaction effect were identified, some notable HexCer species appeared significantly higher in the hippocampus of F1.*Scn1a*$^{+/-}$ compared to 129.*Scn1a*$^{+/-}$ groups including d18:1/16:0, d18:1/24:0-OH and 18:2/18:0-OH, while the same hippocampus d18:1/16:0 and cortex d18:2/20:1 presented significantly higher concentration in the F1.WT compared to 129.WT. Overall, this suggests that mouse strain significantly influences specific HexCer, with a tendency for this difference to be more pronounced in the *Scn1a*$^{+/-}$ mice -an interaction that the two-way ANOVA may not have been powered to detect. Overall, this suggests that HexCer species may contribute to the seizure-susceptibility seen in the F1 strain, with potential further modulations driven by *Scn1a* genotype and seizure-presentation.

Of relevance to this hypothesis is that neuronal activity dynamically regulates HexCer content and trafficking. For instance, in non-pathological conditions, neuronal firing modulates HexCer epimer GlcCer by impacting both its synthesis and degradation via a glial cell-facilitated pathway (Wang et al., 2022). Alternatively, in disease, elevated levels of ceramides and HexCer promote neuronal stress and inflammatory damage, as seen when the acid sphingomyelinase (ASMase), an enzyme that hydrolyzes SM into Cer, is upregulated by seizures (Lee et al., 2025). Further, in a mouse model of PTZ-induced seizures, acute PTZ exposure decreased concentrations of SM and HexCer species in the hippocampus, whilst chronic PTZ exposure showed increased concentration of these mediators in this brain region, suggesting that excess HexCer species are associated with long-term seizure exacerbation (Chen et al., 2025b). This evidence begs the question as to the directionality of the presently observed HexCer differential pattern.

There exists additional evidence for the notion that heightened HexCer brain content confers increased seizure susceptibility, consistent with the greater content of HexCer species observed in the F1 seizure-susceptible strain in the present study. Van Zyl et al. (2010) found that mice engineered to over-express neuronal GalCer together with its sulfatide counterpart displayed increased neuronal hyper-excitability, audiogenic-seizures and a reduced lifespan (Van Zyl et al., 2010). In humans, patients with lysosomal storage disorders (LSDs), conditions that give rise to drug-resistant seizures, have modulated lipid metabolism, including in relation to the HexCer species

family (Abed Rabbo et al., 2021; Dodge, 2017). LSDs are a heterogenous group of rare genetic and metabolic disorders characterized by an accumulation of lipids in organelles of the endosomal-autophagic-lysosomal pathway (Dodge, 2017). ~70% of LSD patients have neurological ailments such as cognitive and motor developmental delays, psychiatric disorders, and seizures (Pará et al., 2020). The connection between lysosomal homeostasis disruption and nervous system dysregulation has not been fully elucidated, but it is clear that lysosomal disorders promote synaptic dysfunction via alternations in myelin, synaptic spines, proteins, vesicles and postsynaptic densities (Pará et al., 2020). LSDs related to HexCer-associated dysregulation include Gaucher's disease, Krabbe's disease, and Fabry's disease (Dodge, 2017; Mencarelli et al., 2013). Krabbe's disease for instance, is a type of LSD, characterized by absence of galactosylceramidase, which dysregulates the lysosomal GalCer metabolism pathway, such that GalCer is metabolised by other minor hydrolytic enzymes, and a toxic metabolite, psychosine, accumulates. The pathology of Krabbe's disease includes widespread demyelination, gliosis and disrupted recruitment of signalling molecules to lipid-rafts which help support neuronal signalling (Abed Rabbo et al., 2021; Dodge, 2017). Notably, Krabbe's disease shares some pathological characteristics of Dravet syndrome, for instance, it often appears in infancy, is associated with seizures, as well as cognitive and motor deficiencies (Dodge, 2017). In contrast, Gaucher's disease is characterized by a mutation in the glucosylceramide hydrolase and thus shows lysosomal accumulation of GlcCer. The major effect is seen in neurons, and displays vast demyelination, and a vast series of clinical symptoms that also include seizures and cognitive and motor deficiencies (Dodge, 2017; Mencarelli et al., 2013). Additionally, there is a growing body of research that associates the disruption of lysosomal homeostasis to other neurological diseases, for instance, amyotrophic lateral sclerosis, atypical forms of spinal muscular atrophy, Charcot–Marie–Tooth disease, hereditary spastic paraplegia, multiple system atrophy, Parkinson's disease and spinocerebellar ataxia (Dodge, 2017). In conclusion, although no lysosomal dysfunction has been directly associated with Dravet syndrome thus far, our results suggest that dysregulation and often accumulation of brain HexCer species and associated pathways might confer an increased susceptibility to seizures and developmental pathologies, this is particularly alluded to the fact that most of the strain-effects were collectively seen in both presence and

absence of the *Scn1a* mutation. Further studies could conduct enzymatic profile assessment, particularly of lysosomal nature, on the Dravet mice as well as Dravet syndrome patient samples to test for potential enzymes dysregulation; assessments should engulf both enzyme expression assays, like western blots or enzyme-linked immunosorbent assays (ELISAs), as well as test for enzyme activity, like with an LSD enzyme activity panel test (Yu et al., 2013).

Not all HexCer species were increased in the seizure-susceptible F1 strain, and there appeared to be tissue-specific HexCer structural pattern alterations in sphingolipid backbones, fatty acyl chain lengths and hydroxylation state. Sphingolipid structural differences profoundly influence geometric shape, biophysical properties and biological functionality (Hannun et al., 2018; Quinville et al., 2021). For instance, fatty acyl chain lengths and saturation levels display different thermostability and solubility levels. Sphingolipids with short chain C16 and unsaturated structures tend to have greater heat stability and ability to dissolve than longer C24 and saturated chain (McCluskey et al., 2022). In general, longer and more saturated chains decrease membrane fluidity due to tighter membrane packing, while unsaturated and shorter chains create more fluid, loosely packed membranes (Mencarelli et al., 2013). Moreover, levels of saturation of the sphingoid base determine their head-group tilts; extra saturation enables more hydrogen bonding which can impact their substrate specific binding and metabolism by enzymes (Mencarelli et al., 2013). In our results, F1 strain mice had a greater abundance of short C16 acyl chains compared to 129 strain mice in both the hippocampus and cortex. Although, the effect of strain in the cortex was limited to short acyl chains, the hippocampus additionally had an increased concentration of HexCer species of all chain lengths. Additionally, each region showed variability in chain length trends, while the hippocampus showed a consistent upregulation of HexCer in F1 strain compared to 129 mice, the cortex showed a dual dynamic, where F1 mice had increased levels of HexCer d18:1/16:0 and d18:1/18:1, while d18/18:0 showed to be significantly higher in the 129 strain. Additionally, hydroxylation states are also important determinants of lipid thermal properties, for instance, hydroxylation tends to make membranes more fluid at lower temperatures, thus reducing the gel-to-fluid transition temperature compared in comparison to non-hydroxylated analogues (Linington et al., 1981). Interestingly, brain

hydroxylated sphingolipid concentrations vary with developmental age, where most notably, OH-GalCer levels increase dramatically during postnatal myelination, (Alderson et al., 2006). Notably, in our results, the strain effects observed in the cortex were limited to non-hydroxylated species, while in the hippocampus there were changes in HexCer of both hydroxylated states. Collectively, these findings suggest that the seizure-susceptible F1 strain mice are not simply characterized by a large upregulation of HexCer compared to the 129 strain, but rather there are specific alterations in HexCer species with more precise structural motifs that also vary by brain region. Altogether given the biophysical implications of HexCer structural patterns on membrane properties, the observed modulation in between the brain region and within regions may be leading to membrane composition dysregulation in F1 that is then associated with its susceptibility and/or seizure presentation.

Coincidently a reported association between sphingolipid CerS enzymatic function and epilepsy has been previously established. There are six known CerS isoforms, each with a preferred chain length and specific cellular distribution. CerS1 prefers C18 chains and is enriched in neurons, while CerS2 prefers C22-C24 and is prevalent in oligodendrocytes (Leipelt et al., 2004). Interestingly, both of these enzyme's genes, CERS1 and CERS2, have been associated with myoclonic epilepsies via reduced expression levels of their corresponding chain length C18-sphingolipids (Mosbech et al., 2014; Vanni et al., 2014). Interestingly, a pattern of d18:1/18:0 downregulation was observed in the F1 cortex compared to 129, suggesting a potential strain-driven modulation of CerS expression or activity. Additionally, CerS dysregulation may also underly the only genotype-specific effect observed in our results, where cortical samples of *Scn1a*$^{+/-}$ mice had significantly higher HexCer content of d18:2/18:0 compared to WT. This suggests an altered CerS1 profile, perhaps in conjunction with DES2, which is responsible for the double saturation in the sphingoid base. So, although heterozygous deletion of the *Scn1a* gene does not affect HexCer metabolism, the specific increase in the C18:0 species could still have a major functional impact, particularly given that C18 species are one of the most highly abundant HexCer in the brain (McCluskey et al., 2022). All-together, these results suggest the modulation of HexCer pathway enzymes like CerS and DES2 might be associated with the strain-driven facilitation of the F1.*Scn1a*$^{+/-}$

epileptic phenotype, warranting further investigation into their sphingolipid enzymatic function profile.

Our results suggest a connection between the tissue region-specific HexCer modulation, myelin function, and the epileptic phenotype. Brain regions differ in cellular and molecular makeup (Muse et al., 2001). Interestingly, our HexCer results showed a disproportionately higher strain-driven HexCer modulation in the hippocampus than in the cortex. The tissue-specific metabolic shift may be the key to understanding seizure susceptibility in our F1 background strain mice. The hippocampal circuits in Dravet mouse models show particularly pronounced hyperexcitability in both pre-epileptic and epileptic periods of the mice's life (Liautard et al., 2013). Moreover, maladaptive myelination within hippocampal circuits have not only been characterized in Dravet syndrome mouse models (Satta et al., 2023), but have also been found to promote epilepsy progression (Knowles et al., 2022). Moreover, oligodendrocytes damage and demyelination has been associated with both excess and lack of sphingolipid production (Alaamery et al., 2021). Here we propose that strain-driven disruptions in myelin-associated HexCer species could alter neuronal excitability and, together with the *Scn1a*$^{+/-}$ genotype, synergise to drive the seizure-prone phenotype in a tissue-region selective manner. Future studies could examine how myelination influences region- and strain-specific HexCer distribution in the Dravet syndrome mouse model, clarifying the links between myelin disruption and HexCer dysregulation. This could be achieved by utilizing via myelination biomarkers, such as myelin basic protein and myelin oligodendrocyte glycoprotein (Satta et al., 2023) and/or the spatial localization of lipid distribution such as using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI-MSI) (Kaya et al., 2023; Murphy et al., 2009).

This study has several limitations that highlight important directions for future research. First, the broader sphingolipid pathway was not assessed, which limits our understanding on how the observed HexCer modulations fit in the wider lipid landscape, especially because a lot of the molecules in the pathway also display critical roles in neuronal survival, inflammation, autophagy, and lysosomal integrity, all of which are relevant to epilepsy. A more comprehensive targeted lipidomic analysis examining not only HexCer but Cer, SM, glycosphingolipids, the other downstream metabolites, and the

expression and activity of related enzymes, in brain tissue and/or cerebrospinal fluid, could offer deeper insight into the pathophysiology of Dravet syndrome. Another limitation of the present study is the collective measurement of GalCer and GlcCer despite their distinct expression patterns. Although GalCer is the predominant HexCer in the brain, both have been reported to be present in the CNS while differing in cellular localisation and function, i.e. GalCer being primarily in oligodendrocytes and GlcCer in neurons (Reza et al., 2021). In the past, particular increase of GalCer in neurons has been associated with seizure-susceptibility (Van Zyl et al., 2010), which begs the question as whether the HexCer dysregulation observed in this study, particularly their upregulation in F1 strain, could be localized to a specific cell and/or a HexCer type, especially given the fact that Dravet syndrome pathophysiology is mostly linked to disrupted GABAergic interneurons. Due to their structural similarity, they require specialized techniques to be reliably distinguished (Reza et al., 2021). Future studies could address the distinction of the two enantiomers, which can be achieved by hydrophilic interaction chromatography (HILIC) as previously demonstrated (Marian et al., 2025), as well as their cellular localization to further characterize the found effects. Finally, while we showed that modulated HexCer species are associated with the seizure-prone mice, whether this reflects a causal mechanism in seizure generation remains unresolved. More broadly, the genotype effect could be likely associated to secondary factors linked to the interplay of the F1 strain background, seizure burden, or a compensatory stress response. Supporting this, Hawkins et al. (2019) suggested that strain differences in the Dravet mice set the tone for the baseline metabolism and thus seizure susceptibility, with the *Scn1a* mutation amplifying these differences. Future studies are needed to further explore this concept and prove mechanistically the link between HexCer and seizure presentation and susceptibility, this idea will be further discussed in the next chapter.

3.5 Conclusion

Lipids, and particularly sphingolipids, play a central role in maintaining homeostasis in lipid-lipid and lipid-protein interactions, neural membrane structural architecture, and signal transduction in the brain. Given that these connections provide

a clear mechanistic basis for how sphingolipid dysregulation can be involved in pathophysiology, their modulation is unsurprisingly linked to epileptic phenotype.

Region- and strain-specific HexCer modulation was observed, with significant upregulation of a wide structural variety of HexCer species in the hippocampus of the seizure-prone F1 strain compared with the non-seizure-prone 129 strain. In contrast, the cortex showed only minor differences, limited to short-chain HexCer species. Additionally, a significant upregulation of the di-saturated short chain C18 species was found in the cortex of *Scn1a*$^{+/-}$ compared to WT. Altogether, these findings provide strong evidence for alterations in specific lipid species, which suggest that an altered lipid pathway is potentially contributing to the seizure-susceptibility seen in the F1.*Scn1a*$^{+/-}$ mouse, with a potential for additional modulations driven by seizure-presentation. Future work should focus on dissecting more of the sphingolipid pathway, both its other metabolites and involved enzymes, to better understand these strain-dependent differences in HexCer content, and their relevance to our understand of the disease as well as potential novel therapeutic targets.

3.6 Appendix

Table S3.1. MS conditions for sphingolipids of the HexCer family of interest.

Compound	Precursor (*m/z*)	Product (*m/z*)	RT (min)
GalCer/HexCer(d18:1/12:0)	644.6	264.3	12.58
HexCer(d18:1/16:0)	700.6	264.3	14.24
HexCer(d18:1/16:0)OH	716.7	264.3	14.03
HexCer(d18:1/16:1)	698.6	264.3	13.47
HexCer(d18:1/16:1)OH	714.7	264.3	13.17
HexCer(d18:1/18:0)	728.6	264.3	14.95
HexCer(d18:1/18:0)OH	744.7	264.3	14.75
HexCer(d18:1/18:1)	726.7	264.3	14.53
HexCer(d18:1/18:1)OH	742.7	264.3	14.26
HexCer(d18:1/20:0)	756.6	264.3	15.55
HexCer(d18:1/20:0)OH	772.7	264.3	15.39
HexCer(d18:1/20:1)	754.6	264.3	15.14
HexCer(d18:1/20:1)OH	770.7	264.3	14.95
HexCer(d18:1/22:0)	784.7	264.3	16.08
HexCer(d18:1/22:0)OH	800.7	264.3	15.91
HexCer(d18:1/22:1)	782.7	264.3	15.69
HexCer(d18:1/22:1)OH	798.7	264.3	15.69
HexCer(d18:1/24:0)	812.7	264.3	16.65
HexCer(d18:1/24:0)OH	828.8	264.3	16.46
HexCer(d18:1/24:1)	810.7	264.3	16.20
HexCer(d18:1/24:1)OH	826.7	264.3	16.01
HexCer(d18:2/16:0)	698.6	262.3	13.71
HexCer(d18:2/16:1)	696.6	262.3	13.14
HexCer(d18:2/18:0)	726.6	262.3	14.51
HexCer(d18:2/18:0)OH	742.7	262.3	14.29
HexCer(d18:2/18:1)	724.7	262.3	14.05
HexCer(d18:2/20:0)	754.6	262.3	15.19
HexCer(d18:2/20:0)OH	770.7	262.3	14.98
HexCer(d18:2/20:1)	752.6	262.3	14.50
HexCer(d18:2/22:0)	782.7	262.3	15.75
HexCer(d18:2/22:0)OH	798.7	262.3	15.58
HexCer(d18:2/24:0)	810.7	262.3	16.26
HexCer(d18:2/24:0)OH	826.7	262.3	16.11
HexCer(d18:2/24:1)	808.7	262.3	15.87
HexCer(d18:2/24:1)OH	824.7	262.3	15.69

Table S3.2. Mean HexCer concentrations (pmol/mg of Tissue) ± SEM by brain region (cortex and hippocampus), strain (129 and F1) and genotype (*Scn1a*$^{+/-}$ and WT), including individual lipid and total HexCer concentration.

Lipid	Cortex								Hippocampus							
	129				F1				129				F1			
	WT		*Scn1a*$^{+/-}$		WT		*Scn1a*$^{+/-}$		WT		*Scn1a*$^{+/-}$		WT		*Scn1a*$^{+/-}$	
	Mean concentration (pmol/mg of Tissue)	SEM	Mean concentration (pmol/mg of Tissue)	SEM	Mean concentration (pmol/mg of Tissue)	SEM	Mean concentration (pmol/mg of Tissue)	SEM	Mean concentration (pmol/mg of Tissue)	SEM	Mean concentration (pmol/mg of Tissue)	SEM	Mean concentration (pmol/mg of Tissue)	SEM	Mean concentration (pmol/mg of Tissue)	SEM
Total	216.17	15.50	193.55	11.02	201.18	13.80	196.32	15.59	1396.05	317.07	1069.71	114.78	1558.38	177.86	1682.75	294.42
HexCer(d18:1/16:0)	2.82	0.16	3.09	0.15	3.38	0.20	3.24	0.08	7.63	0.89	7.61	1.28	10.93	0.86	9.62	1.01
HexCer(d18:1/16:1)	0.05	0.00	0.05	0.00	0.05	0.00	0.06	0.00	0.05	0.01	0.04	0.01	0.05	0.01	0.04	0.01
HexCer(d18:1/18:0)	24.11	1.80	23.01	1.77	21.56	2.33	21.39	1.50	174.99	29.76	151.71	19.92	194.21	23.28	171.74	20.64
HexCer(d18:1/18:0)OH	5.21	0.57	4.54	0.40	4.27	0.44	4.75	0.50	40.19	9.42	31.09	3.83	42.50	5.84	44.72	6.73
HexCer(d18:1/18:1)	0.41	0.03	0.43	0.02	0.49	0.02	0.50	0.01	0.84	0.08	0.84	0.15	0.94	0.09	0.99	0.10
HexCer(d18:1/18:1)OH	0.21	0.01	0.22	0.01	0.22	0.01	0.22	0.01	0.55	0.07	0.52	0.11	0.59	0.07	0.58	0.06
HexCer(d18:1/20:0)	1.56	0.19	1.25	0.12	1.33	0.13	1.24	0.13	14.48	3.84	10.54	2.11	16.01	2.75	16.12	3.53
HexCer(d18:1/20:0)OH	0.29	0.04	0.23	0.03	0.21	0.04	0.27	0.04	9.05	3.20	5.27	0.70	8.57	1.63	10.33	2.32
HexCer(d18:1/20:1)	0.03	0.00	0.03	0.00	0.02	0.00	0.02	0.00	0.25	0.06	0.22	0.05	0.32	0.07	0.30	0.05
HexCer(d18:1/20:1)OH	0.13	0.01	0.12	0.01	0.10	0.01	0.12	0.01	0.69	0.12	0.68	0.19	0.76	0.12	0.68	0.09
HexCer(d18:1/22:0)	16.50	1.90	13.44	1.74	16.03	0.94	13.26	1.60	123.71	28.85	88.35	8.04	136.64	17.27	149.64	31.40
HexCer(d18:1/22:0)OH	6.69	1.24	5.41	0.95	4.41	0.32	6.60	1.52	88.25	30.83	53.63	8.38	77.92	15.28	104.18	22.82
HexCer(d18:1/22:1)	1.47	0.17	1.44	0.12	1.42	0.15	1.52	0.11	14.48	4.27	11.14	2.63	17.43	3.36	17.51	3.59
HexCer(d18:1/22:1)OH	0.55	0.04	0.52	0.04	0.46	0.05	0.53	0.04	6.00	1.95	5.05	1.51	7.20	1.36	7.68	1.53
HexCer(d18:1/24:0)	47.11	5.76	39.97	5.66	49.17	4.20	45.20	5.45	321.49	67.66	246.97	17.81	370.05	34.79	395.87	75.17
HexCer(d18:1/24:0)OH	8.14	2.07	6.75	1.82	4.46	0.52	8.78	2.58	67.90	23.89	42.43	8.04	64.51	14.69	97.70	19.76
HexCer(d18:1/24:1)	78.06	4.27	71.99	2.32	71.99	4.83	67.06	3.30	405.64	90.89	324.44	43.85	484.35	60.50	509.26	92.46
HexCer(d18:1/24:1)OH	13.89	1.18	11.72	0.91	12.28	1.22	12.21	1.00	77.67	21.81	53.05	8.44	80.00	10.45	95.88	19.17
HexCer(d18:2/16:0)	0.06	0.00	0.06	0.00	0.06	0.00	0.06	0.00	0.09	0.01	0.10	0.01	0.12	0.01	0.14	0.02
HexCer(d18:2/18:0)	3.88	0.16	4.32	0.15	4.45	0.32	4.35	0.08	9.77	0.75	9.62	0.87	10.25	0.50	9.19	0.90
HexCer(d18:2/18:0)OH	1.38	0.06	1.45	0.07	1.46	0.05	1.58	0.05	4.19	0.36	4.10	0.54	4.77	0.32	4.99	0.40
HexCer(d18:2/20:0)OH	0.19	0.01	0.19	0.01	0.17	0.02	0.18	0.01	1.40	0.18	1.25	0.18	1.39	0.13	1.38	0.15
HexCer(d18:2/20:1)	0.07	0.02	0.08	0.01	0.10	0.01	0.10	0.01	0.65	0.08	0.61	0.08	0.64	0.04	0.72	0.06
HexCer(d18:2/22:0)	0.32	0.02	0.29	0.01	0.28	0.02	0.29	0.01	2.60	0.55	1.95	0.21	2.69	0.35	3.30	0.61
HexCer(d18:2/22:0)OH	0.77	0.05	0.70	0.04	0.70	0.08	0.70	0.05	6.73	1.62	5.04	0.70	6.74	0.79	8.30	1.45
HexCer(d18:2/24:0)	1.12	0.04	1.08	0.05	1.08	0.09	1.05	0.05	7.64	1.39	5.87	0.51	7.82	0.71	9.17	1.57
HexCer(d18:2/24:0)OH	0.59	0.04	0.57	0.04	0.52	0.07	0.56	0.03	4.21	0.91	3.17	0.34	4.48	0.45	5.26	0.91
HexCer(d18:2/24:1)	0.43	0.05	0.47	0.04	0.38	0.05	0.39	0.02	3.86	0.98	3.57	0.46	5.17	0.54	6.03	1.01
HexCer(d18:2/24:1)OH	0.11	0.01	0.12	0.01	0.11	0.01	0.12	0.01	1.06	0.28	0.86	0.14	1.32	0.17	1.45	0.28

Table S3.3. Statistical results of two-way ANOVA with multiple comparisons for total and individual HexCer concentrations by brain region (cortex and hippocampus), showing effect of strain, genotype and their interaction. Significant differences were established at *p < 0.05, **p < 0.0,1 and ***p < 0.001.

	Cortex							Hippocampus						
	Two-way ANOVA P-value			Multiple Comparisons Adjusted P-value				Two-way ANOVA P-value			Multiple Comparisons			
Lipid	Interaction	Strain	Genotype	129.WT vs 129.*Scn1a*$^{+/-}$	F1.WT vs F1.*Scn1a*$^{+/-}$	F1.*Scn1a*$^{+/-}$ vs 129.*Scn1a*$^{+/-}$	F1.WT vs 129.WT	Interaction	Strain	Genotype	129.WT vs 129.*Scn1a*$^{+/-}$	F1.WT vs F1.*Scn1a*$^{+/-}$	F1.*Scn1a*$^{+/-}$ vs 129.*Scn1a*$^{+/-}$	F1.WT vs 129.WT
Total	0.183	0.251	0.820	0.618	0.922	1.000	0.364	0.913	0.082	0.478	0.956	0.990	0.546	0.684
HexCer18:1/16:0	0.184	*0.026	0.678	0.570	0.953	0.926	0.064	0.976	***0.001	0.142	0.753	0.750	*0.038	*0.044
HexCer18:1/16:1	0.133	0.182	0.449	0.960	0.447	0.172	>0.999	0.503	0.365	0.540	>0.999	0.853	1.000	0.734
HexCer18:1/18:0	0.214	*0.037	0.521	0.980	0.643	0.928	0.117	0.876	0.133	0.224	0.788	0.913	0.648	0.814
HexCer18:1/18:0-OH	0.250	0.464	0.840	0.771	0.948	0.996	0.588	0.872	0.166	0.574	0.972	0.998	0.706	0.861
HexCer18:1/18:1	0.964	**0.002	0.452	0.958	0.982	0.087	0.104	0.271	*0.029	0.542	0.631	0.995	0.076	0.885
HexCer18:1/18:1-OH	0.775	0.784	0.838	0.993	>0.999	>0.999	0.992	0.285	0.070	0.193	0.319	1.000	0.153	0.970
HexCer18:1/20:0	0.483	0.434	0.194	0.440	0.990	>0.999	0.773	0.596	0.276	0.103	0.389	0.901	0.687	0.991
HexCer18:1/20:0-OH	0.106	0.603	0.974	0.645	0.708	0.880	0.460	0.728	0.110	0.768	0.982	>0.999	0.502	0.847
HexCer18:1/20:1	0.298	0.112	0.640	0.988	0.770	0.204	0.992	0.701	*0.044	0.566	0.933	1.000	0.292	0.670
HexCer18:1/20:1-OH	0.443	0.088	0.754	0.995	0.921	0.926	0.319	0.839	0.068	0.153	0.843	0.691	0.655	0.490
HexCer18:1/22:0	0.934	0.849	0.094	0.553	0.731	>0.999	1.000	0.612	0.250	0.205	0.568	0.974	0.656	0.985
HexCer18:1/22:0-OH	0.293	0.755	0.985	0.898	0.922	0.970	0.821	0.601	*0.026	0.556	>0.999	0.903	0.157	0.649
HexCer18:1/22:1	0.679	0.904	0.794	>0.999	0.985	0.992	0.999	0.836	*0.013	0.245	0.931	0.810	0.305	0.202
HexCer18:1/22:1-OH	0.237	0.354	0.671	0.963	0.735	1.000	0.479	0.897	**0.005	0.407	0.978	0.940	0.168	0.134
HexCer18:1/24:0	0.777	0.515	0.324	0.809	0.982	0.935	0.998	0.715	0.161	0.214	0.654	0.958	0.597	0.917
HexCer18:1/24:0-OH	0.117	0.819	0.840	0.747	0.660	0.779	0.627	0.139	**0.001	0.483	0.966	0.419	**0.004	0.544
HexCer18:1/24:1	0.879	0.145	0.145	0.637	0.849	0.805	0.710	0.965	0.067	0.459	0.975	0.973	0.570	0.561
HexCer18:1/24:1-OH	0.182	0.841	0.160	0.171	>0.999	0.877	0.738	0.602	0.077	0.546	0.875	>0.999	0.343	0.844
HexCer18:2/16:0	0.562	0.841	0.834	0.998	0.974	0.966	0.998	0.732	*0.027	0.359	0.988	0.860	0.220	0.561
HexCer18:2/18:0	0.432	0.326	*0.025	0.082	0.776	1.000	0.661	0.947	0.541	0.160	0.798	0.764	0.991	0.983
HexCer18:2/18:0-OH	0.680	0.093	0.126	0.862	0.577	0.420	0.845	0.231	**0.006	0.564	0.596	0.987	*0.020	0.654
HexCer18:2/20:0-OH	0.608	0.222	0.526	>0.999	0.904	0.974	0.657	0.332	0.388	0.301	0.484	>0.999	0.570	>0.999
HexCer18:2/20:1	0.269	**0.007	0.232	0.323	>0.999	0.573	*0.040	0.189	0.259	0.956	0.841	0.798	0.308	1.000
HexCer18:2/22:0	0.194	0.276	0.616	0.541	0.971	1.000	0.356	0.365	0.265	0.455	0.635	>0.999	0.476	1.000
HexCer18:2/22:0-OH	0.126	0.093	0.957	0.684	0.751	>0.999	0.129	0.553	0.124	0.735	0.932	1.000	0.418	0.937
HexCer18:2/24:0	0.311	0.091	0.894	0.935	0.909	0.974	0.258	0.376	0.096	0.745	0.849	0.992	0.235	0.968
HexCer18:2/24:0-OH	0.219	0.134	0.061	0.977	0.146	0.999	0.273	0.633	0.079	0.531	0.882	>0.999	0.365	0.831
HexCer18:2/24:1	0.450	0.372	0.347	0.627	1.000	0.652	>0.999	0.975	**0.007	0.576	0.987	0.994	0.171	0.204
HexCer18:2/24:1-OH	0.900	0.254	0.232	0.802	0.915	0.911	0.855	0.884	*0.033	0.700	1.000	0.994	0.459	0.367

Chapter 4

Evaluation of the Interaction of the Cannabis Constituents CBC and CBCA with ABCG2 and ABCB1 Transporters

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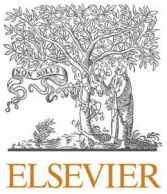

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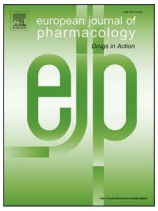

In vitro evaluation of the interaction of the cannabis constituents cannabichromene and cannabichromenic acid with ABCG2 and ABCB1 transporters

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ABSTRACT

Cannabichromene (CBC) and cannabichromenic acid (CBCA) are cannabis constituents currently under evaluation for their therapeutic potential, but their pharmacological properties have not been thoroughly investigated. The most studied ATP-binding cassette (ABC) transporters, ABC subfamily G member 2 (ABCG2) and ABC subfamily B member 1 (ABCB1) limit absorption of substrate drugs in the gut and brain. Moreover, inhibitors of these proteins can lead to clinically significant drug-drug interactions (DDIs). The current study sought to examine whether CBC and CBCA affect ABCB1 and ABCG2 to advance their basic pharmacological characterisation. The plant cannabinoids CBC and CBCA were screened *in vitro* in a bidirectional transport assay to determine whether they were substrates and/or inhibitors of ABCB1 and ABCG2. Transwell assays with polarized epithelial Madin-Darby Canine Kidney II (MDCK) cells expressing ABCB1 or ABCG2 were used. Samples were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS). CBCA was found to be an ABCB1 substrate, but not an ABCG2 substrate. CBC was not a substrate of either transporter. Neither CBCA nor CBC inhibited ABCB1 transport of prazosin or ABCG2 transport of digoxin. *In silico* molecular docking suggested CBCA binds ABCB1 in the access tunnel and the central binding pocket. CBC, an agent with anticonvulsant, anti-inflammatory and anti-depressant properties, is not a substrate or inhibitor of ABCB1 or ABCG2, which is favourable to its therapeutic development. CBCA is an ABCB1 substrate *in vitro* which might contribute to its poor absorption. These findings provide important basic pharmacological data to assist the therapeutic development of these cannabis constituents.

1. Introduction

Medicinal cannabis has been legalised by an increasing number of countries around the world. As a result, scientists have been exploring the therapeutic potential of the cannabis plant and its multitude of phytochemical constituents. Cannabis contains more than 100 terpenophenolic cannabinoids, however, most research has focussed on the major cannabinoids; cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC). Pharmacological research on other cannabinoids is growing but the molecular actions of these compounds are underexplored (ElSohy and Gul, 2015).

After Δ^9-THC and CBD, cannabichromene (CBC, Fig. 1A) is the third most abundant cannabinoid in the cannabis plant (Andre et al., 2016; Izzo et al., 2009). Non-enzymatic decarboxylation of cannabichromenic acid (CBCA, Fig. 1B), which is synthesised enzymatically by the plant, produces CBC (Morimoto et al., 1998). Preclinical research shows both CBC and CBCA have emerging therapeutic potential. CBC exhibits anti-inflammatory properties in mouse models of edema and colitis, and anti-cancer properties in *in vitro* cancer models (Anis et al., 2021; de Petrocellis et al., 2012, 2013; DeLong et al., 2010; Izzo et al., 2012;

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Ligresti et al., 2006b; Romano et al., 2013). CBC also displays antidepressant activity in mouse models (El-Alfy et al., 2010). Additionally, CBC and CBCA were anticonvulsant in a mouse model of intractable childhood epilepsy (Anderson et al., 2021a). Finally, CBCA has been reported to have antibacterial properties against methicillin-resistant *Staphylococcus aureus* (MRSA) (Galletta et al., 2020).

Characterisation of the pharmacological actions of CBC is ongoing; however, several therapeutically-relevant molecular targets have already been identified. CBC has been shown to activate cannabinoid CB_2 receptors and transient receptor potential vanilloid (TRPV) channels (de Petrocellis et al., 2008, 2012; Udoh et al., 2019). CBC also inhibits lactate dehydrogenase and anandamide uptake (Ligresti et al., 2006a; Martin et al., 2021). The pharmacological targets of CBCA are unknown.

Our knowledge of the pharmacodynamic targets of CBC and CBC-related cannabinoids is expanding, however little is known about the effects of these cannabinoids on molecular sites relevant to their pharmacokinetic profile. Understanding the effects of cannabinoids on proteins that influence pharmacokinetics is important for the therapeutic development of this class because such actions can influence drug disposition into target tissues and mediate drug-drug interactions (DDIs). Candidate drugs are now routinely assessed for their effects on drug metabolising enzymes and drug transporters as an important aspect of drug development towards the clinic. It is noteworthy that CBC had negligible to weak inhibitory effects on an array of the major drug metabolising cytochrome P450 (CYP450) enzymes (Doohan et al., 2021). However, the effects of CBC and CBCA on ATP-binding cassette (ABC) transporters is unknown.

ABC transporters are a superfamily of drug efflux proteins that mediate the movement of compounds across physiological membranes. They are distributed widely in apical membranes of tissues, with greatest expression in the intestinal epithelium, liver, and endothelial cells of the blood-brain barrier (ter Beek et al., 2014). These transporters contribute xenobiotic defence in humans by limiting xenobiotic bioavailability. Moreover, they are a major source of DDIs (Chen et al., 2016; Glavinas et al., 2005). ABC subfamily G member 2 (ABCG2) and ABC subfamily B member 1 (ABCB1) are the most studied ABC transporters. Both ABCG2 and ABCB1 have been shown to limit absorption of substrate drugs in the gut and the brain (Glavinas et al., 2005). Moreover, inhibitors of these proteins can lead to clinically significant DDIs (Liu, 2019; Marquez and van Bambeke, 2011). Various plant cannabinoids, including CBD and Δ^9-THC, were shown to affect ABCB1 and ABCG2 by acting as substrates and/or inhibitors (Anderson et al., 2021b; Arnold et al., 2012; Bonhomme-Faivre et al., 2008; Brzozowska et al., 2017; Feinshtein et al., 2013; Holland et al., 2006, 2007; Spiro et al., 2012; Tournier et al., 2010; Zhu et al., 2006). However, whether CBC or CBCA affect ABCB1 and ABCG2 is unknown. The present study therefore aimed to characterise CBC and CBCA as substrates and/or inhibitors of ABCB1 and ABCG2 using *in vitro* transwell assays.

2. Materials and methods

2.1. Drugs

Elacridar, digoxin, and loratadine were purchased from MedChem Express LLC (Princeton, USA). Prazosin and Lucifer Yellow CH dipotassium salt were purchased from Sigma-Aldrich (St. Louis, USA). CBC was synthesised as previously described (Anderson et al., 2021a). CBCA was generously provided by Professor Michael Kassiou at the University of Sydney (AUS).

2.2. Cell culture

Madin-Darby Canine Kidney II (MDCK) cell lines were generously provided by Dr. Alfred Schinkel (Netherlands Cancer Institute, NLD) via Associate Professor Joseph Nicolazzo (Monash University, AUS). Cell lines included wildtype MDCK cells and MDCK cells stably expressing either human ABCB1 or ABCG2 (Evers et al., 2000; Pavek et al., 2005). Cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (p/s) at 37 °C, 5% CO_2 and 95% humidity.

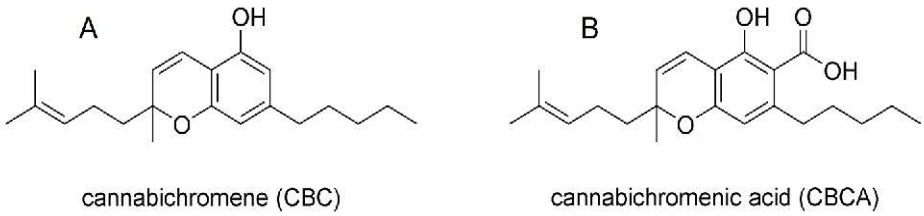
2.3. Bidirectional transport assays

Bidirectional transport assays were conducted as previously described (Anderson et al., 2021b). Briefly, cells were plated on Corning Transwell polycarbonate membrane cell culture inserts (Corning Inc.; Corning, USA) at a density of 2.5×10^5 cells/well or 2.0×10^5 cells/well for 12 mm or 6.5 mm diameter 0.4 μm pore inserts, respectively. Cells were plated 72 h prior to transwell assays.

For substrate assays, cells were first rinsed with phosphate buffered saline (PBS), then DMEM supplemented with 10% FBS containing vehicle (dimethyl sulfoxide (DMSO)) was added to both the apical and basolateral chambers. Following a 15 min incubation at 37 °C in a 5% CO_2, with 95% humidity, media in the donor chamber was replaced with media containing CBC or CBCA (10 μM) and returned to 37 °C. Aliquots (25 or 50 μL) were taken from the acceptor chamber every 60 min for 4 h. CBCA experiments in ABCB1 expressing cells also had an inhibitor condition. In these experiments, cells were incubated with loratadine (10 μM) for 15 min. Media containing CBCA and loratadine then replaced the media in the donor chamber.

For inhibition assays, cells were rinsed with PBS then DMEM supplemented with 10% FBS containing either vehicle, CBC or CBCA that was added to both the apical and basolateral chambers. Following a 15 min incubation at 37 °C in a 5% CO_2, with 95% humidity, media in the donor chamber was replaced with media containing 1 μM substrate (prazosin, ABCG2; digoxin, ABCB1) in the presence of vehicle, CBC or CBCA. Aliquots (25 or 50 μL) were taken from the acceptor chamber every 60 min for 4 h.

Concentrations of CBC, CBCA, prazosin, and digoxin in the aliquots sampled from the acceptor chamber were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Anderson et al., 2021a). Samples were spiked with diazepam as an internal standard. Then either 0.1% formic acid in water (cannabinoids and digoxin) or 0.5 M sodium hydroxide (prazosin) was added for supported-liquid extraction (SLE) using Biotage Isolute SLE + columns (Uppsala, SWE). The SLE solvent used for cannabinoid and digoxin experimental samples was methyl tert-butyl ether, and ethyl acetate was used for prazosin experimental samples. Samples were evaporated to



cannabichromene (CBC) cannabichromenic acid (CBCA)

Fig. 1. Chemical structures of (**A**) CBC and (**B**) CBCA. See Supplementary Table 1 for chemical and physical properties.

dryness with N_2 and reconstituted in 1:1 v/v mobile phase. Mobile phases were as follows: acetonitrile and 0.1% formic acid in water (cannabinoids), methanol and 0.1% formic acid in water (prazosin), or methanol and 0.1% formic acid in 10 mM ammonium acetate (digoxin). Quantification of CBC, CBCA, digoxin, and prazosin was achieved by comparing samples to standards prepared with known amounts of compound. See Supplementary Fig. 1 for representative chromatograms of tested compounds.

2.4. Permeability assay

A Lucifer yellow permeability assay was performed at the completion of each transwell assay to assess monolayer integrity. High potassium Hank's balanced salt solution (HBSS) replaced the media in both chambers and Lucifer yellow (250 μM) was added to the apical chamber. Cells were incubated at 37 °C for 60 min. Samples were then taken from the basolateral chamber and a CLARIOstar plate reader (BMG Labtech; Offenburg, GER) was used to take fluorescence readings (excitation 485 nm, emission 535 nm). Monolayer integrity was considered intact if Lucifer yellow permeability was less than 5% calculated by subtracting the HBSS only fluorescence and normalizing to that of 250 μM Lucifer Yellow (Ivanova et al., 2018).

2.5. Data analysis

The apparent permeability (P_{app}) of each substrate across MDCK cell monolayers in both the basolateral to apical (B > A) and apical to basolateral (A > B) directions were calculated using the following formula:

$$P_{app} = \frac{V_a}{C_0 \times S} \times \frac{\Delta C}{\Delta t}$$

Where V_a is the volume of the acceptor chamber (B > A: 0.5 mL and 0.2 mL; A > B: 1.5 mL and 0.6 mL; for 12 mm and 6 mm inserts, respectively), C_0 is the initial concentration of substrate in the donor chamber (10 μM CBC and CBCA, 1 μM digoxin and prazosin), S is the monolayer growth surface area (1.12 cm^2, 12 mm inserts; 0.33 cm^2, 6.5 mm inserts), and $\frac{\Delta C}{\Delta t}$ is the rate of substrate transport, which was determined by linear regression of concentration-time curves using GraphPad Prism (La Jolla, California, USA).

Transport efflux ratios (r) were calculated by dividing P_{app} in the B > A direction by P_{app} in the A > B direction. Transport ratios could not be calculated when the slope for concentration-time curves in the A > B direction were not significantly different from zero (r = n.d.). Comparisons of curve fits for concentration-time curves were calculated using the Extra sum-of-squares F-test, with $p < 0.05$ considered significantly different.

To determine whether CBC or CBCA were substrates, curve fits for their concentration-time curves in wildtype MDCK cells were compared to those of the ABCG2 and ABCB1 expressing cells. To determine whether CBC or CBCA were inhibitors of ABCB1 or ABCG2, curve fits for the concentration-time curves for digoxin (ABCB1) and prazosin (ABCG2) in the presence of vehicle were compared to those in the presence of 10 μM CBC or CBCA.

2.6. Molecular docking

In silico calculations to investigate binding of CBCA to human ABCB1 used the tariquidar-bound structure PDB ID 7A69 (Nosol et al., 2020). Coordinates and partial charges for CBCA were from NIH PubChem CID 3084339. MGLTools and Autodock Vina packages (Trott and Olson, 2010) were used to calculate partial charges for receptor (ABCB1) atoms and to perform docking, respectively. The entire transmembrane cavity was investigated in the docking search.

AutoDock Vina is designed to maximise the accuracy of docking small ligands and drugs to proteins (Trott and Olsen, 2010). Docking attempts to approximate the chemical potentials of the system. Vina uses a sophisticated gradient optimization algorithm in its local optimization to determine the bound conformation preference and free energy minima and shape of the binding profile. The quasi-Newton optimization method uses the scoring function and its gradient for fixing the position and orientation of the ligand, as well as the values of the torsions for the active rotatable bonds in the ligand and flexible residues. The AutoDock Vina output files are provided (Supplementary Table 2) with the best 8 modes listed with energy terms (kcal/mol) and rmsd distances for the best modes. Figures were rendered with PyMOL (PyMOL Molecular Graphics System, V2, Schrodinger, LLC).

3. Results

3.1. CBC and CBCA do not inhibit ABCG2 or ABCB1 transporter function

Transwell assays were performed to assess bidirectional transport of prazosin and digoxin, known substrates of ABCG2 and ABCB1, respectively. We first confirmed that MDCK cells stably expressing ABCG2 or ABCB1 showed enhanced transport of the substrates compared to wildtype MDCK cells. The permeability of the ABCG2 substrate prazosin was significantly higher in the B > A direction ($p < 0.0001$) and lower in the A > B direction ($p < 0.0001$) in ABCG2 expressing cells compared to wildtype cells (Fig. 2A and B; Tables 1 and 2). The efflux ratio of prazosin in the ABCG2 cells was r = 11 ± 0.2 (Fig. 2B), above the generally accepted thresholds of 1.5 for substrates of ABCG2 (Doan et al., 2002). The permeability of the ABCB1 substrate digoxin in the B > A direction was significantly higher in the ABCB1 cells compared to wildtype cells ($p < 0.0001$) (Fig. 3A and B; Tables 1 and 2). A transport ratio could not be calculated for digoxin since the rate of transport in the A > B direction was not significantly different from zero.

The enhanced transport of prazosin and digoxin in ABC transporter overexpressing cells was mediated by ABCG2 and ABCB1, as the well-established transporter inhibitors elacridar and loratadine inhibited the enhanced permeabilities observed in transporter overexpressing cells. That is, both elacridar and loratadine inhibited the B > A direction transport of prazosin and digoxin in the ABCG2 and ABCB1 cells respectively ($p < 0.0001$) (Figs. 2C and 3C). Because digoxin permeability in the A > B direction was so close to zero the inhibitory effect of loratadine is not reflected in the transport ratio. Elacridar also enhanced transport of prazosin in the A > B direction in the ABCG2 cells ($p < 0.0001$), emulating the transport of prazosin in wildtype cells.

We then examined the inhibitory potential of CBC and CBCA on prazosin transport by ABCG2 and digoxin transported by ABCB1 (Figs. 2 and 3, Table 2). Neither CBC nor CBCA affected prazosin transport in the B > A direction, suggesting these cannabinoids are not inhibitors of ABCG2 (Fig. 2D and E). Interestingly, CBC significantly inhibited transport of prazosin in the A > B direction ($p = 0.0472$) and CBCA significantly increased transport of prazosin in the B > A direction ($p = 0.0208$). Although, both of these latter effects were very subtle so may have limited physiological relevance. CBC and CBCA had no effect on ABCB1 transporter function (Fig. 3D and E).

3.2. CBCA, but not CBC, is a substrate of the ABC transporter ABCB1

We assessed whether CBC and CBCA were substrates of ABCG2 and ABCB1 *in vitro* using MDCK cells expressing human ABCG2 or ABCB1. Transwell assays were performed to assess bidirectional transport of CBC and CBCA across wildtype, ABCG2 and ABCB1 MDCK cell monolayers. ABCG2 and ABCB1 preferentially transport substrates in the basolateral to apical direction (B > A). Permeability of CBC and CBCA in the basolateral to apical (B > A) and apical to basolateral (A > B) directions were determined for each transporter and compared to respective permeabilities in the wildtype control cells.

CBC was not a substrate of either ABCG2 or ABCB1. The cell

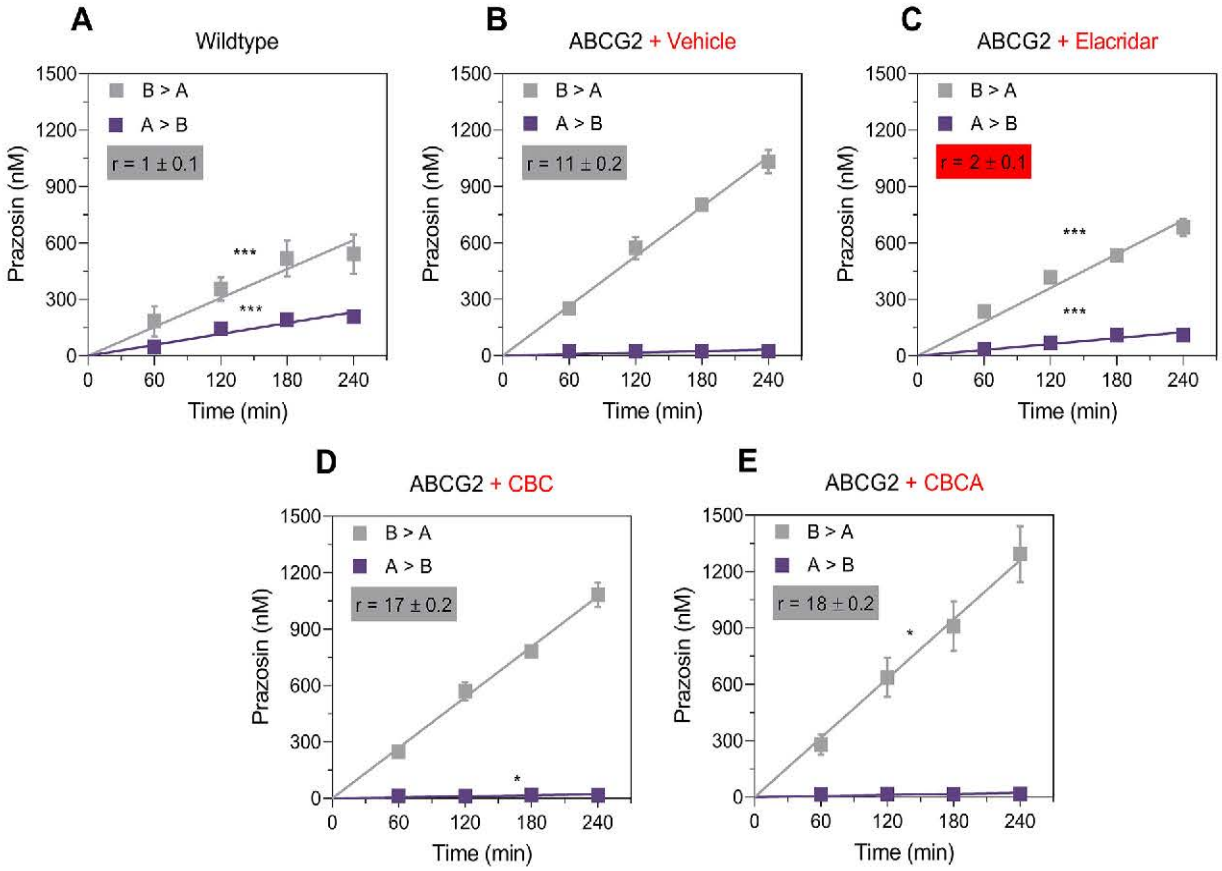


Fig. 2. ABCG2-mediated transport of prazosin is not inhibited by CBC nor CBCA. Concentration-time curves for prazosin in the basolateral to apical (B > A) and apical to basolateral (A > B) directions in (**A**) MDCK wildtype cells, (**B**) MDCK cells expressing ABCG2 in the presence of vehicle, (**C**) elacridar (**D**) CBC, and (**E**) CBCA (n 4–8 per time point). Cannabinoid and elacridar concentrations were 10 μM. Elacridar significantly inhibited transport of prazosin. Data are expressed as means ± SEM. Curves fit to linear regression and transport efflux rations (r) are displayed (*p < 0.05), ***p < 0.0005 compared to vehicle; Extra sum-of-squares F test). See Supplementary Table 3 for the fitting formulas for each graph.

Table 1
Permeabilities of prazosin and digoxin, in wildtype MDCK cells.

	Wildtype		
	P_{app}		r
	B > A	A > B	
Prazosin	1.6 ± 0.1***	1.8 ± 0.1	0.9 ± 0.1
Digoxin	0.2 ± 0.0***	0.1 ± 0.0***	3.3 ± 0.3

P, apparent permeability (cm/s); n.d., not determined; slope of concentration-time curve not significantly different from zero.
***p < 0.0005 compared to vehicle; Extra sum-of-squares F test.

permeabilities of CBC in the B > A direction in ABCG2 or ABCB1 expressing cells were not different than in wildtype cells (Fig. 4, Table 3). Furthermore, the transport ratios for CBC were below the generally accepted thresholds of 1.5 and 2.5 for substrates of ABCG2 and ABCB1, respectively (Doan et al., 2002; Giri et al., 2009).

CBCA was not a substrate of ABCG2. There was no measured transport of CBCA in either direction (B > A and A > B) in ABCG2-expressing cells (Fig. 5B, Table 3). CBCA, however, was a substrate of ABCB1. The permeability of CBCA in the B > A direction was significantly greater in cells expressing ABCB1 compared to wildtype cells ($p = 0.0273$) without affecting the A > B direction (Fig. 5C). The efflux ratio of r = 3.5 ± 0.3 further supports CBCA as an ABCB1 substrate. Moreover, ABCB1 mediated transport of CBCA was significantly inhibited by loratadine, the positive control ABCB1 inhibitor ($p = 0.0008$, Fig. 5D).

Table 2
Permeabilities of prazosin and digoxin, in ABCG2 and ABCB1 MDCK cells, respectively.

	ABCG2			ABCB1		
	P_{app}		r	P_{app}		r
	B > A	A > B		B > A	A > B	
Substrate	2.7 ± 0.1	2.4 ± 0.1	11 ± 0.2	1.3 ± 0.2	n.d	n.d
Substrate + Inhibitor	1.8 ± 0.1***	1.0 ± 0.1***	1.9 ± 0.1	0.5 ± 0.0***	0.1 ± 0.0	8.9 ± 0.2
Substrate + CBC	2.0 ± 0.0	0.1 ± 0.0*	17 ± 0.2	0.8 ± 0.0	0.1 ± 0.0	12 ± 0.3
Substrate + CBCA	2.3 ± 0.1*	0.1 ± 0.3	18 ± 0.2	1.0 ± 0.1	n.d.	n.d.

The ABCG2 substrate used was prazosin and the inhibitor elacridar; the ABCB1 substrate used was digoxin and inhibitor loratadine.
P, apparent permeability (cm/s); n.d., not determined; slope of concentration-time curve not significantly different from zero.
*p < 0.05, ***p < 0.0005 compared to vehicle; Extra sum-of-squares F test.

3.3. *Molecular docking of CBCA to ABCB1*

Given our finding that CBCA is an ABCB1 substrate, an *in silico* molecular docking was performed to investigate the binding of CBCA with human ABCB1. Docking suggested that the inhibitor molecules tariquidar and elacridar bound in the access tunnel and vestibule connecting the two cavities (Fig. 6A). Additionally, CBCA, like tariquidar and elacridar, docked both in the access tunnel and the central binding pocket (Fig. 6B). The top 8 poses generated in the docking search had

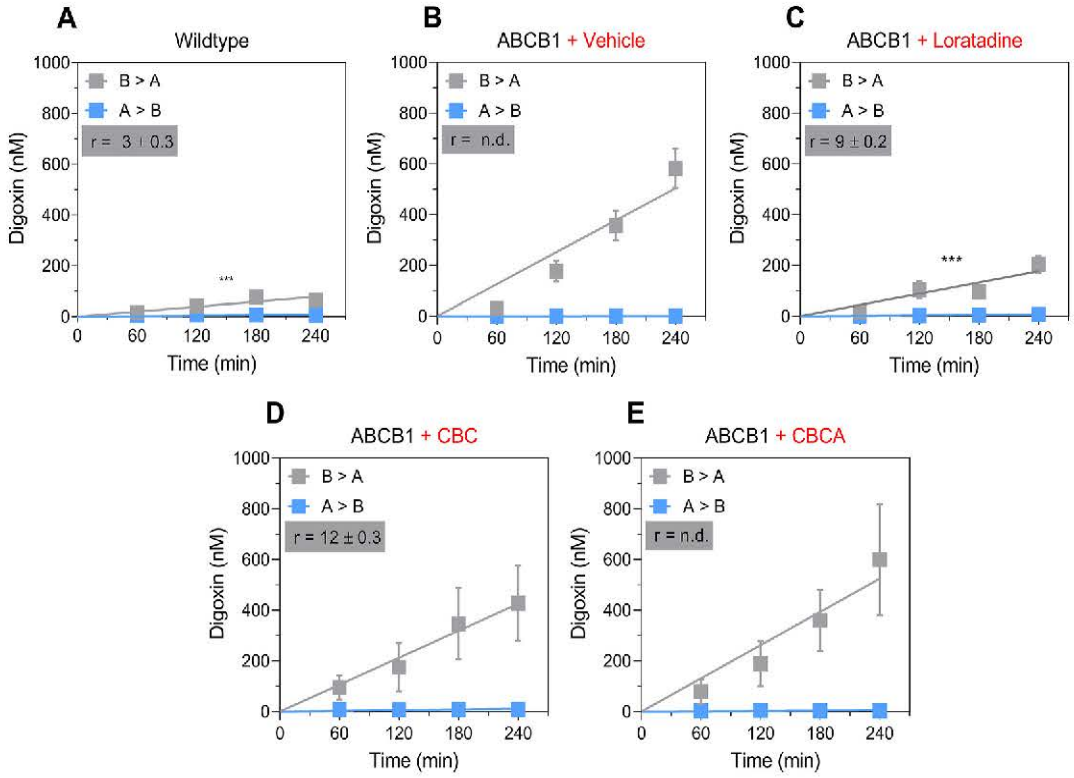


Fig. 3. ABCB1-mediated transport of digoxin is not inhibited by CBC nor CBCA. Concentration-time curves for digoxin in the basolateral to apical (B > A) and apical to basolateral (A > B) directions in (**A**) MDCK wildtype cells, (**B**) MDCK cells expressing ABCB1 in the presence of vehicle, (**C**) loratadine (**D**) CBC, and (**E**) CBCA (n 4–9 per time point). Cannabinoids and loratadine concentrations were 10 μM. Loratadine significantly inhibit transport of digoxin. Data are expressed as means ± SEM. Curves fit to linear regression and transport efflux rations (r) are displayed (***$p < 0.0005$ compared to vehicle; Extra sum-of-squares F test). See Supplementary Table 3 for the fitting formulas for each graph.

calculated binding affinities of −8.3 to −7.0 kcal/mol. Poses 1–4 and 6–7 were in the access tunnel, while poses 5 and 8 bound in the central binding cavity. Notably, similarly to the inhibitors, CBCA contains an aromatic ring system, which positioned in the "top" part of the central cavity, close to residues Y953 and F983.

4. Discussion

Two cannabinoids found in cannabis, CBC and CBCA, have therapeutic potential with preclinical research suggesting they have anticonvulsant, antidepressant, anti-inflammatory, anti-cancer and antibacterial activity (Anderson et al., 2021a; Anis et al., 2021; de Petrocellis et al., 2012, 2013; DeLong et al., 2010; Galletta et al., 2020; Izzo et al., 2012; Ligresti et al., 2006b; Romano et al., 2013). The current study advances our understanding of these compounds by examining their interaction with the ABC transporters ABCB1 and ABCG2. Both these transporters influence drug bioavailability and can mediate DDIs. Using *in vitro* transwell assays in cells expressing human transporters, CBCA was found to be an ABCB1 but not ABCG2 substrate. CBC was not a substrate of either transporter. Neither CBCA nor CBC inhibited ABCB1 or ABCG2 transport of the well-characterised substrates prazosin and digoxin, respectively.

Because ABC transporters affect bioavailability of substrates, determining whether a drug candidate is a substrate is important for predicting plasma exposures and disposition in target tissues. For example, ABC transporter substrates may have low oral bioavailability as a result of ABCG2 and ABCB1 expressed in the gut. Substrates are actively extruded back into the intestinal lumen, thereby reducing plasma concentrations (Glavinas et al., 2005). Similarly, ABC transporter substrates have reduced brain uptake due to drug efflux at the blood-brain barrier (Chaves et al., 2014). The fact that CBC is not an ABCB1 or ABCG2 substrate is a useful property for its therapeutic development. However, CBCA was an ABCB1 substrate, consistent with a previous study showing that the concentrations of CBCA in brain tissue were substantially increased in a Tween80 vehicle (Anderson et al., 2019, 2021a). Tween80 has been shown to inhibit ABCB1 (Nguyen et al., 2021), so an interaction between Tween80 and CBCA at the blood-brain barrier could account for the increased CBCA brain concentrations observed. While being an ABCB1 substrate could be a disadvantage for the development of CBCA as a therapeutic, it does not preclude it since there are many ABCB1 and ABCG2 substrates on the market, and peripheral restriction may be useful for non-CNS applications (Chen et al., 2016). Recently several cannabinoids including CBD, CBDA, CBG, and Δ^9-THC were characterised as ABCG2 substrates in *in vitro* transwell assays (Anderson et al., 2021b).

ABCB1 and ABCG2 transport compounds from a wide variety of drug classes. Substrates of these ABC transporters include analgesics, antiarrhythmics, antihistamines, antibiotics, chemotherapeutics, and immunosuppressive drugs (Chen et al., 2016). Co-administration of one of these substrates with an ABCB1 or ABCG2 inhibitor has the potential to result in clinically significant DDIs (Busti et al., 2008; Liu, 2019; Sadeque et al., 2000; Trivedi et al., 2021; Zhou, 2008). Therefore, drug regulatory agencies require that the inhibitory potential of new drug candidates at ABC transporters be evaluated (Giacomini et al., 2010). CBC and CBCA did not inhibit ABCB1 and ABCG2-mediated transport of conventional substrates, which is favourable for further drug development of these cannabinoids. Whilst CBC and CBCA were not inhibitors of these substrates, future studies could examine whether this holds for other ABCB1 and ABCG2 substrates. Moreover, DDIs can be mediated by other ABC and solute carrier transporters, so future studies could examine the effects of CBC and CBCA on these additional transporters (Liu, 2019). Interestingly, we had recently observed evidence of cannabis-based products inhibiting organic cation transporters (OCTs), organic anion transporters (OATs), and organic anion transporting polypeptides (OATPs) (Anderson et al., 2020). Another area of future investigation would be to assess whether these cannabinoids induce

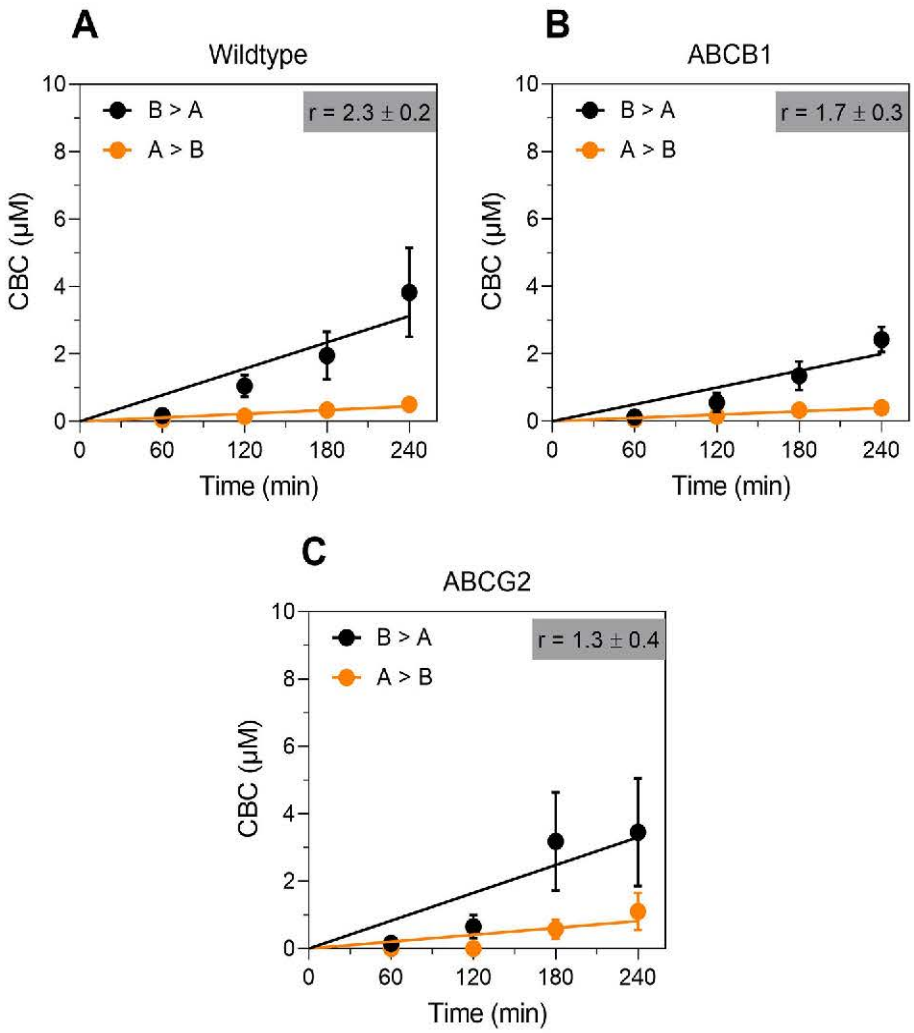


Fig. 4. CBC is not a substrate of either ABCG2 or ABCB1. Concentration-time curves for CBCA in the basolateral to apical (B > A) and apical to basolateral (A > B) directions in (**A**) wildtype MDCK cells and those expressing (**B**) ABCG2 or (**C**) ABCB1 (n 4 per time point). Transport of CBC was not different across any of the cell lines; thus, CBC was not a substrate of either ABC transporter. Data are expressed as means ± SEM. Curves fit to linear regression and transport efflux ratios (r) are displayed. See Supplementary Table 3 for the fitting formulas for each graph.

Table 3
Permeabilities of cannabinoids CBC and CBCA in wildtype, ABCG2 and ABCB1 MDCK cells.

Substrate	Wildtype			ABCG2			ABCB1		
	P_{app}		r	P_{app}		r	P_{app}		r
	B > A	A > B		B > A	A > B		B > A	A > B	
CBC	58 ± 10	25 ± 3.8	2.3 ± 0.2	61 ± 14	46 ± 13	1.3 ± 0.4	37 ± 4.7	22 ± 5.8	1.7 ± 0.3
CBCA	14 ± 1.3	9.4 ± 0.9	1.4 ± 0.1	n.d.	n.d.	n.d.	26 ± 5.0*	7.3 ± 1.9	3.5 ± 0.3
CBCA + Loratadine	–	–	–	–	–	–	n.d.	4.9 ± 1.2	n.d.

P, apparent permeability (× 10^{-5} cm/s).
n.d., not determined; slope of concentration-time curve not significantly different from zero.
*p < 0.05, **p < 0.001, ***p < 0.0005 compared to corresponding wildtype condition; Extra sum-of-squares F test.

expression of these transporters. Previous work has shown that Δ^9-THC induced the expression of ABCB1 at the blood-brain barrier in mice, which reduced the brain uptake and effects of the antipsychotic drug risperidone, an ABCB1 substrate (Brzozowska et al., 2017).

Given our findings suggesting that CBCA is an ABCB1 substrate, an *in silico* molecular docking was performed to investigate the binding of CBCA with human ABCB1. A recent cryo-EM structural study of human ABCB1 bound to substrate and inhibitors revealed a central binding pocket located within the transmembrane (TMD) channel (Nosol et al., 2020). Another cavity, offset with respect to the central pseudosymmetry axis of the TMDs, extends from the central drug-binding pocket to a cytoplasmic gate, dubbed the access tunnel. The inhibitors bound in pairs, with one inhibitor in the central binding pocket and the second extending into the phenylalanine rich access tunnel. Thus, while the transported drug vincristine bound in the central binding pocket, we found that the inhibitor molecules tariquidar and elacridar bound in the access tunnel and vestibule connecting the two cavities, which is consistent with structural studies (Alam et al., 2019; Nosol et al., 2020). It has been suggested that the overlap of substrate and inhibitor binding sites within the central pocket explains how inhibitors act as substrates at low concentrations but interfere with the early steps of the peristaltic extrusion mechanism for substrates at higher concentrations (Nosol et al., 2020). In this study, *in silico* docking using ABCB1 as the transporter (Nosol et al., 2020) revealed that similarly to the inhibitors tariquidar and elacridar, CBCA binds in both the central binding cavity and the access tunnel of ABCB1, roughly overlapping the binding sites for substrates and inhibitors.

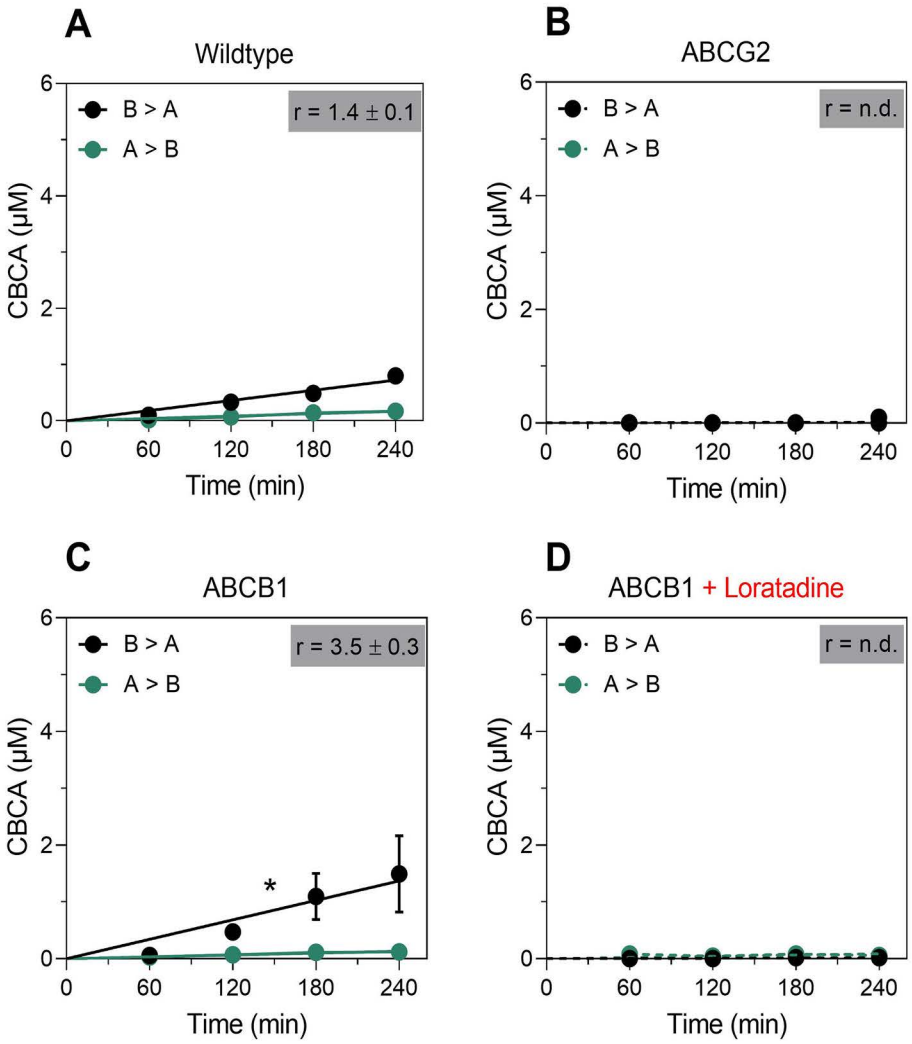


Fig. 5. CBCA is an ABCB1 substrate. Concentration-time curves for CBCA in the basolateral to apical (B > A) and apical to basolateral (A > B) directions in (**A**) wildtype MDCK cells and those expressing (**B**) ABCG2 or (**C**) ABCB1 (n = 4 per time point). CBCA was a substrate of ABCB1 as its transport in the B > A direction was significantly greater than that of wildtype cells (*$p < 0.05$ compared to wildtype; Extra sum-of-squares F test). (**D**) Concentration-time curves for CBCA transport in ABCB1 expressing cells in the presence of 10 μM loratadine. Loratadine significantly inhibited ABCB1 mediated transport of CBCA. Data are expressed as means ± SEM. Curves fit to linear regression and transport efflux ratios (r) are displayed. See Supplementary Table 3 for the fitting formulas for each graph.

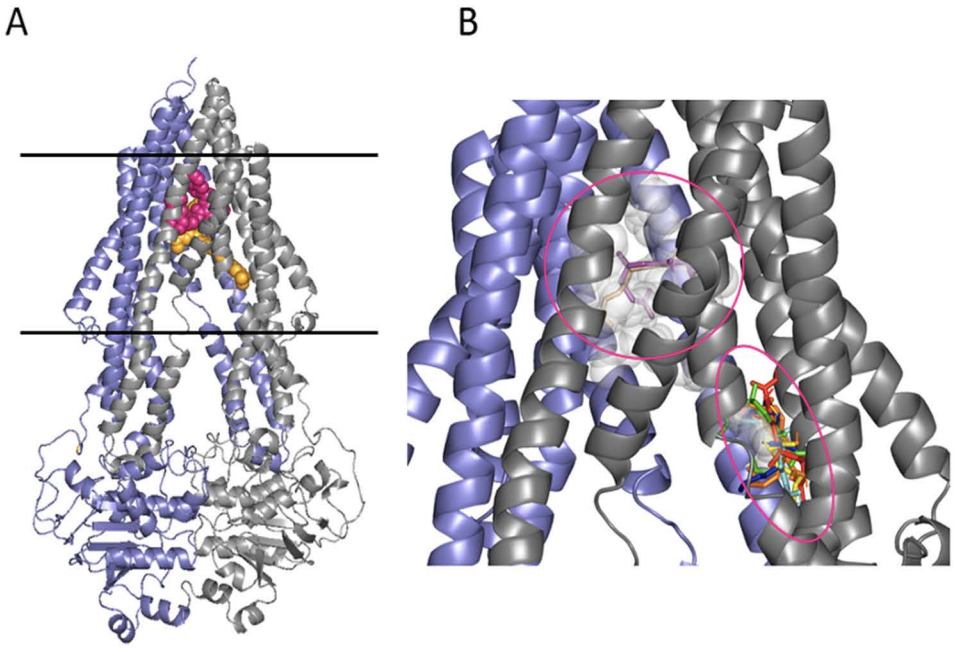


Fig. 6. ABCB1 (PDB 7A69) showing inhibitor binding cavities and docked CBCA. ABCB1 is shown in cartoon representation with N-terminal half slate purple and C-terminal half grey. (**A**) Two bound molecules of the inhibitor tariquidar are shown in van der Waals sphere representation with that bound in the central binding cavity coloured magenta, and the other, which straddles the central binding cavity and the access tunnel, coloured orange. (**B**) The 8 lowest energy poses calculated for CBCA docking to ABCB1 are shown with CBCA in stick form. Poses 5 and 8 bind in the central cavity while the others bind in the access tunnel (both sites circled in magenta). Bound tariquidar molecules, as depicted in panel (**A**), are shown in transparent van der Waals sphere representation coloured grey.

5. Conclusion

Our findings show that the phytocannabinoid CBCA, a compound with anticonvulsant and antibacterial properties, is a substrate of ABCB1. Despite CBCA acting as an ABCB1 substrate, it did not inhibit transport of the ABCB1 substrate digoxin. CBCA was neither a substrate

nor inhibitor of ABCG2. Additionally, CBC was not an ABCB1 or ABCG2 substrate and did not inhibit these transporters. An *in silico* molecular docking model confirmed CBCA's binding with ABCB1, showing binding to both the central binding cavity and the access tunnel. The findings of the present study improve our understanding of the basic pharmacology of phytocannabinoids, which are subject to increasing interest with the rise of medicinal cannabis around the world.

Author contributions

MGE, LLA and JCA contributed to the conception and design of the study. AA and SDB contributed to the synthesis of cannabichromene. MGE and LLA contributed to the acquisition and analysis of data. PMJ and AMG performed the docking study. All authors contributed to drafting the manuscript.

Declaration of competing interest

Associate Professor Arnold has served as an expert witness in various medicolegal cases involving cannabis and cannabinoids and served as a temporary advisor to the World Health Organization (WHO) on their review of cannabis and the cannabinoids in 2018. He has received consulting fees from Creo Inc. The remaining authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2022.174836.

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Chapter 5

General Discussion

5.1 Summary of Findings

The present thesis examined whether lipid dysregulation occurs in a mouse model of Dravet syndrome and expanded upon the pharmacological properties of cannabinoids. Chapter 1 provided a review of the relevant literature and summarised the aims of the present thesis. Chapter 2 and 3 present a lipidomic analysis of brain tissue in the *Scn1a*$^{+/-}$ mice model of Dravet syndrome. It began with an untargeted approach in Chapter 2 which identified various lipids (sphingolipids, glycerophospholipids and glycolipids) with the ceramide HexCer species being the most notably altered in response to the heterozygous deletion of *Scn1a* on the seizure-sensitive F1 background strain which. Following this, a targeted LC-MS/MS analysis was conducted in chapter 3 seeking to confirm the changes on HexCer species and expand on their quantification. Chapter 4 investigated the lipid-like cannabinoids, CBC and CBCA that have anti-seizure effects in the *Scn1a*$^{+/-}$ mice model of Dravet syndrome for their interactions with ABC transporters, drug efflux pumps found in the BBB that can limit brain disposition of substrate compounds.

In Chapter 2 the untargeted lipidomic analysis of cortical and hippocampal brain tissue of the *Scn1a*$^{+/-}$ mice at the seizure-prone developmental age of P24, discovered: 1) a distinct genotype separation between F1.*Scn1a*$^{+/-}$ mice (that express the severe seizure phenotype) versus F1.WT mice (non-seizure controls), where in the cortex but not in the hippocampus, the most significant lipid differences underpinning the observed segregation were reductions in HexCer(d42:2), TG(54:6), PC(33:1), PC(35:1), PC(37:1), and PC(40:4e), and increased PC(38:3) in F1.*Scn1a*$^{+/-}$ relative to F1.WT; and 2) a significant cortical separation between 129 seizure-resistant strain and the F1 seizure-susceptible strain which was driven by decreases in Cer(34:1), Cer(36:2), Cer(38:2), LPC(18:0), PC(42:2), PS(38:4), and PS(44:10) concentrations, and significantly higher dMePE(30:0), PC(28:0), PC(29:0), and PC(30:0) concentration, with the last two also appearing significantly differentiated in the hippocampus. These findings propose that unique lipidomic changes in sphingolipids, glycerophospholipids and glycolipids are associated with the heterozygous deletion of *Scn1a* and the seizure-susceptible F1 strain.

Chapter 3 sought to confirm the identified changes in the HexCer lipid sub-class using targeted LC-MS/MS methods across the wide array of within class structural variants. The results failed to confirm the significant decrease in cortical F1.*Scn1a*$^{+/-}$ HexCer(d42:2) species found using this lipidomics approach. However, the analysis identified a broad array of differentially expressed HexCer species that were mostly upregulated in brain tissue of the seizure-prone F1 strain, and more markedly displayed in the hippocampus than in cortex. However, not all HexCer species were increased in the seizure-susceptible F1 strain, and there appeared to be tissue-specific HexCers structural pattern alterations. F1 strain mice had a greater abundance of short C16 acyl chains compared to 129 strain mice in both the hippocampus and cortex. Although, the effect of strain in the cortex was limited to short acyl chains, the hippocampus additionally had an increased concentration of HexCer species of all chain lengths. Additionally, each region showed variability in chain length trends, while the hippocampus showed a consistent upregulation of HexCers in F1 strain compared to 129 mice, the cortex showed a dual dynamic, where F1 mice had increased levels of HexCer d18:1/16:0 and d18:1/18:1, while d18/18:0 showed to be significantly higher in the 129 strain. Notably, the strain effects observed in the cortex were limited to non-hydroxylated species, while in the hippocampus there were changes in HexCers of both hydroxylated states. Additionally, although all strain-effects appeared as the collective aggregation of both genotypes and no gene and strain interaction effect were identified, some notable HexCer species appeared significantly higher in the hippocampus of F1.*Scn1a*$^{+/-}$ compared to 129.*Scn1a*$^{+/-}$ groups, including d18:1/16:0, 18:2/18:0-OH and d18:1/24:0-OH, while the same hippocampus d18:1/16:0 and cortex d18:2/20:1 presented significantly higher concentration in the F1.WT compared to 129.WT. Additionally, we observed a single genotype-driven upregulation of the d18:2/18:0 species in the cortex of *Scn1a*$^{+/-}$ mice compared to WT mice on the F1 background strain. Overall, the results suggests that HexCer species may be associated with seizure-susceptibility seen in the F1 strain, with potential further modulations driven by *Scn1a* genotype and seizure-presentation.

The plant cannabinoids CBC and CBCA, which are structurally distinct to the known anti-seizure agent CBD, also display anti-seizure effects in the *Scn1a*$^{+/-}$ mouse

model of Dravet syndrome, through unknown mechanism(s) of action (Anderson et al., 2021a). Chapter 4 sought to advance the understanding of these actions through characterisation of interaction with ABCB1 and ABCG2 transporters. The examination involved an *in vitro* pharmacological screen in a bidirectional transport assay to determine substrate and/or inhibitor activity. Transwell assays with polarized epithelial MDCK cells expressing ABCB1 or ABCG2 were used, and samples were measured using LC-MS/MS. The key results are: 1) CBCA is an ABCB1 substrate, but not an ABCG2 substrate; 2) CBC was not a substrate of either transporter; 3) Neither CBCA nor CBC inhibited ABCB1 transport of prazosin or ABCG2 transport of digoxin; 4) *In silico* molecular docking suggested CBCA binds ABCB1 in the access tunnel and the central binding pocket. These findings provide important basic pharmacological data on these cannabis constituents in their evaluation as next-generation cannabinoid anti-seizure agents.

5.2 Broader Potential Implications and Future Directions

5.2.1 Lipidomics - Chapter 2 and 3

These two chapters entailed a lipidomic investigation that began using an exploratory untargeted approach followed by a targeted confirmatory LC-MS/MS analysis of an extended series of HexCer species. Chapter 2 found the heterozygous deletion of *Scn1a* in the seizure-susceptible F1 strain altered lipids of the glycolipid (TG), glycerophospholipid (PC, LPC, dMePE, and PS) and sphingolipid (Cer and HexCer) families. As discussed in the chapter, all such changes in lipid pathways are plausible based on prior studies that have linked them to neuronal structure and function and the pathophysiology of epilepsy. This provides clues on exciting new investigational avenues. Future research could focus on the targeted quantification of glycerolipid, glycerophospholipid and the expanded sphingolipid family in the search to expand on the underexplored Dravet syndrome pathophysiology and potentially find alternative therapeutic targets that have the potential for increased efficacy.

A valuable next step could be to perform lipidomic pathway- and network-level analysis of the untargeted results. This refers to a novel computational approach that

aims to find broader patterns of biological dysregulation beyond individual compounds; it works by getting fed lipidomic data, most commonly from untargeted analysis, which get screened against statistical systems together with molecular databases of compound relationships, and leads to the identification and visualization of potential relationships between molecules appearing in the data (Gaud et al., 2021; Yang et al., 2016). It stands on the basis that perturbation in biological compounds is often accompanied by broad shifts across entire families, especially true in lipids given that many lipids share common biosynthetic and metabolic enzymes (Gaud et al., 2021; Yang et al., 2016). Different applications exist, a widely used option is MetaboAnalyst 5.0 which has enrichment, pathway, joint-pathway and network analysis modules for metabolomic analysis (Du et al., 2023; Pang et al., 2021). Alternatively, LIPID MAPS' has its own application called BioPAN which is lipid specific and utilizes their mammalian lipidome database together with computational statistical scoring to flag pathways or enzymes that are activated or supressed (Gaud et al., 2021). Utilizing these methods could indeed further enrich the data obtained from the untargeted analysis and potentially highlight unidentified patterns associated with the disease.

From here, to corroborate the results and further expand the lipidomic exploration, analysis was focused on the flagged sphingolipid class HexCer. This was due to its large genotype-driven significant differentiation in cortical F1.*Scn1a*$^{+/-}$ compared to F1.WT mice, as well as for its vast physiological relevance to the pathophysiology of epilepsy. Unfortunately, the targeted analysis was unable to replicate these results, which was thoroughly discussed in the chapter. This mismatch however further emphasises the importance of the application of targeted validation and extends onto the need for future work that analyses the remaining lipids identified in the untargeted analysis. Nonetheless, the targeted analysis was able to identify various other differentiations that reflect upon the potential contribution of the HexCer species to the seizure-susceptibility seen in the F1 strain, with a potential for additional modulations driven by *Scn1a* genotype and seizure-presentation. This again encourages the future study of the pathway including extended members of the sphingolipid class as well as markers of enzymatic activity. Enzymes of interest to identify sources of potential HexCer dysregulation include those specific to GalCer and GlcCer synthesis, UGT81 and UGCG

respectively, as well as their degradation, GALC and GBA, but also those involved in Cer biosynthesis, namely CerS, DES1, and ASMase (Hannun et al., 2018).

A key finding of the present study is a markedly broader influence of strain compared to *Scn1a* genotype on the observed profile differences. This observation aligns with earlier work by Hawkins et al. (2019), who's global RNA-seq analysis in hippocampal WT and *Scn1a*$^{+/-}$ mice on both F1 and 129 strains revealed minimal to modest expression changes driven by *Scn1a* genotype alone, with more substantial differences driven by strain. This pattern was echoed in the untargeted PLS-DA analysis, where the hippocampus separation was modest compared to the cortex, yet, within it, F1 versus 129 strain comparison was further distinct than the *Scn1a* versus WT genotype comparison. The targeted HexCer analysis reinforced this further, with more strain effects on lipids in not just the cortex, but also the hippocampus. Indeed, there were more marked differences in the seizure-prone F1 strain compared to the non-seizure prone 129 strain in the hippocampus. It is plausible that genes differentially expressed between strains include those involved in sphingolipid metabolism, thus leading to the value of future studies assessing gene expression of the sphingolipid pathway. Notably, Hawkins et al. (2019) investigation found that at P24, both comparisons of F1. *Scn1a*$^{+/-}$ with F1.WT and F1.*Scn1a*$^{+/-}$ with 129.*Scn1a*$^{+/-}$ mice showed prominent differentiation in the expression of genes related to oligodendrocytes, the myelin hubs of the brain. This theory is mainly attributed to the high expression of HexCer species in myelin, which is known to assists with its structural integrity and functionality (Reza et al., 2021), and confirmed by previously identified demyelination damage in Dravet syndrome tissue (Satta et al., 2023). In their study Satta et al. (2023) analysed myelination status in Syn-Cre/Scn1a$^{WT/A1783V}$ mouse model of Dravet syndrome brain via the use immunofluorescence and RT-qPCR techniques of two myelin markers, myelin basic protein and myelin oligodendrocyte glycoprotein. Their results showed myelination reduction in both the prefrontal cortex and the hippocampus by P25, thus elucidating to presence of demyelination as a pathophysiological characteristic of the Dravet mouse model. Future studies could treat *Scn1a*$^{+/-}$ mice with agents know to increase myelination, for example GPR17 antagonists (Chen et al., 2009), to see whether these

agents reduce seizures, but also improve the autism-like features of the model such as cognitive and social memory deficits (Bahceci et al., 2020).

The targeted analysis discovered that an altered HexCer pathway is potentially contributing to the seizure-susceptibility seen in the F1 strain, with possible additional modulations driven by the *Scn1a* mutation. Although there was a strong association between underlying strain and lipid dysregulation, whether these changes reflect a causal mechanism via the underlying genetic background or an interaction with seizure generation remains unresolved. This is because at the day of sample collection, P24, F1.*Scn1a*$^{+/-}$ mice already exhibit spontaneous seizures (Miller et al., 2014). As previously discussed, it is very possible that genotype effect is associated to secondary factors linked to the interplay of the F1 strain background, seizure burden, or a compensatory stress response. Supporting this, Hawkins et al. (2019) suggested that strain differences in the Dravet mice set the tone for the baseline metabolism and thus seizure susceptibility, with the *Scn1a* mutation amplifying these differences in the F1 background strain due to the introduction of the "seizure susceptible" C57BL/6 strain's genetics. Therefore, future studies are needed to investigate the mechanistic link between HexCer dysregulation and seizure presentation and susceptibility. Clarifying this relationship is crucial as it may reveal whether HexCer modulation is a contributing factor to epileptogenesis, a consequence of seizure, or both. Ultimately, this information feeds onto its potential as a therapeutic target. The *Scn1a*$^{+/-}$ model offers a valuable system to explore this, particularly when combined with comparisons of seizure-prone and seizure-resistant genetic backgrounds.

While our investigation and protocols discussed below mainly focus on HexCer, similar approaches could extend to other lipids identified in the untargeted analysis. An initial approach could identify the directional relationship of lipid dysregulation and seizure presentation, i.e., is seizure susceptibility/burden associated with specific lipid dysregulation? Hawkins et al. (2019)'s comparison of F1.*Scn1a*$^{+/-}$ mice with and without seizures revealed changes in genes related to astrogliosis, neurodevelopment, and plasticity, they suggested that this reflected perhaps decreased homeostatic flexibility to combat the seizures (Hawkins et al., 2019). This could be translated to the lipidomic space. A sound methodology is the characterization of seizure frequency, duration and

severity via *in vivo* application of advance seizure monitoring technology paired with post-mortem brain lipidomic quantification. Seizure monitoring could be approached in two previously validated ways: 1) continuous monitoring of spontaneous seizure using video and/or EEG recordings (Griffin et al., 2018); and 2) the experimental model where mice are assessed for temperature thresholds for hyperthermia-induced generalized tonic-clonic seizures (Bahceci et al., 2023; Hawkins et al., 2017; Zhou et al., 2024). This combined with approach with lipidomics has not been previously applied to the Dravet syndrome context, however a close methodology was recently used in an acute and chronic PTZ rat model. Chen et al. (2025b) applied integrated proteomics and lipidomics analysis of the rat's hippocampus and reveal metabolic changes attributed to seizure induction, they found that both glycerophospholipids and sphingolipids were dramatically altered after seizures; most notably, the acute PTZ model yielded decreased hippocampal HexCer levels while the chronic administration displayed upregulation compared to controls, thus suggesting a direct modulation of HexCer species based on intensity and duration of neuronal excitability (Chen et al., 2025b). Altogether, the application of this methodology to the Dravet syndrome context has the potential to identify lipidomic changes that are directly associated with seizure presentation.

An extended investigation could then assess the impact of HexCer modulation on seizure expression. This could be achieved with the *Scn1a*$^{+/-}$ mice via two approaches coupled to monitoring or induction of seizures as explained above: 1) targeted pharmacological intervention of enzymes of interest, and/or 2) injection of AAV vectors or CRISPR manipulation to knockdown or overexpress genes of interest. Viable candidates for the pharmacological approach include: 1) RA 5557, an UGT8A inhibitor that reduces biosynthesis of GalCer (Zaccariotto et al., 2022); 2) Impiramine, a tricyclic antidepressant that reduces Cer production via ASMase inhibition -coincidentally found to promote newborn neuron survival in the hippocampus after seizure in a pilocarpine-induced rat seizure model (Lee et al., 2025); 3) Cyclopropenone PR280, a novel potent DES1 inhibitor that reduces Cer production, yet to be further pharmacologically characterized (Rivero et al., 2024); and 4) Fumonisin B1, a CerS inhibitor, also a disruptor of Cer production, that although is known to cause neurotoxic effects, is a valuable tool to assess the sphingolipid production pathway (Chen et al., 2025b). In parallel, a genetic

and viral manipulation approach could be done as previously performed by Qin et al. (2022), where kainic acid epilepsy model mice were followed by video monitoring of seizures presentation after being injected with AAV to upregulate an unrelated target to the present thesis GPR120, a receptor shown to negatively regulate inflammation and apoptosis; they found that the upregulation alleviated epileptic activity (Qin et al., 2022). HexCer species have not been reported to act as primary signalling molecules, however, a similar approach but on *Scn1a*$^{+/-}$ mice could be performed for enzymatic members of the sphingolipid family that modulate their expression. In light of the observed potentially pathologically linked increase of HexCer concentration on the F1 mice, the upregulation of its degradation via GALC/GBA may be of potential therapeutic relief. GALC, involved in GalCer breakdown, could be a particularly good candidate for this approach, given that AAV1-GALC gene methodology has been previously employed in a mouse model of Krabbe disease with positive therapeutic outcomes (Herdt et al., 2023). Altogether, these explorations might not only provide a mechanistic explanation of how HexCer and the greater sphingolipid family affect seizure presentation, but also potentially identify novel drug candidates that have not been previously explored to treat Dravet syndrome.

As previously mentioned, a future avenue of investigation could be the assessment of myelination biomarkers, such as myelin basic protein and myelin oligodendrocyte glycoprotein (Satta et al., 2023) and/or the spatial localization of lipid distribution through MSI techniques (Kaya et al., 2023; Murphy et al., 2009). However, an additional valuable avenue of future research that could elucidate more of the association between lipid dysregulation and seizure could involve the employment of lipid imaging techniques to spatially map lipid alterations in the Dravet mice and correlate them to sites of seizure-induced tissue damage. The methodology could assess the correlation of 2 methodologies: 1) high resolution Desorption Electrospray Ionization (DESI)- or MALDI-MSI of brain sections for spatial localization of lipid alterations in seizure-prone tissue areas (Claude et al., 2017; Kaya et al., 2023; Zavolskova et al., 2025), and 2) histological marker assessment for identification of seizure-induced tissue damage, these could include: Nissil staining as a marker for neuronal damage and loss (Si et al., 2017), fluoro-Jade B/C staining as a marker of neuronal degeneration (Wang et al., 2008), and glial fibrillary acidic protein (GFAP) for assessment of astrocyte activation

(Syková, 2004; Trautz et al., 2019). MALDI approaches have been previously applied on GalCer identification in tissue with great success (Jones et al., 2017). Altogether, this assessment could observe the correlation between lipid dysregulation, its localization, as well as whether these correspond with sites of seizure-driven damage.

Here we examined the lipidome post-seizure onset, a future investigation could assess the brain lipidomic profile before seizure-onset. This could help distinguish if lipid changes are driven by the developmental nature of the model, i.e., the genotype alone in the absence of the seizure phenotype, and thus elucidate the role of lipids in the process of epileptogenesis. To assess how genotype interferes with lipidomic profile before seizure onset, P24 mice could be compared to younger pre-symptom onset mice (Cheah et al., 2012). This approach has precedence. Hawkins et al. (2019)' global RNA-seq analysis was performed at two age time-points, P14 and P24, and they found that seizure-absent P14 displayed no differentially expressed genes between 129.WT and 129.*Scn1a*$^{+/-}$ mice, while 21 genes appeared differentially expressed between F1.WT and F1.*Scn1a*$^{+/-}$. These findings indeed suggest that potential molecular changes in the lipidome may precede or be independent of seizure activity and driven by the *Scn1a* deletion. This is valuable information on lipidome changes that are important for epileptogenesis and seizure presentation, thus pinpointing what molecular changes coincide with epileptogenesis. This could provide new molecular leads for 'anti-epileptogenic' compounds that target lipid pathways to prevent seizure predisposition (Bromfield et al., 2006).

Future studies could also explore how established therapeutic interventions with unknown mechanisms affect the lipidome in Dravet syndrome. This approach could not only uncover novel mechanism/s underlying the efficacy of treatments like lipid-like cannabinoids such as CBD and the ketogenic diet to uncover novel therapeutic targets related to the brain lipidome. This could be again assessed via pharmacological/treatment administration on the *Scn1a*$^{+/-}$ mice, together with monitoring seizure presentation, and followed by a post-mortem lipidomic analysis. This approach has been previously employed, whilst not for lipids, but for various metabolomic compounds in the ketogenic diet in Dravet syndrome, and indeed found genotype-and treatment driven modulations of catabolic processes (i.e. glucose,

glycolysis and tricarboxylic acid (TCA) cycle intermediates, noradrenaline, corticosterone, and GABA) (Miljanovic et al., 2021). Additionally, while not yet being examined in specifically Dravet syndrome, previous studies in schizophrenia patients have found that CBD treatment changes plasma sphingolipid levels (Jieu et al., 2024); in this study, it was found that, in healthy volunteers, antipsychotics reduced Cer and SM levels in plasma, and that CBD co-administration restored the sphingolipid levels towards baseline.

Another future direction is to track plasma or CSF lipid levels in F1. *Scn1a*$^{+/-}$ mice longitudinally. This is of interest as it could potentially identify lipid metabolic shifts *in vivo* which can lead to discovery of disease biomarkers, as well as markers for therapeutic effectivity. For instance, it has been previously found that intractable childhood epilepsy patients that don't respond to the ASM valproate possess lower blood free fatty acids and higher triglyceride levels; and, in parallel, the ketogenic diet treatment in status epilepticus patients increase blood levels of ceramides and HexCer over time, suggesting that ketosis influences glycosphingolipid metabolism (Guo et al., 2023).

In conclusion, the present study highlights a potential multifaceted association between lipidomic dysregulation, particularly of HexCer, and Dravet syndrome. Although the analysis did produce conflicting results that investigations hereafter need to reconcile, future research could aim to clarify the relationship between lipidomic alterations with seizure activity as well as whether the lipid dysregulation contributes to the epileptogenesis process. Unravelling these gaps in knowledge may pave the way for the discovery of novel therapeutics.

5.2.2 ABC Transporters and Cannabinoids - Chapter 4

Our research group previously identified that the plant cannabinoids CBC and CBCA display anti-seizure activity in the *Scn1a*$^{+/-}$ mouse model of Dravet syndrome, with comparable potency to the registered anti-seizure drug CBD (Anderson et al., 2021a). CBC and CBCA however have undergone little pharmacological characterisation, and thus mechanism(s) contributing to the observed effects are unclear. In parallel, previous studies have characterised various cannabinoids as ABC transporter substrates and

inhibitors. However, the interactions of CBC and CBCA at these transporters were yet to be determined. Chapter 4 therefore characterized the interaction of CBC and CBCA with ABCB1 and ABCG2 transporters *in vitro* using transwell assays. It was found that CBCA was an ABCB1 substrate, potentially partially helping to explain its modest distribution into the brain after administration in mice and indicating a potential source of pharmacodynamic interaction. Conversely, CBC was not a transporter substrate which is consistent with its abundant brain uptake (Anderson et al., 2021a), and suggests that ABCB1 is not directly involved in its observed anti-seizure activity. Moreover, this characterization aids on the development of these compounds as potential therapeutic agents, by improving the understanding of the potential targets involved in the anti-seizure activity, and potential for drug-drug interactions.

Epilepsies are tightly connected to modulation of ABC transporter expression. Patients with refractory epilepsies commonly overexpress ABC transporters in endothelial cells that comprise the BBB, and in astrocytes and neurons to a minor extent (Lazarowski et al., 2007; Leandro et al., 2019). Indeed, the over-expression of ABC transporters due to the pathophysiology of epilepsy has been put forth as a theory for the development of drug-resistance in epilepsy treatment with ASM (Lazarowski et al., 2007). Sources of transporter overexpression in epilepsy have been attributed to several factors, including increased inflammation and oxidative stress involved in the epilepsy pathophysiology together with uncontrolled recurrent seizures, and genetic polymorphisms (Czornyj et al., 2022; Leandro et al., 2019). So, anti-seizure drugs that are ABC transporter substrates (e.g., clobazam (Nakanishi et al., 2013), and as observed here, CBCA), potentially interfere with ABC transporter functionality, and can also be further limited from brain uptake, leading to reduced treatment efficacy.

Czornyj et al. (2022) hypothesized that ABCB1 expression induction could play a pivotal role in epileptogenesis, heart failure and SUDEP via cardiac and neuronal-transporter mediated membrane depolarization modulation. Consequently, an open question exists as to whether the pharmacological manipulation of expression and functionality of ABC transporters can itself be an anti-seizure strategy. An investigation could assess cannabinoid-driven excitatory modulation of ABC-overexpressing cells. The methodology could use patch-clamp electrophysiology protocols on control and

ABC-transporter-overexpressing cell lines to test whether ABC proteins modulate cellular excitation in the absence and/or presence of cannabinoids. Electrophysiological approaches have been previously employed in members of the ABC transporter family, including ABCC8 and ABCC9 (Bryan et al., 2007), plant ABCB4 (Deslauriers et al., 2021), as well as ABCB1 (Vanoye et al., 1997), but not ABCG2 transporters. For instance, Vanoye et al. (1997) tested chloride currents of mouse fibroblast cells transfected to over express ABCB1; they found that while transporter expression didn't change current properties in comparison to non-transfected cells, an anti-ABCB1 antibody significantly reduced the current, evidencing an ABCB1 involvement in cellular excitability. Overall, this approach together with pharmacological intervention of ABC transporter-interacting cannabinoids like CBCA could reveal indirect effects of cannabinoid stimulation via direct contact with ABC transport modulation of membrane excitability and could potentially unravel a mechanism of action tied to their reported anti-seizure effects.

Moreover, many ABC transporters have overlapping substrates due to protein structural similarities. Whilst ABC transporters like ABCB1 and ABCG2 are most studied due to their ability to influence drug disposition (e.g., drug absorption in the gut, or brain retention), many ABC transporters play a role in regulating the tissue concentrations of lipids, providing a potential convergence of action of the cannabinoids on brain lipid concentrations. ABC transporters transport cholesterol, bile acids, glycerophospholipids (such as PC, PE and PS), sphingolipids, as well as lipid mediators of inflammation (Neumann et al., 2017; Tarling et al., 2013; Ye et al., 2020), and have been found to be involved in phagocytosis and lipid raft composition modulation (Kotlyarov et al., 2022; Wu et al., 2020). For instance, ABCB1 exerts anti-inflammatory effects in macrophages by downregulating lipid rafts rich in immune signalling receptors toll-like receptor 4 (TLR4) via the active efflux of localized cholesterol (Kotlyarov et al., 2022). In parallel, ABCG1 functionality is heavily influenced by lipid membrane composition, as its ATPase activity was absent when reconstituted in egg lecithin, but became active when in lipid bilayer rich in cholesterol, glycerophospholipid, and sphingolipids, alluding to the functional modulation of lipids on ABC transporter function (Neumann et al., 2017). Consequently, it would be of interest for future research to examine whether cannabinoids as substrates, inhibitor or expression modulators of ABC transporters are

able to influence transporter-lipid interactions. This might uncover a novel anti-seizure mode of action of the cannabinoids that seeks to normalise the dysregulation in lipids, such as the one observed here in the lipidomic analysis of a mouse model of Dravet syndrome. This hypothesis has precedent. For instance, He et al. (2024) identified a protective CBD-driven inhibition of foam cell formation via CBD-induced elevated levels of PPARγ and its associated target ABCA1/ABGG1, which in turn reducing foam cell formation via increasing cholesterol efflux within macrophages. Additionally, CBD and Δ^9-THC 'fluidise' lipid-rich synaptic vesicles (Hillard et al., 1985), suggesting a biophysical interaction that could potentially be related to ABC transporters involved in membrane lipid regulation. Future research could investigate this hypothesis by exploring how cannabinoids modulate ABC transport-mediated lipids modulation. A viable methodology could assess whether pharmacological intervention of cannabinoids differentiates lipid distribution in normal cells compared to ABC transporter-overexpressing cells with the use of mass spectrometry imaging techniques such as DESI and MALDI to quantify spatial lipid composition. Although these technologies are more commonly used in tissue sections, they are also well suited for cultured cells, and even to the single-cell and single-organelle level (Li et al., 2023). This approach has not been published before, but it is a plausible method to identify ways in which cannabinoids could interact with ABC transporter endogenous functionality and potentially pinpoint a novel mechanism of action in epilepsy which involves modulation of lipid biology.

Drug-associated transporter induction have been vastly found in the cancer research space (Glavinas et al., 2004); however, some investigations have also alluded to ASM-driven modulation of transporter expression. For instance, Grewal et al. (2017) found that the ASMs valproate and carbamazepine caused a significant induction in ABCB1 and/or ABCC2 expression in cell lines, both at the transcript and protein level, as well as increased functional activity. Chapter 4 only examined whether CBC and CBCA are substrates or inhibitors of ABCB1 and ABCG2, but cannabinoids have been previously described to also modulate the expression of these transporters. Holland et al. (2006) found that 72-hour exposure of CBD and Δ^9-THC in ABCB1 overexpressing cell line conferred moderate effects of multi-drug resistance reversal by decreasing ABCB1 expression. However, Brzozowska et al. (2017) demonstrated that Δ^9-THC exposure in

mice increased ABCB1 expression in various brain regions, and that such effect decreases the bioavailability of the ABCB1 substrate and antipsychotic drug risperidone. The data is conflictive, however, the mere possibility of pharmacologically driven overexpression is particularly concerning in epilepsy, as it could directly and indirectly contribute to the pathology and further development and maintenance of multi-drug resistance (Leandro et al., 2019; Riquelme-Alcazar et al., 2020). Future studies could further characterize whether cannabinoids like CBC or CBCA modulate an increase or reduction in ABC transporter expression in the brain following methodologies as described above, as any modulation would present a liability of such treatments.

Like CBCA, phytocannabinoids CBD, Δ^9-THC, CBDA, CBDVA, and CBG have been characterised as ABCB1 and ABCG2 substrates of varying binding character, as well CBD, Δ^9-THC, and CBG exerting inhibitory activity (Anderson et al., 2021b; Holland et al., 2007; Holland et al., 2006) (Table 1.3). Thus, the fact that CBCA uptake could be limited further by epilepsy-induced ABCB1 over-expression at the BBB, and thus, reduce its efficacy to a greater extent. In contrast, CBC itself does not have this character, theoretically making it a more desirable candidate anti-seizure drug. This characterization is also of further importance given ABC transporters grave involvement in drug-drug interactions (Liu, 2019). Further assessments could investigate the pharmacokinetic interactions of CBCA in the presence of other ASM drugs such as clobazam that have been identified to interact with ABC transporters (Nakanishi et al., 2013). This has great clinical relevance as any pharmacokinetic modulation might predict modulation in drug brain concentrations of co-administered anti-seizure substrate drugs that might either lead to their increase and potential toxicity or lowering which would lead to treatment failure. Altogether, this information is of great importance for the potential development of these compounds as therapeutic drugs.

In conclusion, these findings provide important information on basic pharmacological data on these cannabis constituents in their evaluation as next-generation cannabinoid anti-seizure agents as well as advancing on the potential role of ABC transporters in their anti-seizure properties. Altogether, the described future directions could elaborate the knowledge of pharmacokinetic and pharmacodynamic

properties of cannabinoids CBC and CBCA and consequently assist in their development as therapeutic drugs.

5.2.3 Conclusion

The research presented in this thesis provides novel information about the pathophysiology of Dravet syndrome, which helps to inform future therapeutic development. This study is the first to describe HexCer species alterations in the *Scn1a*$^{+/-}$ model of Dravet syndrome, identifying them as potential contributing factors to the F1 strain seizure-susceptibility, with an additional possible modulation driven by the *Scn1a* mutation and seizure-presentation. However, the conflicting findings across the untargeted and targeted approaches needs to be reconciled in future research. Additionally, two phytocannabinoids with lipid-like properties and anti-seizure activity, CBC and CBCA, were used to explore the role of ABC transporters as a target for the treatment of Dravet syndrome and further characterize their pharmacological properties for their potential development as a therapeutic. We report for the first time that CBCA is an ABCB1 substrate, but not an ABCG2 substrate, CBC was not a substrate of either transporter, and that neither CBCA nor CBC show inhibitory activity on either transporter. These findings suggest CBCA's interaction with ABC transporters as a potential treatment pathway, and postulates CBC as a desirable lipid candidate whose scaffold could be used for further drug development. It is hoped that the results presented in this thesis will help advance the knowledge of Dravet syndrome pathophysiology and cannabinoids pharmacology and thus advance the development of novel treatments for refractory epilepsies which to this day stand with great unmet clinical need.

Chapter 6

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