Preclinical validation of endocannabinoid system drug targets for the treatment of Dravet syndrome

Dilara Bahceci

A thesis submitted to fulfil requirements for the degree of Doctor of Philosophy

Faculty of Medicine and Health
The University of Sydney
2020
Statement of originality and author attribution

This thesis is submitted to the University of Sydney in fulfilment of the requirements for the Degree of Doctor of Philosophy. This thesis has not been submitted for any degree or other purposes. I certify that to the best of my knowledge the content of this thesis is the product of my work and that all the assistance received in preparing this thesis and sources have been acknowledged.

The investigations in this thesis were carried out at the University of Sydney, specifically within the Brain and Mind Centre, the Discipline of Pharmacology and the Brain and Mind Centre Animal Facility unless otherwise specified. I completed the majority of this thesis; any assistance is detailed below for specific experiments.

For the work in Chapter 2 and Chapter 3, I designed the experiments with A/Prof Jonathon Arnold and Dr Lyndsey Anderson and conducted the majority of the experimental work. I performed the statistical analyses and interpretations of all data. I solely conducted all western blot studies. The quantification of mRNA with ddRT-PCR was conducted by Dr Nicole Hawkins, under the direction from A/Prof Jennifer Kearney from Northwestern University, IL, USA, on the request of A/Prof Arnold, Dr Anderson and I. For the analyses of endocannabinoid and LPI concentration, I solely collected and dissected tissue samples, and prepared samples for analysis. HPLC/MS/MS analyses and data analyses were conducted with the assistance of Dr Jordyn Stuart. For the work in Chapter 2, Mr Peter Doohan performed the hyperthermia-induced seizure experiments with ABX-1431 and GAT229, including analytical analyses, under the supervision of A/Prof Arnold, Dr Lyndsey Anderson and I. For the experimental work in Chapter 3, for the Gpr55 genetic deletion investigations, A/Prof Arnold designed the experiments and I solely conducted all experimental work except for the quantification of spontaneous seizures, which was completed by Dr Lyndsey Anderson.

For the publication in Chapter 4, I was primary author and solely designed and conducted all behavioural experiments under the supervision of A/Prof Arnold. I analysed all of the behavioural measures except for the manual scoring, which was conducted by Miss Cassandra Ocelli Hanbury-Brown under my supervision. Chapter 4 has been published upon submission of this thesis (Bahceci et al., 2019). A/Prof Jonathon Arnold is corresponding author and has granted permission to include the published material.

For the generation of manuscripts for all chapters I undertook statistical analysis and writing of the manuscripts introduction, methods, results and discussion under the guidance and feedback from of my supervisor A/Prof Jonathon Arnold and with feedback from Dr Lyndsey Anderson.

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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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Acknowledgements

They say it takes a village to raise a child, and I believe this sentiment rings true for the completion of a PhD thesis. Although I am the sole author on this document, this thesis would not have been possible without the input of countless people, many who will have to go unnamed but not unappreciated.

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Lastly, I offer my gratitude to the mice that contributed to this research. Thank you.
HAVE YOU BEEN HELPED, SIR?

BY SO MANY PEOPLE MY ENTIRE LIFE
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Abbreviations

129 129S6/SvEvTac
2-AG 2-arachidonoylglycerol
ABHD12 α/β-hydrolase-12
ABHD6 α/β-hydrolase-6
ACEA arachidonyl-2′-chloroethylamide
AEA N-arachidonoylethanolamide, anandamide
AGPAT8 1-acylglycerol-3-phosphate acyltransferase 8
ANOVA analysis of variance
ATF-2 activating transcription factor 2
B6 C57BL/6
cAMP cyclic adenosine monophosphate levels
CB1 cannabinoid receptor 1
CB2 cannabinoid receptor 2
CBC cannabichromene
CBD cannabidiol
CBE cannabielsoin
CBG cannabigerol
CBL cannabicyclol
CBN cannabinol
CBND cannabinodiol
CBT cannabitriol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CE</td>
<td>collision energy</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>DAG</td>
<td>diacylglycerols</td>
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<td>DAGL</td>
<td>diacylglycerol lipase</td>
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<td>ddRT-PCR</td>
<td>digital-drop real-time PCR</td>
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<tr>
<td>DHEA</td>
<td>docosahexaenylethanolamide</td>
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<tr>
<td>DI</td>
<td>discrimination index</td>
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<tr>
<td>DSI/DSE</td>
<td>depolarization-induced suppression of inhibition/excitation</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EEG</td>
<td>electroencephalogram</td>
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<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinase</td>
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<td>ESI+</td>
<td>electrospray ionization in positive mode</td>
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<tr>
<td>FAAH</td>
<td>fatty acid amine hydrolase</td>
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<tr>
<td>FABP5</td>
<td>fatty-acid binding protein 5</td>
</tr>
<tr>
<td>GEFS+</td>
<td>generalised epilepsy with febrile seizures plus</td>
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<td>GIRK</td>
<td>inwardly-rectifying potassium channels</td>
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<td>GPR55</td>
<td>G protein-coupled receptor 55</td>
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<td>GTCS</td>
<td>generalized tonic-clonic seizure</td>
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<tr>
<td>HPLC/MS/MS</td>
<td>high-performance liquid chromatography tandem mass spectrometry</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>ILAE</td>
<td>International League Against Epilepsy</td>
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<td>IPSC</td>
<td>induced pluripotent stem cell</td>
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<tr>
<td>LEA</td>
<td>dihomo-γ-inolenylethanolamine</td>
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LPI  L-α-lysophosphatidylinositol
LPIAT1  LPI-acyltransferase-1
MAGL  monoacylglycerol lipase
MAPK  mitogen-activated protein kinase
MBOAT7  membrane-bound O-acyltransferase 7
MES  maximal electroshock
MRM  multiple reaction monitoring
MSI/MSE  metabotropic-induced suppression of inhibition/excitation
MTBE  methyl tert-butyl ether
NAPE  N-arachidonoyl phosphatidylethanolamine
NAPE-PLD  N-acyl phosphatidylethanolamine phospholipase D
Nav  voltage-gated sodium
Nav1.1  voltage-gated sodium channel, type I, alpha subunit
NFAT  nuclear factor of activated T-cells
NF-κβ  nuclear factor kappa-light-chain-enhancer of activated B cells
NHMRC  National Health and Medical Research Council
NMDA  non-N-methyl-D-aspartate
OEA  oleoylethanolamine
P  postnatal day
PACAP-27  pituitary adenylate cyclase activating peptide-27
PAM  positive allosteric modulator
PA-PLA₁  phosphatidic acid-preferring phospholipase A1
PCR  polymerase chain reaction
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<td>palmitoylethanolamide</td>
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<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>PPI</td>
<td>prepulse inhibition of startle</td>
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<td>PTZ</td>
<td>pentylenetetrazol</td>
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<tr>
<td>RhoA-ROCK</td>
<td>Ras homolog family member A - Rho-associated protein kinase</td>
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<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>SLE</td>
<td>supported-liquid extraction</td>
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<tr>
<td>SSI/SSE</td>
<td>synaptically-evoked suppression of inhibition/excitation</td>
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<td>SUDEP</td>
<td>sudden unexpected death in epilepsy</td>
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<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor potential vanilloid 1</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
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<tr>
<td>$\Delta^8$-THC</td>
<td>($-\Delta^8$-trans-tetrahydrocannabinol)</td>
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<tr>
<td>$\Delta^9$-THC</td>
<td>($-\Delta^9$-trans-tetrahydrocannabinol)</td>
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Abstract

Dravet syndrome is a severe, early-onset epileptic encephalopathy associated with multiple seizure types, behavioural impairments, and significant risk of sudden unexplained death in epilepsy (SUDEP). Patients exhibit poor prognosis due to pharmacoresistance, with no specific therapeutic options for the associated behavioural comorbidities that have a greater impact on patients’ quality of life than seizures. Thus, there is an urgent need for the development of better therapeutic options that more comprehensively address the multiple aspects of Dravet syndrome.

Over 80% of patients with Dravet syndrome carry mutations in the *SCN1A* gene, which disable voltage-gated sodium channels located on inhibitory neurons and lead to global hyperexcitability through disinhibition. The genetic aetiology of Dravet syndrome has been harnessed to develop mouse models that employ heterozygous deletion of *Scn1a* (*Scn1a<sup>+/−</sup>*) and recapitulate many features of the condition, including hyperthermia-induced seizures, spontaneous seizures, premature death and associated comorbidities. Additionally, the *Scn1a<sup>+/−</sup>* mouse model has been pharmacologically validated for the preclinical screening of potential therapeutics.

In Chapters 2 and 3 we utilise a strategy that exploits the background strain-dependent determination of the epilepsy phenotype in a Dravet syndrome mouse model to uncover potential novel drug targets. *Scn1a<sup>+/−</sup>* mice on a 129S6/SvEvTac (129) background display a normal phenotype, whereas forward-crossing 129.*Scn1a<sup>+/−</sup>* mice with C57BL/6 mice to generate *Scn1a<sup>+/−</sup>* mice on a mixed F1(129 x C57BL/6) background strain uncovers the severe seizure phenotype. This implies the presence of phenotype-modifying genes (genetic modifiers) that may serve as therapeutic targets. By comparing gene transcription and functional protein levels between the seizure-resistant and seizure-susceptible strains of mice, differentially-expressed molecular targets are identified as potential genetic modifiers. Then, the phenotype-modifying ability of these
candidate targets can be determined by evaluating their impact on the epilepsy phenotypes in the $Scn1a^{+/−}$ mouse model through pharmacological or genetic manipulation; thus, providing causal evidence for their potential therapeutic significance. A preliminary RNA-seq analysis that compared the transcriptome expression between the seizure-resistant and seizure-susceptible strains highlighted that the genes encoding the cannabinoid type 1 (CB1) receptor ($Cnr1$) and G protein-coupled receptor 55 (Gpr55; $Gpr55$) might be genetic modifiers, as mice on the seizure-susceptible background strain expressed less $Cnr1$ mRNA but more $Gpr55$ mRNA compared to mice on the seizure-resistant background strain.

Drugs targeting the endocannabinoid and Gpr55-lyso phosphatidylinositol (LPI) systems hold potential for treating Dravet syndrome as these systems are known to regulate neuronal excitability. Our investigation of the endocannabinoid system focussed on the CB1 receptor, which is the main cannabinoid receptor of the brain, and its two main endogenous ligands, anandamide and 2-AG. While the Gpr55-LPI system investigations centred around the Gpr55 receptor and its most selective, abundant, potent and efficacious endogenous ligands, 2-arachidonoyl LPI and 1-stearoyl LPI. Enhanced endocannabinoid signalling is typically associated with reduced neuronal excitability, whereas increased Gpr55 signalling was shown to enhance neuronal excitability. Moreover, potentiating endocannabinoid signalling is known to have anticonvulsant properties in conventional preclinical seizure models, while there is accumulating evidence to suggest Gpr55 antagonism may be anticonvulsant. Yet, these systems have not been specifically investigated for the treatment of paediatric epilepsies like Dravet syndrome. Therefore, this thesis utilises a $Scn1a^{+/−}$ mouse model of Dravet syndrome to explore the endocannabinoid and Gpr55-LPI systems for novel druggable targets that may have the potential to address not only the seizure phenotypes, but also the behavioural abnormalities associated with Dravet syndrome.
The work in Chapter 2 investigates the endocannabinoid system of a Dravet syndrome mouse model to determine its therapeutic potential. We confirmed that seizure-susceptible mice had reduced hippocampal expression of CB1 receptor mRNA and protein with quantitative PCR (qPCR) and western blotting, respectively. We also measured lower hippocampal concentrations of the endogenous cannabinoid 2-AG in seizure-susceptible mice compared to seizure-resistant mice using high-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS). Moreover, when we examined the hippocampal expression of 2-AG’s metabolising enzymes with qPCR, we identified that mRNA expression of diacetyl glycerol lipase beta (Dagl-β) was selectively increased in the phenotypic F1.Scn1a+/− mice. Dagl-β is the enzyme responsible for synthesising 2-AG in the brain’s resident immune microglial cells, which highlights it as a potential therapeutic target that warrants further investigation. To explore the functional significance of the observed endocannabinoid system alterations, we investigated the phenotype-modifying potential of pharmacologically potentiating endocannabinoid signalling using ABX-1431, a monoacylglycerol lipase (MAGL) inhibitor that increases 2-AG levels, and with GAT229, a CB1 receptor positive allosteric modulator. We demonstrated that both methods of enhancing endocannabinoid signalling had anticonvulsant actions against hyperthermia-induced seizures. The work in Chapter 2 supports that augmenting the endocannabinoid system, putatively through CB1 receptors, has anticonvulsant effects and warrants the further examination of targeting the 2-AG-CB1 receptor axis in the treatment of Dravet syndrome.

The research in Chapter 3 explores the expression and therapeutic captivity of the Gpr55-LPI system in a Dravet syndrome mouse model. We confirmed with qPCR that hippocampal mRNA expression of Gpr55 was significantly greater in mice on the seizure-susceptible background strain, then demonstrated with HPLC/MS/MS that hippocampal concentrations of the
LPI analogues were overall less in the seizure-susceptible strain of mice. Moreover, we observed that heterozygous deletion of Scn1a reduced hippocampal 2-arachidonoyl LPI concentrations in mice specifically on the seizure-resistant but not seizure-susceptible background strain, suggesting potential maladaptation in seizure-susceptible mice. Furthermore, examination of LPI synthetic and degradative enzymes with qPCR revealed that heterozygous deletion of Scn1a downregulated the mRNA expression of membrane Bound O-Acyltransferase Domain Containing 7 (Mboat7), which has been associated with epilepsy and behavioural impairments, and requires further investigation. As selective and brain penetrant Gpr55 antagonists do not exist, Chapter 3 then sought to genetically validate whether downregulation of Gpr55 could influence the seizure and mortality phenotypes of the Dravet syndrome mouse model. We demonstrated that heterozygous deletion of Gpr55 was anticonvulsant against hyperthermia-induced seizure and rescued the premature mortality in the phenotypic Scn1a+/− mice. Therefore, the data presented in Chapter 3 supports Gpr55 as a novel anticonvulsant drug target to pursue in the treatment of Dravet syndrome.

The behavioural comorbidities associated with Dravet syndrome are a detrimental aspect of the condition that are critically unaddressed with current treatment strategies. Therefore, the research in Chapter 4 expands the utility of the Scn1a<sup>tm1Kea</sup> mouse model by characterising behavioural impairments in order to provide a screening platform for the development of drugs that specifically address this aspect of Dravet syndrome. The behavioural phenotypes of other mouse models of Dravet syndrome have been characterised only during adulthood. The work presented in Chapter 4 advances on the other models by characterizing for the first time the behavioural phenotype during adolescence, which better models paediatric Dravet syndrome patients. The behaviour of adolescent Scn1a<sup>+/−</sup> and WT mice were compared across several
behavioural domains that as assessed motor function (open field test), sociability and social recognition memory (three-chambered social preference and social interaction tests), memory function (novel object recognition, Barnes maze, fear conditioning paradigm), anxiety-related behaviour (elevated plus maze and open field thigmotaxis), startle reflex and sensorimotor gating (prepulse inhibition of startle tests), and repetitive compulsive behaviour (marble burying test). We demonstrated that adolescent Dravet syndrome mice displayed increased anxiety-related thigmotactic behaviour, atypical fear expression, blunted acoustic startle responses, and impaired social recognition and spatial memory. This work has established a behavioural assessment platform to investigate the therapeutic potential of pharmacological agents, and to assess the impact of any genetic modifier candidates. The absence of additional and more severe behavioural modalities that have previously been reported for adult Dravet syndrome mice suggests that cognitive decline may be progressive, further supporting the necessity for an early-intervention platform.

Chapter 5 summarises the main findings of this thesis and discusses implications of these findings. Furthermore, we suggest future directions to expand on the work presented in this thesis in order to assist with the development of effective therapies for the management of all aspects of Dravet syndrome.
Chapter 1

Introduction
1. Introduction to cannabis and cannabinoids

‘Cannabis’ is a colloquial term referring to plants from the Cannabis genus, which are commonly treated as a single, highly diverse species. Historically, cannabis has evolved alongside humans for millennia with human interest in the plant arising due to its material, spiritual, recreational and medicinal utilities (Bonini et al., 2018). Cannabis is most commonly known for its psychoactive use and abuse as consumption of cannabis is associated with feelings of relaxation, euphoria, spontaneous laughter, increased sociability and increased appetite. However, cannabis can also induce dissociation, paranoia, psychosis, cognitive impairments and lead to dependence. Due to its propensity to be abused, cannabis use was prohibited in the early 20th century (Bonini et al., 2018). Despite its restriction, cannabis remains the most commonly consumed illicit drug worldwide (Peacock et al., 2018). The legalisation of cannabis for medical and recreational use has been increasing worldwide and attitudes towards cannabis are becoming more positive (Carliner et al., 2017). This has also contributed to the increase in research regarding the medicinal benefits of cannabis, especially for epilepsy.
The 11 subclasses are (−)-Δ9-trans-tetrahydrocannabinol (Δ9-THC), (−)-Δ8-trans-tetrahydrocannabinol (Δ8-THC), cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabinoiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), cannabinol (CBT), and miscellaneous types (no structure). The most prevalent acid components are presented for each type of phytocannabinoid. The letters represent the structural components of CBGA; (A) a resorcinol (benzene-1,3-diol) ring, (B) a terpene moiety, (C) an alkyl chain, and (D) a carboxylic group. The numbers represent the conversion methods.
Investigations into the chemical composition of the cannabis plant has identified over 560 chemical constituents, with 120 being classified as plant-derived cannabinoids (phytocannabinoids) based on structural similarity to (−)-Δ⁹-trans-tetrahydrocannabinol (Δ⁹-THC), the main psychoactive component in cannabis (ElSohly et al., 2017). Phytocannabinoids are defined as products isolated from the cannabis plant that exhibit the typical C21 terpenophenolic skeleton, including derivatives and transformation products. Phytocannabinoids have been categorised into 11 general types (Figure 1): (−)-Δ⁹-trans-tetrahydrocannabinol (Δ⁹-THC), (−)-Δ⁸-trans-tetrahydrocannabinol (Δ⁸-THC), cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabidiol (CBND), cannabinol (CBN), cannabinol (CBN), cannabitriol (CBT), and other miscellaneous types (ElSohly et al., 2017). Identification, separation and synthesis of the phytocannabinoids has allowed investigations into their pharmacology, with Δ⁹-THC and CBD being the best characterised. Δ⁹-THC is the most abundant phytocannabinoid in street cannabis and is responsible for the profound psychotropic effects that drive recreational use. Although it displays therapeutic benefits, its intoxicating effects render it undesirable for clinical use (Dos Santos et al., 2014; Swift et al., 2013). However, CBD and other phytocannabinoids are more desirable therapeutic candidates as they lack psychotropic effects and display improved toxicity while maintaining therapeutic benefits (Mechoulam et al., 2007).

2. Cannabis-based treatments for epilepsy

Historically, the earliest use of cannabis for medicinal purposes was documented to 2700 BCE in Asia, with reports of cannabis improving seizure control (Friedman & Sirven, 2017). The prohibition of cannabis during the early 20th century significantly hindered research into its therapeutic potential. Recently however, research into the medicinal benefits of cannabis and
cannabinoids has reignited, primarily due to the emergence of anecdotal reports claiming dramatic benefits of cannabis-based products for children with treatment-resistant epilepsy (Porter & Jacobson, 2013; Press, Knupp & Chapman, 2015; Suraev et al., 2018; Suraev et al., 2017). One striking example was a case study reporting that the use of cannabis extract oil, high in CBD, drastically reduced the seizure incidence of a child with Dravet syndrome from “50 convulsive seizures per day to now 2–3 nocturnal convulsions per month” (Maa & Figi, 2014).

These anecdotal reports identified that CBD was a commonality in these cannabis products; which led to preclinical and clinical investigation of CBD in severe refractory epilepsies. CBD was shown to significantly reduce seizures when administered in conjunction with conventional anticonvulsant medications in three phase III trials, in addition to demonstrating an acceptable safety profile (Devinsky et al., 2017; Devinsky et al., 2019; Devinsky et al., 2018b). Consequently, CBD has been approved by US and European drug approval agencies for the treatment of refractory childhood epilepsies, including Dravet syndrome and Lennox-Gastaut syndrome. The exact mechanisms by which CBD is improving seizure control has not been ascertained and is likely multifaceted due to CBD’s multimodal nature. One likely mechanism is the potentiation of concurrently administered conventional anticonvulsants. In patients with Dravet syndrome, CBD was shown to elevate plasma concentrations of other anticonvulsants and their metabolites, particularly with clobazam, presumably via inhibition of their metabolising enzymes cytochrome P450s (Geffrey et al., 2015; Morrison et al., 2019). Work from our own lab supported a pharmacokinetic interaction between CBD and clobazam, while also demonstrating a pharmacodynamic interaction through GABA_A receptors in a mouse model of Dravet syndrome (Anderson et al., 2019a). However, further evaluation of the clinical trials assessing adjuvant CBD in refractory paediatric epilepsies suggest that CBD has anticonvulsant activity independent of its
interaction with clobazam (Bialer & Perucca, 2020; Devinsky et al., 2020; Savage et al., 2020). CBD has also demonstrated direct anticonvulsant actions through increasing inhibitory and decreasing excitatory neuronal activity in a Dravet syndrome patient-derived induced pluripotent stem cell (IPSC) model (Sun & Dolmetsch, 2018). Additionally, CBD’s anticonvulsant activity in a mouse model of Dravet syndrome was attributed, at least partially, to inhibition of G-protein receptor 55 (GPR55) (Anderson et al., 2019a; Kaplan et al., 2017). CBD has additional molecular targets that could be contributing to its anticonvulsant efficacy (Table 1) but these yet to be confirmed for Dravet syndrome. Based on the intersection between CBD concentrations observed in patients and the concentrations at which CBD’s pharmacological engagements are observed, functional antagonism of GPR55 receptors, desensitization of transient receptor potential cation channel subfamily V member 1 (TRPV1) receptors and inhibition of adenosine transport are CBD’s mechanisms of action that have been proposed to be responsible for the control of epileptic seizures in humans (Gray & Whalley, 2020). For example, CBD’s anticonvulsant activity in a mouse model of generalised seizures was attributed to its interaction with Trpv1 receptors as CBD exhibited a blunted anticonvulsant response in Trpv1 knock-out mice (Gray et al., 2019). Despite CBD’s efficacy in some cases, not all patients responded to the treatment. Furthering our understanding of the pharmacology behind CBD’s anticonvulsant molecular mechanisms would better inform and direct the development of future cannabis-based anticonvulsants.

CBD is only one of many phytocannabinoids present in the cannabis plant and there is emerging preclinical evidence to show that some of these phytocannabinoids, such as cannabidiolvarin (CBDV), also elicit anticonvulsant actions (Hill et al., 2012; Huizenga, Sepulveda-Rodriguez & Forcelli, 2019). Additionally, there is evidence to suggest that the phytocannabinoids may produce improved anticonvulsant actions in combination rather than individually (Hill et al.,
2013; Pamplona, da Silva & Coan, 2018). These findings suggest that the cannabis plant could hold great therapeutic potential for the treatment of epilepsy and warrants thorough investigation if cannabinoid pharmacology. Many of the cannabinoids elicit their pharmacological actions through the endogenous cannabinoid (endocannabinoid) system. Therefore, there is also a need to improve our understanding of the endocannabinoid system and its role in the aetiology of epilepsy, as this may also yield druggable molecular targets for improved anticonvulsant therapeutics.

3. The endocannabinoid system

The endocannabinoid system has been implicated in many physiological systems; including energy metabolism, cardiovascular and reproductive functions, inflammation, glaucoma, cancer, liver and musculoskeletal disorders, appetite, learning and memory, anxiety, depression, schizophrenia, stroke, multiple sclerosis, neurodegeneration, addiction, and epilepsy (Zou & Kumar, 2018). The endocannabinoid system formally consists of two main receptors, cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2) receptors, and two main endogenous ligands (endocannabinoids), $N$-arachidonoyl-ethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG). However, there are several other receptors, including G protein-coupled receptors, ion channel and nuclear receptors, that also interact with endocannabinoids and thus might be considered part of the broader endocannabinoid system. Given the current thesis is devoted to the study of childhood epilepsy, our overview of the endocannabinoid system will largely be confined to the central nervous system (CNS).

3.1 Cannabinoid receptors 1 and 2

CB1 and CB2 receptors are both seven-transmembrane G protein-coupled receptors that are encoded by the genes $CNR1$ and $CNR2$, respectively. CB1 receptors are most highly expressed
in the brain but also are present in adipose tissue, and peripheral organs at varying levels (Fagerberg et al., 2014). Within the CNS, CB1 receptors are the most abundant G protein-coupled receptor, with highest expression in the basal ganglia, hippocampus, cortex and cerebellum in humans and rodents (Howlett & Abood, 2017).

In the brain, CB1 receptors almost always present on presynaptic GABAergic interneuron synapses while only being found at low density on glutamatergic synapses (Howlett & Abood, 2017; Kano, 2014; Kawamura et al., 2006). Moreover, CB1 receptors are almost exclusively expressed on cholecystokinin-expressing interneurons in relation to parvalbumin- and calretinin- and calbindin-expressing interneurons (Marsicano & Lutz, 1999). Due to this selectivity, despite the higher expression at GABAergic synapses, CB1 receptor-positive interneurons represent a smaller portion of all GABAergic interneurons (10-20%) and therefore there may be more CB1 receptor-sensitive excitatory synapses than CB1 receptor-sensitive GABAergic synapses in the brain (Katona, 2015). There is some evidence to show CB1 receptors are also present at much lower but still functionally-relevant levels on astrocytes, but there is some controversy around this (Oliveira da Cruz et al., 2016; Stella, 2010). CB2 receptors, on the other hand are mainly expressed peripherally in organs associated with the immune system (Fagerberg et al., 2014). The expression of CB2 receptors in the CNS remains controversial with conflicting evidence regarding its low expression on neurons and/or microglia (Chen et al., 2017; Li & Kim, 2017).
Figure 2. The synthesis and metabolism of anandamide and 2-AG, adapted from Parsons and Hurd (2015). Endocannabinoids are synthesised in the post-synaptic neuron in response to activation. Anandamide is synthesised from N-arachidonoyl-phosphatidylethanolamine (NAPE) via phospholipase D (NAPE-PLD), while 2-AG is hydrolysed from diacylglycerol (DAG) via the α and β DAG lipase (DAGL) isoforms. Endocannabinoids travel in a retrograde manner across the synaptic cleft to stimulate CB1 receptors located on presynaptic GABAergic or glutamatergic neurons. Endocannabinoid signalling is terminated with the degradation of the endocannabinoids after cellular uptake. 2-AG is hydrolysed predominantly with monoacylglycerol lipase (MAGL) but also with α/β-hydrolase domain-6 (ABHD6) into glycerol and arachidonic acid (AA). Whereas, anandamide is hydrolysed by fatty acid amide hydrolase (FAAH) into ethanolamine and AA.

### 3.2 Endocannabinoids

The arachidonic acid derivatives anandamide and 2-AG are the best characterised endocannabinoids. Anandamide was identified in 1992 and was named based on the Sanskrit word for ‘bliss’ (Devane et al., 1992). When assessed pharmacologically, anandamide did not replicate all the effects of Δ⁹-THC. This prompted explorations for other endocannabinoids, which led to
the discovery of 2-AG in 1995 (Mechoulam et al., 1995). A schematic depiction of endocannabinoid synthesis and metabolism can be found in Figure 2. Within the CNS, endocannabinoid synthesis occurs in the postsynaptic neuron, in an ‘on-demand’ or ‘activity-dependent’ manner as it is triggered by increased intracellular Ca\(^{2+}\) concentrations (Di Marzo et al., 1994). Anandamide is synthesized from N-arachidonoyl phosphatidylethanolamine (NAPE) through cleavage by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) (Di Marzo et al., 1994). While 2-AG is synthesized from diacylglycerol (DAG) by the diacylglycerol lipases (DAGLs), DAGL-\(\alpha\) and DAGL-\(\beta\) (Murataeva, Straiker & Mackie, 2014). Although both isoforms synthesise 2-AG, the \(\alpha\) isoform is the predominant synthesiser as the \(\beta\) isoform is predominantly expressed on microglia (Viader et al., 2016), and Dagl-\(\beta\) knock-out mice produce normal global 2-AG concentrations and exhibit normal endocannabinoid signalling (Tanimura et al., 2010; Wilkerson et al., 2016).

Following intracellular synthesis, the endocannabinoids pass through the plasma membrane with evidence to support both passive diffusion and active transport (Chicca et al., 2012; Fowler, 2013; Nicolussi & Gertsch, 2015). Endocannabinoids then undergo ‘retrograde signalling’, whereby they travel back across the synapse, at least partially transported by protein fatty-acid binding protein 5 (FABP5) (Haj-Dahmane et al., 2018), to bind to CB1 receptors located on the presynaptic neuron. Afterwards, the endocannabinoids enter the presynaptic cell where they are rapidly catabolized by several intracellular enzymes. The degradation of the two endocannabinoids is also achieved via distinct pathways. Monoacylglycerol lipase (MAGL) is the enzyme responsible for \(~85\%\) of 2-AG’s hydrolysis into arachidonic acid and glycerol, while \(\alpha/\beta\)-hydrolase-6 (ABHD6) and \(\alpha/\beta\)-hydrolase-12 (ABHD12) account for \(~9\%\) and \(~4\%\) of the degradation, respectively (Blankman, Simon & Cravatt, 2007). Anandamide on the other hand is
hydrolysed predominantly by fatty acid amine hydrolase (FAAH) into arachidonic acid and ethanolamine (Cravatt et al., 1996). FABP5 has the additional function of assisting anandamide’s degradation due to intracellular shuttling of anandamide to FAAH (Bjorklund et al., 2014; Kaczocha et al., 2014).

Anandamide and 2-AG display sophisticated selectivity in their regulation of several brain functions. For example, through the use of selective gene-deletion mouse models and pharmacological studies, it has been established that 2-AG is the main endocannabinoid responsible for the retrograde suppression of neural activity and synaptic plasticity, while anandamide serves a more “fine-tuning” function (Luchicchi & Pistis, 2012). For this reason, 2-AG is sometimes referred to as the “main cannabinoid”. This notion is supported by 2-AG being present in concentrations that are orders of magnitude more abundant than anandamide; along with 2-AG displaying full agonism at CB1 receptors, while anandamide is a partial agonist at CB1 receptors (Sugiura et al., 2006).

Additional to the endocannabinoids exist structurally related fatty acids amides, including oleoylethanolamine (OEA) and palmitoylethanolamide (PEA), which can also influence neuronal activity. PEA, for example, has been shown to increase concentrations and efficacy of anandamide and 2-AG, while also displaying intrinsic anticonvulsant properties (Iannotti, Di Marzo & Petrosino, 2016; Petrosino et al., 2016; Post et al., 2018).

### 3.3 CB1-mediated signalling

Both CB1 and CB2 receptors predominantly couple to the Gαi/o proteins (Zou & Kumar, 2018). CB1 receptors, however, have also been shown to couple to Gαz, Gαq/11, and Gα12/13 in certain cell types and under certain conditions (Diez-Alarcia et al., 2016; Prather et al., 2000).
Cannabinoid receptor signalling mediated by Ga\textsubscript{i/o} proteins leads to inhibition of adenylyl cyclase, which suppresses cellular cyclic adenosine monophosphate (cAMP) levels and consequently the activity of protein kinase A (Howlett et al., 2002). In addition, Ga\textsubscript{i/o} is linked to activation of inwardly-rectifying potassium channels (GIRK) or mitogen-activated protein kinase signalling cascades (Howlett et al., 2002). Moreover, presynaptic CB1 receptors coupled to Ga\textsubscript{i/o} inhibit N-type and P/Q type voltage-gated calcium channels. Overall, these actions reduce membrane excitability, which in turn inhibits the release of neurotransmitters such glutamate and GABA into the synapse (Farrell & Soltesz, 2019; Soltesz et al., 2015).

Interestingly, CB1 receptors are more efficiently coupled to G proteins at glutamatergic synapses than at GABAergic synapses (Steindel et al., 2013), implying that despite the receptor expression data demonstrating greater CB1 receptor expression on GABAergic synapses, CB1 receptor-mediated control over glutamatergic synapses may be stronger. This is supported by numerous accounts where stimulation of CB1 receptors resulted in suppression of excitatory transmission in the hippocampus (Kawamura et al., 2006; Sugaya et al., 2016; Takahashi & Castillo, 2006). Auxiliary to Ga\textsubscript{i/o}-mediated signalling, cannabinoid receptors activation also associate with β-arrestins 1 and 2, which divert signalling along β-arrestin-mediated pathways and are responsible for desensitisation and internalisation of the receptors (Zou & Kumar, 2018). Interestingly, CB1 receptors exhibited biased signalling patterns dependent on the ligand bound (Diez-Alarcia et al., 2016). The ligand-specific biased signalling, in conjunction with the varied cellular and regional expression of cannabinoid receptors, demonstrates how the endocannabinoid system can elicit specific regulation and homeostasis of multiple biological functions.

As activation of CB1 receptors reduce neuronal excitability and consequently neurotransmitter release in an activity-dependent manner, the endocannabinoid system essentially
functions as a “circuit-breaker” in the brain, regulating and dampening excess neuronal activity where needed. The endocannabinoid system has been shown to mediate multiple forms of synaptic plasticity that may explain its role in controlling learned behaviour, memory function and neuroadaptive changes that might occur as a result of disease including brain hyperexcitability observed in epilepsy. There are multiple forms of synaptic plasticity that have been characterised to be mediated by endocannabinoid-mediated retrograde signalling in response to different patterns of neural stimulation. The first type is depolarization-induced suppression of inhibition/excitation (DSI/DSE), which is the transient suppression of neurotransmitter release in response to strong neuronal activation which suppresses inhibitory or excitatory signalling dependent on the synapse (Kreitzer & Regehr, 2001; Pitler & Alger, 1992). Interestingly, inhibitory synapses are more sensitive to depolarization-induced suppression of synaptic transmission than excitatory synapses (Ohno-Shosaku et al., 2002). Other forms of retrograde signalling are short-term depression, which is also referred to as metabotropic-induced suppression of inhibition/excitation (MSI/MSE) or synaptically-evoked suppression of inhibition/excitation (SSI/SSE) (Kano et al., 2009). Finally, endocannabinoids also mediate long-term depression, which is a long-lasting inhibition of synaptic plasticity at both excitatory and inhibitory synapses (Chevaleyre & Castillo, 2003; Gerdeman, Ronesi & Lovinger, 2002). This neural-regulating function of CB1-mediated signalling suggests it may be useful in dampening the neuronal hyperactivity elicited by epileptic brains.

3.4 G protein-coupled receptor 55 (GPR55)

As mentioned earlier, there are additional receptors to CB1 and CB2 receptors that show cannabinoid reactivity. GPR55 is the receptor with the most evidence to support its role in the endocannabinoid system and will be discussed in detail. GPR55 was originally referred to as the third cannabinoid receptor due to its affinity and activation by cannabinoids with no known
endogenous ligand at the time (Ryberg et al., 2007). Interestingly, GPR55 binds to cannabinoids despite only sharing 13.5% and 14.4% sequence homology with CB1 and CB2 receptors, respectively (Ross, 2009). Additionally, the formation of CB1-GPR55 heteromers supports a strong interaction between CB1 and GPR55 receptors and further implicates a place for GPR55 in the broader endocannabinoid system (Kargl et al., 2012; Marichal-Cancino et al., 2013; Martinez-Pinilla et al., 2014).

However, GPR55 left orphan status with the discovery of its endogenous ligand, the lipid signalling molecule L-α-lysophosphatidylinositol (LPI) (Oka et al., 2007; Oka et al., 2009), which does not react at cannabinoid receptors (Kapur et al., 2009). This discovery established the GPR55-LPI system, which has since been implicated in a number of pathologies. The peripheral physiological functions of GPR55 receptors have been more extensively studied, with GPR55 being investigated as a target for vasculature health, metabolism, gastrointestinal function, bone health, inflammation, cancer and pain (Alhouayek, Masquelier & Muccioli, 2018; Simcocks et al., 2018). Centrally, GPR55 is currently being investigated for its function in anxiety, Parkinson’s disease and pain (Marichal-Cancino et al., 2017).

GPR55 has also been shown to be activated by the “endocannabinoid-like” fatty acids PEA and OEA, and by various peptides, most notably pituitary adenylate cyclase activating peptide-27 (PACAP-27) (Foster et al., 2019; Ryberg et al., 2007). However, the full implications and physiological functions of these endogenous ligands at this receptor are unknown as our understanding of GPR55’s function and its role in various physiological processes are still developing. Unsurprisingly, both Δ⁹-THC and CBD have affinity for the GPR55 receptor, but while Δ⁹-THC activates GPR55, CBD was an antagonist at GPR55 (Lauckner et al., 2008; Ryberg et al., 2007; Whyte et al., 2009). Moreover, there are also numerous synthetic compounds that
modulate GPR55, including CBD derivatives and synthetic cannabinoid agonists (Simcocks et al., 2018). Although selective agonists and antagonists have been developed for GPR55, they do not readily cross the blood brain barrier, which further limits investigation of GPR55’s CNS function (Heynen-Genel et al., 2010; Kargl et al., 2012; Kotsikorou et al., 2013).

GPR55 receptors have been shown to couple to Ga_{12/13} and/or Ga_{q} proteins in a ligand-dependent manner and activate intracellular cascades that have functionally opposite outcomes to CB1/CB2 receptors (Henstridge et al., 2009; Lauckner et al., 2008; Ryberg et al., 2007). Stimulation of the Ga_{12/13} subunit activates the Ras homolog family member A - Rho-associated protein kinase (RhoA-ROCK) pathway. This stimulates the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and then subsequently increases phosphorylation of activating transcription factor 2 (ATF-2). ROCK also activates phospholipase C (PLC), which increases intracellular Ca^{2+} concentrations and triggers Nuclear factor of activated T-cells (NFAT)-induced nuclear translocation (Henstridge et al., 2009; Lauckner et al., 2008; Oka et al., 2010; Ryberg et al., 2007). Alternatively, Ga_{q}-mediated PLC activation results in the release of diacylglycerol and Ca^{2+}, which result in phosphorylation of protein kinase C and extracellular signal-regulated kinases (ERK_{1/2}) and subsequently activates cAMP-response element binding protein (CREB) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) (Henstridge et al., 2009; Lauckner et al., 2008). Moreover, GPR55 also exhibits β-arrestin-mediated receptor internalization (Kapur et al., 2009). Generally, stimulation of GPR55 and activation of these G-protein-mediated pathways ultimately increase cell excitability and alter gene expression.

Recently, there has been evidence showing that GPR55 can modulate neuronal excitability, suggesting that GPR55 may hold therapeutic potential in the treatment of epilepsy. Peripherally, GPR55 receptors are located in the stomach, intestines, spleen and bone marrow; while in the
brain, GPR55 is expressed in the basal ganglia, hippocampus, hypothalamus, frontal cortex and cerebellum (Henstridge et al., 2011; Ryberg et al., 2007; Wu et al., 2013). Centrally, GPR55 has been shown to be expressed on both neurons and glia, with hippocampal GPR55 located presynaptically on glutamatergic neurons (Kallendrusch et al., 2013; Ryberg et al., 2007; Sylantyev et al., 2013). The sub-cellular locations or knowledge of GPR55 receptor distribution on specific neuron types in other regions of the brain has not yet been characterised.

Based on GPR55 distribution in the hippocampus and its activation resulting in increased cell excitability, it is consistent that GPR55 activation promoted neural excitability and glutamate release in the hippocampus (Hurst et al., 2017; Kramar et al., 2017; Sylantyev et al., 2013). In contrast, PEA enhanced GABAergic transmission in the striatum in a GPR55-dependent manner, as determined with electrophysiological recordings from mouse brain slices (Musella et al., 2017). This contrasting effect may be due to GPR55 being expressed on GABAergic neurons in the striatum but is yet to be investigated and confirmed. The functional outcome of GPR55 activation in other brain regions is yet to be determined, but GPR55 does not appear to be a receptor critical for neuronal function based on Gpr55 deletion (Gpr55−/−) mouse models. Gpr55−/− mice exhibited intact short-term and long-term synaptic plasticity (Hurst et al., 2017; Wu et al., 2013). Moreover, when assessed in a comprehensive array of behavioural tests, Gpr55−/− mice exhibited reduced locomotor activity and metabolism, while displaying comparable performance in cognitive and mood-related behaviours (Bjursell et al., 2016; Wu et al., 2013).

Despite GPR55 not displaying a critical role in neural functioning, it may still hold therapeutic potential as the GPR55-LPI system may be altered in disease states and because seizure activity can be attenuated by altering metabolism and mediating inflammation, other GPR55-mediated functions (Clanton et al., 2017; Dey et al., 2016; Korff & Dale, 2017; Rho, 2017;
Simeone, Simeone & Rho, 2017). Moreover, CBD was recently demonstrated to have anticonvulsant activity against multiple seizure types in two mouse models of Dravet syndrome, and CBD’s anticonvulsant efficacy was suggested attributed, at least partly, to antagonism of GPR55 (Anderson et al., 2019a; Kaplan et al., 2017). Hence, GPR55 may be contributing to the endocannabinoid system’s control of neurotransmission and it may be implicated in the pathophysiology of epilepsy. Therefore, GPR55 will be investigated as potential anticonvulsant target for Dravet syndrome in this thesis.

4. Targeting the endocannabinoid system for epilepsy

Due to the endocannabinoid system functioning as a regulator of neuronal transmission, especially in an activity-dependent manner, it is not surprising that the endocannabinoid system could play a role in the pathophysiology and subsequently the treatment of epilepsy disorders. There is evidence to suggest pathophysiological reorganisation of the endocannabinoid system in epilepsy conditions (De Caro et al., 2017; Katona, 2015). For example, reduced mRNA expression of the 2-AG synthesising DAGL-α was observed in post-mortem hippocampal sections from patients with temporal lobe epilepsy, suggesting patients had reduced 2-AG concentrations (Romigi et al., 2010). Additionally, temporal lobe epilepsy patients also exhibited significantly lower anandamide concentrations in cerebrospinal fluid, and significantly downregulated hippocampal CB1 receptor mRNA, specifically in glutamatergic synapses (Ludanyi et al., 2008). Moreover, variants of the gene encoding DAGL-α were found to be significantly associated with seizures and neurodevelopmental disorders (Smith et al., 2017), implicating 2-AG alterations in the pathophysiology of epilepsy conditions.
Animal models of seizure and epilepsy have also provided evidence to suggest that the endocannabinoid system is altered by seizures. Seizure activity appears to upregulate CB1 receptor expression in the hippocampus, as elevated hippocampal CB1 protein expression was reported one year after pilocarpine-induced seizures in one study and 3 h after kainic acid-induced seizures in another study (Bojnik et al., 2012; Wallace et al., 2003). Moreover, hyperthermia-induced seizures also upregulated CB1 receptors in the hippocampus of juvenile rats, and upregulation was shown to be specific to interneurons (Chen et al., 2007; Chen et al., 2003; Feng et al., 2016). Interestingly, CB1 receptor activation was critical for the adulthood seizure-susceptibility that developed in response to hyperthermia-induced seizures, suggesting that CB1 receptor activation can promote epileptogenesis and potentially be proconvulsant (Chen et al., 2007; Feng et al., 2016). This may be a barricade to the development CB1 receptor-related therapeutics for epilepsy that requires careful consideration and exploration when pursuing cannabinoid-related therapeutics.

Changes in endocannabinoid concentrations in response to seizures seem to be more varied and this may be due to the activity-dependent, transient nature of endocannabinoid signalling. For example, kainic acid-induced seizures have been associated with increased hippocampal concentrations of only anandamide, both anandamide and 2-AG or neither endocannabinoid in rats (Lerner et al., 2017; Marsicano et al., 2003; Wettshureck et al., 2006). Elevated hippocampal 2-AG concentrations have also been reported in rats shortly after pilocarpine-induced seizures (Wallace et al., 2003). Moreover, age also seems to influence the endocannabinoid system’s response to seizure. One study demonstrated age-dependent alteration in endocannabinoid levels in response to kainic acid-induced seizures, with P14 rats exhibiting increased hippocampal anandamide and 2-AG levels, while adult rats had reduced anandamide and 2-AG levels (Fezza et al., 2014). Although this does complicate the story, it also suggests that there may be beneficiary
effects of endocannabinoid system modulation specific to certain aged population, i.e. paediatric vs adulthood epilepsy disorders.

The role of CB1 receptors in seizure aetiology has been investigated using both genetic and pharmacological methods. CB1 receptor knock-out mice do not display innate spontaneous seizures (Rowley et al., 2017) but have displayed increased susceptibility to and severity in kainic acid-induced and pilocarpine-induced seizure models (Kow et al., 2014; Marsicano et al., 2003; Sugaya et al., 2016). Moreover, the specific deletion of CB1 receptors in the hippocampus was sufficient to increase susceptibility to kainic acid-induced seizures, highlighting the integral role of cannabinoid signalling in the hippocampus (Monory et al., 2006). Consistent with the CB1 receptor-mediated “circuit-breaking” function was the finding that conditional deletion of CB1 receptors from glutamatergic but not GABAergic neurons increased seizure susceptibility in a kainic acid-induced seizure model and a kindling model of temporal lobe epilepsy (Monory et al., 2006; von Ruden et al., 2015b). Interestingly, despite greater CB1 receptor expression at inhibitory synapses, global CB1 receptor deletion exacerbates seizures, suggesting that CB1 receptors seem to have a stronger effect at glutamatergic synapses.

Considering endocannabinoid signalling functions as a “circuit-breaker” in conjunction with the CB1 receptor deletion studies demonstrating proconvulsant effects, there is considerable evidence to support the anticonvulsant potential of potentiating endocannabinoid signalling. Thus, there is a plethora of preclinical evidence to suggest that modulation of the endocannabinoid system may hold anticonvulsant potential and was reviewed in detail by Rosenberg, Patra and Whalley (2017). Due to the multi-component nature of the endocannabinoid system, there are numerous pharmacological approaches that could be employed to augment endocannabinoid signalling. The most obvious approach is direct CB1 receptor agonism. In experimental acute models of seizure
and chronic models of epileptogenesis, CB1 receptor agonism typically had anticonvulsant effects. Rosenberg et al. (2017) summarised that CB1 receptor-activating drugs, including Δ⁹-THC, displayed anticonvulsant actions in 34 of the 50 assessments (68.0%) in acute seizure models such as the maximal electroshock and pentylenetetrazol models (Rosenberg, Patra & Whalley, 2017). CB1 receptor-activating drugs were also anticonvulsant in 25 of the 34 incidences (73.5%) testing in models of chronic epileptogenesis, including the pilocarpine-induced and amygdala kindling models (Rosenberg, Patra & Whalley, 2017). For example, the CB1 receptor agonist WIN55,212 was anticonvulsant in the maximal electroshock and pentylenetetrazol acute models of seizure and in the pilocarpine model of status epilepticus in rodents (Suleymanova et al., 2016; Vilela et al., 2013; Wallace et al., 2001). Interestingly, CB1 receptor agonism had mixed effects on seizure outcomes in 4 of the acute seizure assessments and 2 of the chronic seizure models. For example, WIN55,212 was anticonvulsant at a low dose (0.5mg/kg), but proconvulsant at a higher dose (5 mg/kg) in a kainic acid seizure model (Rudenko et al., 2012). Moreover, there were no significant effects in 7 of the acute seizure model assessments and 5 of the chronic epilepsy assessments (Rosenberg, Patra & Whalley, 2017).

Although CB1 receptor agonists mostly proved anticonvulsant, there were incidences of CB1 receptor agonism displaying proconvulsant activity. For example, the selective CB1 receptor agonists WIN55,212 and arachidonyl-2′-chloroethylamide (ACEA), reduced seizure thresholds and increased seizure duration in the pentylenetetrazol seizure model in rats (Vilela et al., 2013). Likewise, Δ⁹-THC reduced latency to pentylenetetrazol-induced seizures in pregnant female mice and reduced the threshold to seizure in the 60 Hz electroshock model (de Salas-Quiroga et al., 2015; Karler & Turkanis, 1980). More recently, synthetic cannabinoid receptor agonists (SCRAs) have become popular drugs of abuse that have been associated with serious toxicity, including
seizures (Adamowicz et al., 2017; Louh & Freeman, 2014; Schep et al., 2015). Pharmacological evaluation of these compounds in mice confirmed that they do indeed display intrinsic proconvulsant activity and increase susceptibility to seizures through CB1 receptor agonism (Funada & Takebayashi-Ohsawa, 2018; Kevin et al., 2019; Malyshevskaya et al., 2017; Wilson et al., 2019).

Due to the potential CB1 receptor-mediated toxicity, additional methods of promoting endocannabinoid signalling for anticonvulsant purposes are being investigated. One approach is to increase endocannabinoid concentrations, which can be achieved through inhibition of anandamide’s and 2-AG’s hydrolysing enzymes, FAAH and MAGL/ABHD6, respectively; along with inhibition of anandamide’s synaptic re-uptake. Again, from the afore-mentioned review by Rosenberg et al. (2017), pharmacologically increasing endocannabinoid concentrations was generally found to be anticonvulsant, with 9 out of 16 cases showing endocannabinoid-enhancing drugs to have anticonvulsant effects in acute seizure models. Moreover, these agents displayed mixed effects in 2 cases and had no significant effect in 5 cases of the total 16 assessments in acute seizure models, therefore generally proving anticonvulsant (Rosenberg, Patra & Whalley, 2017). In chronic models of epilepsy, elevating endocannabinoid concentrations was summarised to be anticonvulsant in 7 cases and proconvulsant in 1 of the total 8 assessments (Rosenberg, Patra & Whalley, 2017). Overall, indirect modulation of CB1 receptors may be a safer approach to augmenting endocannabinoid signalling. Specifically, MAGL inhibitors that elevate 2-AG concentration have been demonstrated to display anticonvulsant effects in pentyleneetrazol, status epilepticus and kindling models of seizure and epilepsy in rodents (Terrone et al., 2018; von Ruden et al., 2015a; Zareie et al., 2018). As the pharmacological activity of MAGL inhibitors are dependent on the presence of 2-AG, MAGL inhibitors theoretically function to enhance the 2-AG-
mediated “circuit-breaking” function. Moreover, as 2-AG is only produced on-demand where neural activity is present, MAGL inhibition has the added advantage of eliciting its actions in a spatiotemporally specific and necessary manner.

Figure 3. Cooperation between the GPCRs orthosteric and allosteric binding sites to induce intracellular protein coupling, from Scott & Kendall, 2017. Schematic illustration showing how binding of the allosteric modulator to an allosteric binding site distinct from the orthosteric binding site may influence binding of the orthosteric ligand to its respective site and how both ligands may impact binding of intracellular proteins to their respective binding sites. β-Arrestin binding can involve different isoforms not indicated and other G protein isoforms may also be impacted. Further, some ago-allosteric modulators can function without an orthosteric compound.

More recently, the development of CB1 receptor positive allosteric modulators (PAMs) has opened up another avenue of endocannabinoid system modulation that avoids direct CB1 receptor agonism and is yet to be assessed for its anticonvulsant potential. Allosteric modulators
act by binding to an allosteric site, i.e. at a site distinct from the orthosteric site, that theoretically has no intrinsic efficacy but induces conformational changes in the receptor that increases the affinity and/or intrinsic efficacy of an orthosteric ligand (Figure 3; Price et al., 2005).

This pharmacological approach is therapeutically superior to CB1 receptor agonism because, like MAGL inhibitors, it should enhance the endocannabinoid system’s intrinsic “circuit-breaking” function in an on-demand, region specific manner, whilst avoiding adverse off-target effects or overstimulation. This has been demonstrated by CB1 receptor PAMs, ZCZ011 and GAT229, reducing neuropathic pain in mouse models without inducing cannabimimetic adverse effects (Ignatowska-Jankowska et al., 2015; Slivicki et al., 2018). There are no published studies investigating the anticonvulsant potential of CB1 receptor PAMs, but theoretically these hold anticonvulsant potential. Thus, the anticonvulsant potential of positively modulating the allosteric site of CB1 receptors will be investigated in this thesis.

Interestingly, endocannabinoid system modulators have been assessed only in experimental seizure and epilepsy models that use adult rodents and predominantly in models that induce seizures in healthy animals. Due to the developmental changes that occur in the endocannabinoid system (Meyer, Lee & Gee, 2018) and variation of endocannabinoid signalling caused by disease state (Kaur, Ambwani & Singh, 2016), it is possible that endocannabinoid system modulation could have different effects in different patient populations. Thus, there is a need to assess the therapeutic potential of potentiating endocannabinoid signalling in paediatric models of epilepsy, including Dravet syndrome, that will be explored in this thesis.
5. Epilepsy

Epilepsy is the most common, chronic neurological condition with a prevalence rate of 7.6 per 1,000 persons worldwide (Fiest et al., 2017). “Epilepsy” is defined by the International League Against Epilepsy (ILAE) as “a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition” (Fisher et al., 2005). While an “epileptic seizure” is defined as “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain” (Fisher et al., 2005). The definition of “epilepsy” has been updated to be more encompassing and now epilepsy is diagnosed if a condition meets any of the following criteria: “(1) at least two unprovoked (or reflex) seizures occurring > 24 h apart; (2) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; (3) diagnosis of an epilepsy syndrome” (Fisher et al., 2014).
### Focal onset

#### Aware or impaired awareness

<table>
<thead>
<tr>
<th>Motor onset</th>
<th>Non-motor onset</th>
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<tr>
<td>• Automatisms</td>
<td>• Autonomic</td>
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<tr>
<td>• Atonic</td>
<td>• Behavioural arrest</td>
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<tr>
<td>• Clonic</td>
<td>• Cognitive</td>
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<tr>
<td>• Epileptic spasms</td>
<td>• Emotional</td>
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<td>• Hyperkinetic</td>
<td>• Sensory</td>
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<tr>
<td>• Myoclonic</td>
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<td>• Tonic</td>
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#### Generalised onset

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<thead>
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<tr>
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<td>• Atypical</td>
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<tr>
<td>• Clonic</td>
<td>• Eyelid myoclonia</td>
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<tr>
<td>• Tonic</td>
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<tr>
<td>• Myoclonic-tonic-clonic</td>
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<tr>
<td>• Myoclonic-atonic</td>
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<td>• Epileptic spasm</td>
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#### Unknown onset

<table>
<thead>
<tr>
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<th>Non-motor</th>
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<tr>
<td>• Tonic-clonic</td>
<td>• Behavioural arrest</td>
</tr>
<tr>
<td>• Epileptic spasms</td>
<td></td>
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</tbody>
</table>

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**Figure 4.** The expanded ILAE 2017 operational classification of seizure types, modified from Fisher et al. (2017b).
Seizures can present in many different forms and are classified based on initial manifestation then typical features observed during the seizure, as outlined in Figure 5 (Fisher et al., 2017a; Fisher et al., 2017b). Seizures can be divided into three categories based on initial manifestation: focal, generalised and unknown. Focal seizures, formerly called partial seizures, originate in neuronal networks limited to part of one cerebral hemisphere, whereas generalised seizures originate from cortical or subcortical regions of both hemispheres. If the source of seizure activity cannot be identified, then it is classified as having unknown onset. Ancillary to onset, seizures are further classified based on features observed during the seizure. For focal seizures, characterisation is divided based on level of awareness, then on motor or non-motor onset. Whereas for generalised seizures, which are the most commonly observed type of seizure, seizures are divided into motor or non-motor (absence) seizures. Generalised motor seizures can then be categorised as tonic-clonic (formerly referred to as grand mal seizures), myoclonic, atonic or other types that are less common. Tonic-clonic seizures involve bilateral stiffening of limbs during the tonic phase, followed by repetitive, rhythmic convulsions of muscles during the clonic phase. Myoclonic seizures, on the other hand, consist of sudden, brief movements that can involve contraction of one muscle or several muscles. Whereas, atonic seizures, also referred to as “drop seizures”, involve loss of body tone and can result in falls. Finally, generalised non-motor seizures, which are more commonly referred to as absence seizures and were formerly known as petit mal seizures, manifest as episodes of unresponsiveness or lapse of consciousness and can occur with head nodding or eye blinking.

Seizures conceptually result from transient imbalance between excitatory (glutamatergic) and inhibitory (GABAergic) activity within a neuronal network that causes abnormal enhanced synchrony, resulting in network dysfunction (Fisher et al., 2005). This imbalance in transmission
can arise from numerous sources. The aetiologies of epilepsy are grouped by the ILAE into six categories: genetic, structural, infectious, metabolic, immune and unknown (Scheffer et al., 2017), with genetic origins remaining as the leading proposed aetiology (Thomas & Berkovic, 2014). Epilepsies are classified as being genetic in origin if the condition results directly from a single (monogenetic) or number of mutations (polygenetic), with genetic mutations being inherited or occurring de novo (Thomas & Berkovic, 2014). Moreover, it should be noted that even epilepsies categorized as acquired, such as those resulting from trauma, stroke, neoplasm, infection or congenital malformations, can have genetic contributions (Thomas & Berkovic, 2014). Majority of the mutations associated with epilepsy disorders occur in genes encoding ion channels, with ion channel gene mutations accounting for approximately 25 to 56% of the mutations identified for paediatric refractory epilepsies (Anwar et al., 2020; Kaplan, Isom & Petrou, 2016; Liu et al., 2018; Oyrer et al., 2018; Wang et al., 2017a).

6. Ion channels

Ion channels are pore-forming membrane proteins that control the flux of ions across cellular membranes. There are many different types of ion channels, each with specific functions that alter neuronal excitability, including establishing action potentials and maintaining homeostasis. Since ion channels are critical to the excitability of neurons, they appear to play an important role in epileptogenesis. Ion channels can be activated by various stimuli including ligands, temperature or mechanically, and those activated by changes in membrane voltage are characterised as voltage-gated ion channels. Then they are characterised by the ions that flux through the channel, which include sodium ions, potassium ions, calcium ions and protons, and the channels can be selective or non-selective for specific ion types. Of all ion channel types,
voltage-gated sodium channels are the biggest contributors to the aetiology of epilepsy (de Lera Ruiz & Kraus, 2015; Kaplan, Isom & Petrou, 2016).

7. Voltage-gated sodium channels

Voltage-gated sodium channels (Na\textsubscript{v}) are responsible for the generation and propagation of action potential in excitable nerves, skeletal muscle and cardiac cells. Structurally, Na\textsubscript{v} channels are a membrane-bound protein complex comprising of one pore-forming α-subunit (260 kDa) bound to one or two smaller, auxiliary β-subunits (30-45 kDa) (Figure 5) (Catterall, 2000; Kaplan, Isom & Petrou, 2016; Meisler & Kearney, 2005; Wang, Ou & Wang, 2017). There are ten different α-subunit proteins (Na\textsubscript{v}1.1-Na\textsubscript{v}1.9 and Na\textsubscript{v}X; encoded by genes SCN1A-SCN5A, SCN8A-SCN11A and SCN7A, respectively) and 5 β-subunits (β1, β1B, β2-β4; encoded by genes SCN1B-SCN4B, with β1B being a splice variant of SCN1B). Structurally, each α-subunit is comprised of four structurally homologous domains (D1-4), which are composed of six transmembrane α helical segments (S1-S6) (Figure 5). The S4 segment act as a voltage sensor as it contains a high concentration of positively charged arginine residues. When assembled into its 3D confirmation, the α-subunit creates an ion-conducting pore, with ion permeability selectivity controlled by the S5-S6 pore loop of each domain (Catterall, 2000; Kaplan, Isom & Petrou, 2016; Meisler & Kearney, 2005; Wang, Ou & Wang, 2017). The β-subunits are auxiliary and modulate the kinetics and voltage-dependence of α-subunits, while also participating in non-conducting roles, such as channel localisation, cell-to-cell and cell-to-matrix interactions, and modulating pharmacological interactions with compounds (Catterall, 2000; Kaplan, Isom & Petrou, 2016; Meisler & Kearney, 2005; Wang, Ou & Wang, 2017).
Figure 5. A schematic representation of the structure of the α and β subunits of voltage-gated sodium channel, adapted from George (2005). The α-subunit, marked in red, is composed of 4 homologous domains (D1-D4), which themselves are comprised of six transmembrane α helical segments (S1-S6). The S4 segment act as a voltage sensor (shown in black) and ion selectivity is determined by the S5-S6 pore loop of each domain, shown in turquoise. The domains assemble in the membrane to form a Na⁺-permeable pore. The auxiliary β subunit is shown in blue.

The α-subunits of voltage-gated sodium channels that are mostly expressed in the CNS are Nav1.1, Nav1.2, Nav1.3, Nav1.5, and Nav1.6, while the Nav1.7, Nav1.8, and Nav1.9 α-subunits are mainly distributed in the periphery (Wang, Ou & Wang, 2017). Within neurons, Nav channels are expressed ubiquitously throughout neuronal architecture but with higher density at the nodes of Ranvier and axon initial segments, supporting their crucial role in action potential initiation and propagation (Catterall, 2000; Kaplan, Isom & Petrou, 2016; Meisler & Kearney, 2005; Wang, Ou & Wang, 2017).

Activation of Nav channels are responsible for the initiation of action potentials and subsequently contribute to the stimulation or activation of neurons. When a neuronal membrane is at its normal resting potential, the pore of Nav channels are in a closed state, ready to be opened.
Once the membrane voltage surrounding the \( \text{Na}_V \) channels becomes positive enough to reach the activation threshold potential, \( \text{Na}_V \) channels undergo conformational change, opening the ion pore and allowing an influx of positively-charged sodium ions into the cell. This causes depolarization of the neuronal membrane, and the voltage of the neuronal membrane surrounding the channels becomes more positive and in turn activates neighbouring \( \text{Na}_V \) channels by the same mechanism. Once enough \( \text{Na}_V \) channels are activated and there is a large influx of sodium ions to surpass the threshold level, an action potential is generated which results in a sharp rise in membrane voltage. Action potentials travels down the neuronal axons to the axon terminal, where they trigger the release of neurotransmitters. Within milliseconds after activation, \( \text{Na}_V \) channels close and enter an inactivated state. During inactivation, \( \text{Na}_V \) channels are non-responsive to membrane voltage and are responsible for the refractory period of action potentials, during which it is impossible for another action potential to occur (Catterall, 2000; Kaplan, Isom & Petrou, 2016; Meisler & Kearney, 2005; Wang, Ou & Wang, 2017). As \( \text{Na}_V \) channels play such a crucial role in activating and regulating neuronal excitability, it is no surprise that changes in the function of these channels by mutation or drug action can influence global neuronal activity. As mentioned earlier, mutations in genes encoding \( \text{Na}_V \) channels are prevalent in epilepsy, and mutations in \( \text{SCN1A} \), the gene encoding the \( \alpha_1 \)-subunit (\( \text{Na}_{V1.1} \)), constitutes the greatest number of epilepsy-related mutations identified (Kaplan, Isom & Petrou, 2016; Meisler et al., 2001).

### 8. \( \text{SCN1A} \) mutations and \( \text{Na}_{V1.1} \) impairment

Most \( \text{SCN1A} \) mutations identified effect one copy of the gene and render half the \( \text{Na}_{V1.1} \) subunits absent or functionally impaired and are associated with a broad spectrum of epilepsy disorders. Within the brain, \( \text{Na}_{V1.1} \) is expressed almost ubiquitously with relatively high expression observed in the thalamus, superior colliculus, inferior colliculus, deep cerebellar nuclei,
pons, medulla, and spinal cord; whereas lower expression was observed in the hippocampus, cerebral cortex, and cerebellum (Ogiwara et al., 2007). $\text{Na}_V\text{1.1}$ is predominantly distributed on initial axonal segments of parvalbumin-expressing GABAergic inhibitory neurons (interneurons), with some expression on somatostatin-expressing interneurons, calretinin-expressing interneurons and excitatory neurons (Dutton et al., 2013; Ogiwara et al., 2007). As $\text{Na}_V\text{1.1}$ channels are located on GABAergic neurons and are responsible for action potential initiation and propagation, $\text{Na}_V\text{1.1}$ impairment results in impaired sodium currents and neuronal firing, consequently impairing the functioning of GABAergic neurons; as demonstrated by heterozygous deletion of $\text{Scn1a}$ in mice (Kaplan et al., 2017; Rubinstein et al., 2015; Tsai et al., 2015; Yu et al., 2006).

There is evidence to support a genotype-phenotype correlation between the severity or sequence-locations of $\text{SCN1A}$ mutations, which determine $\text{Na}_V\text{1.1}$ protein impairment, and the severity of the epilepsy phenotype observed (Marini et al., 2011). For example, single amino substitutions, which are mostly missense mutations, cause milder $\text{Na}_V\text{1.1}$ impairment and are more frequently associated with the milder form of epilepsy, generalised epilepsy with febrile seizure plus (GEFS+). Whereas nonsense or frame-shift type mutations, that cause truncation of the $\text{Na}_1\text{.1}$ protein, are prevalent in the severe intractable epileptic encephalopathy, Dravet syndrome (Catterall, Kalume & Oakley, 2010; Kaplan, Isom & Petrou, 2016; Marini et al., 2007; Meisler et al., 2001). Moreover, mutations in the pore-forming region of $\text{Na}_1\text{.1}$, a region integral for channel function, are more prevalent in patients with Dravet syndrome than in patients with GEFS+ (Kanai et al., 2004). Further supporting the genotype-phenotype correlation was the report of an infantile epileptic encephalopathy more severe than Dravet syndrome being linked to a severe gain-of-function mutation of $\text{SCN1A}$ that caused depolarization-block and a more profound $\text{Na}_V\text{1.1}$ impairment (Berecki et al., 2019). However, this genotype-phenotype correlation is not always
observed, with the same SCN1A mutations resulting in Dravet syndrome in some patients and GEFS+ in others (Escayg et al., 2000; Guerrini et al., 2010; Suls et al., 2010). This incomplete penetrance of SCN1A mutations suggests other factors can influence epilepsy severity and highlight potential avenues to pursue in the development of novel treatment strategies for SCN1A-mediated epileptic encephalopathies.

9. Dravet syndrome

SCN1A mutations are the leading cause of Dravet syndrome, previously known as severe myoclonic epilepsy in infants, which is a rare but catastrophic form of intractable infant-onset epilepsy that has an incidence of 1 per 15,700 to 1 per 45,800 around the world (Bayat, Hjalgrim & Moller, 2015; Brunklaus et al., 2012; Ishii et al., 2017; Krueger & Berg, 2015; Wu et al., 2015). Dravet syndrome typically presents with febrile (generalised and unilateral, clonic or tonic–clonic) seizures within the first year of life in otherwise healthy infants. Seizure types progress to include generalised tonic-clonic, myoclonic, atypical absences, and focal impaired awareness seizures (Dravet, 2011).

Additional to seizures, delays in cognitive, behavioural and motor development manifest in patients with Dravet syndrome during the second year of life (Dravet, 2011). As Dravet syndrome patients get older, seizures tend to become less frequent and less severe (Genton, Velizarova & Dravet, 2011) but the behavioural comorbidities can worsen and persist well into adulthood (Battaglia et al., 2016; Olivieri et al., 2016). The observed comorbidities include hyperactivity and attention disorders, motor disorders, intellectual disabilities, speech difficulties, and features of autism spectrum disorder including: asocial, impulsive and perseverative behaviours (Acha et al., 2015; Brunklaus, Dorris & Zuberi, 2011; Genton, Velizarova & Dravet,
Patients with Dravet syndrome also exhibit neuropsychological deficits in both visual and auditory sensorimotor processing (Acha et al., 2015; Chieffo et al., 2016), which may precede and contribute to the associated attention, language and cognitive impairments (Brunklaus, Dorris & Zuberi, 2011; Genton, Velizarova & Dravet, 2011; Sinoo et al., 2019; Turner et al., 2017; Villeneuve et al., 2014).

The presence of these behavioural comorbidities, especially hyperactivity and inattention symptoms, are stronger predictors of worse quality of life than seizure frequency (Brunklaus, Dorris & Zuberi, 2011; Lagae et al., 2018; Sinoo et al., 2019; Wolff, Casse-Perrot & Dravet, 2006). Tragically, Dravet syndrome is also associated with a high rate of childhood mortality (approximately 15% by the age of 18), with a particularly high incidence of sudden unexpected death in epilepsy (SUDEP) (Cooper et al., 2016; Shmuely et al., 2016).

Over 80% of Dravet syndrome patients carry a mutation in SCN1A with around 90% of these mutations arising de novo, and the majority of the identified mutations were truncating mutations, followed by splice-site or missense mutations (De Jonghe, 2011; Depienne et al., 2009; Marini et al., 2011). As all these mutations result in NaV1.1 channel malfunction or absence, it suggests that NaV1.1 haploinsufficiency is responsible for the majority of Dravet syndrome cases. NaV1.1 haploinsufficiency leads to the impaired function of GABAergic neurons (Figure 6), as demonstrated by reduced GABAergic function recorded from post-mortem patient cortical tissue explants (Ruffolo et al., 2018). Moreover, patient-derived iPSC studies have confirmed that SCN1A mutations impaired sodium currents and action potentials predominantly in inhibitory neurons; confirming that network hyperexcitability is resulting from disinhibition (Higurashi et al., 2013; Liu et al., 2013; Maeda et al., 2016; Sun et al., 2016).
Imaging studies in Dravet syndrome patients have reported structural abnormalities are observed with variable incidence (11% - 71% cases) and the most commonly reported abnormalities were sclerosis and brain atrophy, predominantly in the hippocampus and cortex (Brunklaus et al., 2012; Gaily et al., 2013; Perez et al., 2014; Siegler et al., 2005; Striano et al., 2007). These reports highlight cortical and hippocampal regions as regions of interest in the pathology of Dravet syndrome. Furthermore, an interesting observation made in majority of these studies was that patients initially presented with normal MRIs but then progressed to exhibit structural abnormalities. This progressive pathology is consistent with Dravet syndrome being an epileptic encephalopathy and supports the need for early pharmacological intervention.

Figure 6. Schematic representation of reduced SCN1A expression in Dravet syndrome, with permission from Doohan (2019). (A) In a healthy patient, Nav1.1 is predominantly expressed on inhibitory interneurons. Inhibitory neurons function to maintain balance between excitatory and inhibitory neurotransmission. (B) In Dravet syndrome there is a heterozygous loss of Nav1.1 expression, leading to reduced interneuron function. The reduced function of interneurons disrupts the balance between inhibitory and excitatory neurotransmission resulting in hyperexcitability.
9.1 Current treatment strategies for Dravet syndrome

Dravet syndrome is notoriously treatment-resistant with most patients responding poorly to available therapies and complete seizure control is rarely attainable. The current first line of treatments are clobazam and valproic acid but pharmacoresistance leads to the addition of second-line treatments such as topiramate, stiripentol and ketogenic diet (Table 1); with even combinations of these therapies still proving ineffective for many patients (Knupp & Wirrell, 2018; Wirrell, 2016). Moreover, there are no specific treatments for the behavioural comorbidities associated with Dravet syndrome, which severely and negatively impact on the quality of life of patients and carers (Brunklaus, Dorris & Zuberi, 2011; Lagae et al., 2018; Sinoo et al., 2019; Wolff, Cassé-Perrot & Dravet, 2006).

Table 1. Drugs used in the treatment of Dravet syndrome and their proposed mechanisms of action.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Chemical structure</th>
<th>Known mechanisms of action</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Clobazam</td>
<td><img src="image" alt="Clobazam Structure" /></td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor PAM</td>
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<tr>
<td>Valproic acid</td>
<td><img src="image" alt="Valproic Acid Structure" /></td>
<td>Decreasing GABA degradation, Voltage-gated sodium, potassium, and calcium channels inhibitor</td>
<td>Ghodke-Puranik et al., 2013; Zhang et al., 2018</td>
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<tr>
<td></td>
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<td>Upregulates SCN1A expression</td>
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<td>Drug</td>
<td>Mechanism</td>
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<td></td>
<td>Synaptosomal GABA uptake inhibitor</td>
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<td></td>
<td>Lactate dehydrogenase inhibitor</td>
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<td>CYP&lt;sub&gt;150&lt;/sub&gt; enzymes inhibitor</td>
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<td>Non-N-methyl-D-aspartate (NMDA) glutamate receptors inhibitor</td>
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<td>Inhibitor of voltage-dependent sodium and calcium channels</td>
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<td>Induces serotonin release from vesicles</td>
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<td>Sodium-dependent serotonin transporter inhibitor</td>
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? Known mechanism of action but unknown if contributor to drug’s anticonvulsant actions.

The recommended pharmacotherapies for Dravet syndrome typically aim to increase inhibitory transmission through various mechanisms, with many of the anticonvulsant medications
having multimodal actions. Clobazam is a GABA<sub>A</sub> receptor PAM (Sankar, 2012), while valproic acid increases GABA concentrations through inhibiting its degradation enzymes, in addition to inhibiting voltage-gated sodium, potassium, and calcium channels, and upregulating SCN1A expression (Ghodke-Puranik et al., 2013; Zhang et al., 2018). Topiramate is also a GABA<sub>A</sub> PAM, in addition to an inhibitor of non-N-methyl-D-aspartate (NMDA) glutamate receptors and an inhibitor of voltage-dependent sodium and calcium channels (Latini et al., 2008; White et al., 2000). Benzodiazepines are known to lose anticonvulsant efficacy due to GABAA receptor internalisation. Interestingly, stiripentol is also a GABAA receptor PAM but it binds at a site separate to that of benzodiazepines that does not result in GABAA receptor internalisation (Grosenbaugh & Mott, 2013). Moreover, stiripentol inhibits the synaptosomal uptake of GABA (Poisson et al., 1984) and inhibits lactate dehydrogenase, which is hypothesised to reduce neuronal excitability by restricting lactate concentrations, the fuel source for neuron metabolism (Sada et al., 2015). Furthermore, stiripentol has the additional action of inhibiting CYP<sub>450</sub> enzymes, consequently increasing the concentrations of concurrent anticonvulsant medications, including clobazam (Giraud et al., 2006). Despite the multimodal actions of the conventional anticonvulsants, Dravet syndrome patients still fail to respond completely to these treatments, highlighting a dire need for the development of better therapeutic options.

Two recent therapeutic developments are fenfluramine and cannabidiol, which were discovered serendipitously. Fenfluramine was originally approved as an anorectic agent but was withdrawn in 1997 due to cardiovascular-associated adverse effects. However, before its withdrawal, fenfluramine proved successful in a trial for refractory epilepsy with self-induced seizures (Boel & Casaer, 1996). When a significant portion of the responders were discovered to carry SCN1A mutations, subsequent trials were conducted in Dravet syndrome patients. Recently,
fenfluramine was shown to be an effective anticonvulsant in Dravet syndrome patients in phase III clinical trials when administered as an adjuvant therapy; with patients maintaining a safe cardiovascular profile (Lagae et al., 2019; Schoonjans et al., 2017a; Schoonjans et al., 2017b). Interestingly fenfluramine’s anticonvulsant actions are proposed to be through augmenting serotonergic signalling, as its primary mode of action is to induce serotonin release from vesicles and consequently enhance synaptic serotonin concentrations (Sourbron et al., 2017). The other novel non-conventional medication to be recently approved for the treatment of Dravet syndrome is cannabidiol, which was described earlier in section 2.

Most anticonvulsant medications can only address the symptoms of epilepsy and not the causal mechanisms. As most Dravet syndrome cases are due to Na\textsubscript{V}1.1 haploinsufficiency, therapies that augment Na\textsubscript{V}1.1 might prove efficacious. There are advances in pharmacotherapy being developed to utilize anti-natural antisense transcripts (antagoNAT), which are single-strand oligonucleotide molecules that enhance gene transcription. There is currently an antagoNAT agent called CUR-1916 (also known as OPK88001) being developed by OPKO Health, which aims to upregulate transcription of the healthy copy of SCN1A. CUR-1916 received orphan drug status by the FDA in 2017 and OPKO Health reported intentions for clinical trials but no progress has been reported since. Although the concept of upregulating Na\textsubscript{V}1.1 is alluring, it may not be a perfect treatment. CUR-1916 significantly reduced spontaneous seizure frequency and duration, while also increasing the temperature thresholds for hyperthermia-induced seizures in a mouse model of Dravet syndrome (Hsiao et al., 2016). However, it did not completely protect against hyperthermia-induced seizures or spontaneous seizures. Furthermore, CUR-1916’s efficacy was only assessed in mice that express a milder phenotype. That is, the mice were tested at 7-11 weeks,
an age at which 70% of the Dravet syndrome mice expressing a severe phenotype have already died. Future studies are needed to assess the agent’s efficacy during the vulnerable period.

Further challenges for the antagoNATs clinical development is that these agents need to be delivered via the invasive intrathecal route (Hsiao et al., 2016). This is likely due to the oligonucleotides being too large to cross the blood brain barrier and having poor bioavailability due to rapid degradation by circulating enzymes (Wahlestedt, 2013). AntagoNATs plasma stability was improved by the addition of a phosphorothioate group, however phosphorothioate-containing oligonucleotides may increase systemic inflammation, leading to fever in some patients (Wahlestedt, 2013). While this may be tolerated by some populations, fevers are known triggers for seizures in Dravet syndrome patients. It is apparent that there are still numerous hurdles and still much development required for Scn1a-antagoNAT therapies.

To further complicate the treatment strategies of Dravet syndrome, there are also contraindicated pharmacotherapies. The use of sodium channel blockers, such as lamotrigine, carbamazepine or phenytoin, should be avoided as they can exacerbate seizures and can induce lasting negative effects on cognitive outcomes (de Lange et al., 2018; Genton, 2000; Shi et al., 2015). It is evident that current treatment strategies for Dravet syndrome are complicated and lack efficacy in addressing the condition’s multiple aspects. Most anticonvulsant drugs are designed as broad-spectrum agents, i.e. to treat all types of seizures. However, seizures and epilepsy disorders can arise from many different aetiologies that cause different pathophysiological mechanism. Although there has been progress in the development of anticonvulsant drugs that utilise novel mechanisms of action and display improved safety profile, they have not reduced the prevalence of drug resistant epilepsy disorders, including Dravet syndrome. The pharmacoresistance observed in patients with Dravet syndrome suggests that seizures are resulting from different or additional
pathophysiological mechanisms to those targeted by current therapeutics. Although novel therapeutics such as fenfluramine and CBD have proven useful, they were discovered serendipitously. A targeted and physiologically-informed strategy is required to develop improved therapeutics for Dravet syndrome. Expanding the understanding of the underlying pathophysiological mechanisms of the disorder, in addition to understanding how variability in phenotype can arise from identical genetic alterations, could provide a platform for the discovery of novel and precise drug targets that this thesis will explore.

9.2 Scn1a+/− mouse models of Dravet syndrome

As haploinsufficiency of SCN1A is the major cause of Dravet Syndrome, numerous genetic mouse models that utilize haploinsufficiency of Nav1.1 (Scn1a+/−) have been generated. These mouse lines include targeted deletion of Scn1a exon 1 (Scn1a<sup>−/−</sup>Kea) (Miller et al., 2014) and exon 26 (Scn1a<sup>−/−</sup>Wac) (Yu et al., 2006), along with specific point insertion of human mutations R1407X (Scn1a<sup>−/−</sup>Kzy) (Ogiwara et al., 2007) and E1099X (Scn1a<sup>−/−</sup>Swl) (Tsai et al., 2015). Scn1a<sup>+/−</sup> mouse models recapitulate many of the seizure-related features seen in Dravet syndrome patients. Scn1a<sup>+/−</sup> mice display increased susceptibility to febrile seizures and exhibit spontaneous seizures and spontaneous death, particularly during the 3<sup>rd</sup> and 4<sup>th</sup> postnatal weeks of life (Cao et al., 2012; Hawkins et al., 2017; Kalume et al., 2007; Miller et al., 2014; Mistry et al., 2014; Oakley et al., 2009; Ogiwara et al., 2007; Tsai et al., 2015; Yu et al., 2006). Two of the mouse models that assessed behaviour identified that Scn1a<sup>+/−</sup> models reproduced many of the comorbidities observed in patients, including hyperactivity, stereotyped behaviours, lowered sociability, poor spatial learning, memory deficits and impaired sleep (Han et al., 2012; Ito et al., 2013; Kalume et al., 2015). As the Scn1a<sup>+/−</sup> model faithfully recapitulates many aspects of what Dravet syndrome
patients exhibit, it has provided a useful model to investigate the impact of Nav1.1 impairment on the pathophysiologies of the disease.

The use of mouse models have informed on Nav1.1 expression patterns, revealing its expression being predominantly on GABAergic interneurons (Dutton et al., 2013; Ogiwara et al., 2007). Moreover, the contribution of Nav1.1 channels to the seizure phenotype was supported by Nav1.1 expression being upregulated during the second and third postnatal weeks, which coincides with the period of spontaneous seizure susceptibility observed in the Scn1a<sup>+/−</sup> mice (Cheah et al., 2013). Electrophysiological studies using the Scn1a<sup>+/−</sup> mouse models have confirmed that Nav1.1 haploinsufficiency causes network hyperexcitability at the network level as recorded by field potentials from brain slices (Liautard et al., 2013). They have also confirmed impaired function in interneurons from the hippocampus, neocortex and cerebellum (Kalume et al., 2007; Kaplan et al., 2017; Ogiwara et al., 2013; Rubinstein et al., 2015; Tai et al., 2014; Tsai et al., 2015; Yu et al., 2006). Interestingly, one study identified increased excitability from cortical excitatory pyramidal neurons (Mistry et al., 2014) while other studies report pyramidal neurons to be unaffected (Rubinstein et al., 2015; Yu et al., 2006). Further supporting the theory that loss of Scn1a from interneurons is the underlying pathophysiological mechanism was work showing that conditional deletion of Scn1a on GABA interneurons globally or specifically in cortical and hippocampal neurons, was sufficient to reproduce the febrile- and spontaneous-seizure phenotypes, as well as the premature lethality phenotype (Cheah et al., 2012; Kuo et al., 2019; Ogiwara et al., 2013). Whereas, Scn1a deletion from excitatory neurons had no detectable effects (Dutton et al., 2013; Ogiwara et al., 2013). Together these studies support impaired interneuron function to be the leading cause of hyperexcitability through disinhibition.
Moreover, *Scn1a*+/- mice respond to common anticonvulsant medications in a manner similar to what is observed clinically. For example, sodium channel blockers exacerbated seizures in these mice while clobazam was the most effective anticonvulsant (Hawkins et al., 2017). Additionally, as mentioned earlier, CBD’s efficacy was also replicated in the *Scn1a*+/- mice (Anderson et al., 2019a; Kaplan et al., 2017). Therefore, in addition to improving understanding of the pathophysiology associated with Na\textsubscript{V}1.1 impairment, the *Scn1a*+/- mouse models also provide a validated platform to assess the efficacy of potential therapeutic agents.

10. **Genetic modifiers: a strategy to identify drug targets**

One strategic approach to identifying novel drug targets is the identification of genetic modifiers. As described earlier, identical SCN1A mutations can lead to a range of epilepsy symptoms and variability in their severity. The variability observed in the symptomatic presentation of patients with Dravet syndrome has also been observed in *Scn1a*+/- mouse models. The severity of the epilepsy phenotype is critically sensitive to the background strain of the mice with the *Scn1a* alternations. Heterozygous *Scn1a* deletion on a 129 background strain (129.*Scn1a*+/-) yields a very mild to non-existent seizure phenotype. However, crossing 129.*Scn1a*+/- mice with wildtype (WT) C57BL/6 mice to generate *Scn1a*+/- mice on a mixed F1 (129 x C57BL/6) background strain uncovers a severe seizure phenotype (Miller et al., 2014; Mistry et al., 2014; Yu et al., 2006). Moreover, the seizure phenotype is exacerbated with continuous backcrossing on to a congenic C57BL/6 background strain (Miller et al., 2014; Rubinstein et al., 2015; Yu et al., 2006). The minimal phenotype expression of *Scn1a*+/- mice on the 129 background is corroborated by these mice exhibiting significantly less impaired, if not normal, sodium current density in inhibitory neurons (Mistry et al., 2014; Rubinstein et al., 2015). The sensitivity of the Dravet syndrome phenotype to background strain indicates that non-*Scn1a*
genes are influencing phenotype severity. Genes secondary to the mutation that alter phenotype severity are referred to as genetic modifiers and hold potential as novel therapeutic targets for the treatment of Dravet syndrome.

There have been several genetic modifiers of Scn1a that have already been identified and validated. Potential genetic modifiers were identified in a preliminary RNA-seq analysis that compared the hippocampal transcriptome between the seizure-resistant and seizure-susceptible background strains of a Dravet syndrome mouse model (Hawkins et al., 2019; Hawkins & Kearney, 2016). After confirmation of varied expression between background strains, the ability of the target gene to influence phenotype can be established through genetic or pharmacological investigations. For example, Gabra2, which codes for the α2 subunit of the GABA_A receptor, was less expressed in mice on the F1 seizure-susceptible background strain compared to the 129 seizure-resistant strain, suggesting it may influence phenotype (Hawkins et al., 2016). Then, the GABA_A receptor’s influence on the phenotype was confirmed when positive allosteric modulation of the GABA_A receptors, with clobazam or AZD7325, improved susceptibility to febrile seizures and spontaneous seizures (Hawkins et al., 2017; Hawkins et al., 2016; Nourbakhsh, Atabaki & Roohbakhsh, 2018). Another example is Scn8a, encoding the voltage-gated sodium channel α-subunit NaV1.6, which was also more expressed in the seizure-susceptible strain and was proved to be a modifier of Scn1a when pharmacological inhibition of NaV1.6 with GS967 significantly improved survival and spontaneous seizures (Anderson et al., 2017; Martin et al., 2007).

It is likely that there are additional genes, from many neurological systems, that are genetic modifiers of Scn1a. As the endocannabinoid system functions to regulate neuronal activity, there may be molecular constituents in this system that could be influencing the Dravet syndrome
phenotype. Utilizing the \( Scn1a^{+/−} \) mouse model to investigate the interaction between the endocannabinoid system and \( N_{aV}1.1 \) impairment could highlight mechanisms by which cannabinoids are eliciting anticonvulsant actions and reveal new cannabinoid-based drug targets for the directed development of cannabinoid-based medicines.

11. Aims

The global objective to this thesis is to assist in the development of novel therapeutics for the more comprehensive treatment of Dravet syndrome by taking a multi-faceted, physiologically-directed approach that is centred around the \( Scn1a^{+/−} \) mouse model. We aim to identify and validate molecular targets by first expanding the understanding of the broader endocannabinoid system in the \( Scn1a^{+/−} \) mouse model, and then by employing genetic and pharmacological techniques to determine the impact of potential molecular targets on the epilepsy phenotype. Moreover, we aim to improve the drug-testing platform of the mouse model to include assessment of the associated behavioural comorbidities.

11.1 Specific aims

1. Validation of CB1 receptors as an anticonvulsant target in a \( Scn1a^{+/−} \) mouse model of Dravet Syndrome (Chapter 2)

The endocannabinoid system plays a major role in regulating neuronal excitability, predominantly through CB1 receptors. Pharmacological potentiation of the endocannabinoid system has shown promise in numerous preclinical models of acute seizure and epilepsy but is yet to be investigated in paediatric epilepsy models, including Dravet syndrome models. A preliminary RNA-seq investigation of the hippocampal transcriptome of a \( Scn1a^{+/−} \) mouse model highlighted
Cnr1, the gene encoding the CB1 receptor, as a potential genetic modifier as it was less expressed in mice on the seizure-susceptible strain. Therefore, in Chapter 2 we aim to assess if Cnr1 could be a genetic modifier of Scn1a. We aim to determine if there are alterations in molecular component of the endocannabinoid system that are consistent with increased excitability in the F1 seizure-susceptible strain of the Scn1a+/− mouse model by measuring the mRNA and protein expression of key constituents with ddRT PCR and western blotting, respectively; and endogenous ligand concentrations will be assessed with mass spectrometry. Additionally, we aim to assess if potentiation of endocannabinoid signalling with a CB1 receptor PAM or MAGL inhibitor will display anticonvulsant efficacy against the hyperthermia-induced seizure phenotype of F1.Scn1a+/− mice. We hypothesise that the mice on the seizure-susceptible background strain will have less expression of endocannabinoid system constituents and that potentiation of the endocannabinoid signalling will elicit anticonvulsant actions.

2. Targeting Gpr55 as a treatment strategy in a Scn1a+/− mouse model of Dravet syndrome (Chapter 3)

There is growing evidence to suggest that Gpr55 can modulate neuronal excitability, including the anticonvulsant actions of CBD being attributed to GPR55 antagonism. Moreover, Gpr55 was also suggested as a potential genetic modifier of Scn1a in the preliminary RNA-seq study as it was expressed more in the hippocampus of seizure-susceptible mice than seizure-resistant mice. Therefore, in Chapter 3 we aim to investigate if Gpr55 could be a modifier of the Scn1a+/− phenotype. Our first objective is to determine if there are differences in the Gpr55-LPI system of the Scn1a+/− mouse model by measuring the gene expression and ligand concentrations of key constituents of the Gpr55-LPI system using ddRT-PCR and mass spectrometry,
respectively. Then, we aim to examine the influence of Gpr55 on the Dravet syndrome phenotype by evaluating the impact of heterozygous deletion of Gpr55 on the hyperthermia-induced seizures, spontaneous seizures and the premature mortality phenotype in Scn1a<sup>+/−</sup> mice. We predict to observe changes in key components of the Gpr55-LPI system that would be consistent with increased Gpr55 signalling in the F1 seizures-susceptible mice, and therefore hypothesise that genetic down-regulation of Gpr55 will improve the Dravet syndrome phenotype.

3. Characterising the adolescent behavioural phenotype of a Scn1a<sup>+/−</sup> mouse model (Chapter 4)

In addition to seizures, patients with Dravet syndrome also exhibit developmental delays in attention, emotional, and cognitive functions that severely impact on the quality of and are not specifically addressed by current therapeutic strategies. The behavioural phenotype of other Scn1a<sup>+/−</sup> mouse models have been characterised to be consistent with impairments seen in patients but behavioural phenotypes have only been characterised in adult mice. The behavioural phenotype of the mouse model we utilise has not been characterised at any stage of development. Characterisation of the impact of heterozygous deletion of Scn1a adolescents will establish a platform to assess the therapeutic effects of earlier pharmacological intervention. Thus, in Chapter 4, we aim to assess the impact of heterozygous Scn1a deletion on adolescent behaviour by comparing the behaviour of Scn1a<sup>+/−</sup> and WT mice across several behavioural domains. We will assess motor function (open field test), sociability and social recognition memory (three-chambered social preference and social interaction tests), memory function (novel object recognition, Barnes maze, fear conditioning paradigm), anxiety-related behaviour (elevated plus maze and open field thigmotaxis), startle reflex and sensorimotor gating (prepulse inhibition of
startle tests), and repetitive compulsive behaviour (marble burying test). We hypothesise that
Scn1a+/− mice will display impairments in numerous behavioural modalities measured.
Chapter 2

Validation of CB1 receptors as an anticonvulsant target in a $Scn1a^{+/−}$ mouse model of Dravet Syndrome
Introduction

Dravet syndrome is a severe, intractable form of paediatric epilepsy with patients exhibiting various seizure-types, behavioural comorbidities and a significant risk for sudden unexplained death in epilepsy (SUDEP) (Cooper et al., 2016; Dravet et al., 2005; Dravet & Oguni, 2013; Shmuely et al., 2016). Current anticonvulsant treatments for Dravet syndrome aren’t sufficient and there is a dire need for the development of novel therapeutics (Cooper et al., 2016; Knupp & Wirrell, 2018). Over 80% of patients have loss-of-function mutations in the SCN1A gene, encoding the $\alpha_1$ subunit of the voltage-gated sodium channel (Nav1.1) (De Jonghe, 2011; Depienne et al., 2009; Marini et al., 2011). As Nav1.1 is predominantly expressed on inhibitory neurons (Dutton et al., 2013; Ogiwara et al., 2007), its loss-of-function results in global network hyperexcitability through disinhibition (Higurashi et al., 2013; Liu et al., 2013; Maeda et al., 2016; Sun et al., 2016).

Despite many of the SCN1A mutations resulting in the same functional outcomes, there is considerable variability in symptom severity amongst patients (Brunklaus et al., 2012; Olivieri et al., 2016; Ragona et al., 2011). This incomplete penetrance of the Scn1a phenotype is also observed in mouse models of Dravet syndrome where the severity of the Scn1a$^{+/-}$ phenotype is critically sensitive to the background strain. Scn1a$^{+/-}$ mice on the 129 background strain (129.Scn1a$^{+/-}$) are phenotypically similar to their wildtype (WT) counterparts. However, crossing 129.Scn1a$^{+/-}$ mice with C57BL/6 mice to create a mixed (129 X C57BL/6J) F1 background strain produces F1.Scn1a$^{+/-}$ mice that recapitulate many of the symptoms observed in patients. F1.Scn1a$^{+/-}$ mice exhibit increased susceptibility to febrile seizures, spontaneous seizures, reduced lifespan, and behavioural comorbidities (Hawkins et al., 2017; Miller et al., 2014; Mistry et al., 2014; Chapter 4). This strain-dependent presentation of the Dravet syndrome phenotype in mice suggests that
phenotype is influenced by non-Scn1a genes, which are termed ‘genetic modifiers’ and may serve as novel drug targets.

One physiological system that may host druggable targets is the endogenous cannabinoid (endocannabinoid) system, which functions in the central nervous system (CNS) as a regulator of neuronal activity (Kawamura et al., 2006; Sugaya et al., 2016; Takahashi & Castillo, 2006). There are two main cannabinoid receptors, cannabinoid receptor type 1 (CB1) that is distributed predominantly in the CNS, and cannabinoid receptor type 2 (CB2) that is mainly located peripherally (Howlett & Abood, 2017). The main endogenous ligands of CB1 and CB2 receptors are N-arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995). In response to post-synaptic neuronal activation, anandamide and 2-AG are synthesised by N-arachidonoyl phosphatidylethanolamine (NAPE) and diacylglycerol lipase (DAGL) isoforms α and β, respectively (Di Marzo et al., 1994; Murataeva, Straiker & Mackie, 2014).

Endocannabinoids travel retrogradely across the synaptic cleft to activate CB1 receptors, which are located presynaptically on both on glutamatergic and GABAergic neurons (Howlett & Abood, 2017; Kano, 2014; Kawamura et al., 2006). CB1 receptors are G protein coupled receptors coupled predominantly to Ga\textsubscript{i/o} proteins (Howlett et al., 2002). Stimulation of the Ga\textsubscript{i/o} proteins results in numerous intracellular actions, including suppression of cAMP, stimulation of potassium channels and inhibition of inward-facing voltage-gated calcium channels, which ultimately reduce presynaptic cell excitability and inhibit further neurotransmitter release (Farrell & Soltesz, 2019; Howlett et al., 2002; Soltesz et al., 2015). Anandamide and 2-AG are then rapidly metabolised by fatty acid amine hydrolase (FAAH) and mainly monoacylglycerol lipase (MAGL), respectively (Blankman, Simon & Cravatt, 2007; Cravatt et al., 1996).
As the endocannabinoid system regulates neuronal activity, we hypothesized that there may be endocannabinoid-related molecular targets that could hold therapeutic potential for the treatment of Dravet syndrome. Further support to investigate the endocannabinoid system was provided by an RNA-seq analysis of the Scn1a+/− model, which showed significantly less hippocampal expression of Cnr1, the gene encoding CB1 receptors, in mice on the seizure-susceptible F1 background strain (129S6/SvEvTac x C57BL6/J) compared to mice on the 129S6/SvEvTac seizure-resistant background strain (Hawkins et al., 2019). Theoretically, reduced CB1 receptor expression in the F1 mice could be decreasing regulation of neuronal transmission. This could be contributing to the excessive neuronal hyperexcitability and the severe seizure phenotype observed in F1.Scn1a+/− mice, making it a potential genetic modifier.

Based on the neuronal type that CB1 receptors are located on, stimulation of CB1 receptors can inhibit the release of glutamate or GABA (Farrell & Soltesz, 2019; Lee, Foldy & Soltesz, 2010; Neu, Foldy & Soltesz, 2007; Soltesz et al., 2015), which can lead to anticonvulsant or proconvulsant effects. This contrasting effect of CB1 receptor stimulation has been observed in preclinical models of seizure. For example, the synthetic CB1 receptor agonists WIN 55,212 and ACEA were anticonvulsant in pentylenetetrazol and maximal electroshock acute models of seizure and in the pilocarpine model of status epilepticus in rodents (Suleymanova et al., 2016; Vilela et al., 2013; Wallace et al., 2001). Whereas CB1 receptor agonism has also demonstrated intrinsic proconvulsant effects and increased susceptibility in conventional seizure models, albeit predominantly with synthetic and potent agonists (Funada & Takebayashi-Ohsawa, 2018; Kevin et al., 2019; Malyshevskaya et al., 2017; Vilela et al., 2013; Wilson et al., 2019).

To avoid the proconvulsant effects of CB1 receptor agonism, endocannabinoid signalling can be pharmacologically enhanced indirectly and specifically. One such approach is the elevation
of endocannabinoid concentrations through inhibition of their degradative enzymes. For example, MAGL inhibitors that elevate 2-AG concentration have been demonstrated to display anticonvulsant effects in pentylentetrazol, status epilepticus and kindling models of seizure and epilepsy in rodents (Terrone et al., 2018; von Ruden et al., 2015a; Zareie et al., 2018). MAGL inhibition has the added advantage of enhancing endocannabinoid signalling in spatiotemporally restricted manner as its effects are dependent on the presence of 2-AG, and 2-AG is only produced on-demand where neural activity is present, which would occur at glutamatergic synapses in a hyperexcitable network.

Another preferred pharmacological approach is positive allosteric modulation (PAM) of CB1 receptors. PAMs bind to a site on the receptor separate to the orthosteric binding site that elicits no intrinsic activation of the receptor (Wootten, Christopoulos & Sexton, 2013). Instead, PAMs increase the efficacy and activity of orthosteric ligands and therefore their function is specific and limited by the presence of orthosteric ligands (Wootten, Christopoulos & Sexton, 2013). Like MAGL inhibitors, CB1 receptor PAMs potentiate the intrinsic neural regulating effect of the endocannabinoid system. This has been demonstrated by the CB1 receptor PAMs ZCZ011 and GAT229 reducing neuropathic pain in mouse models without inducing cannabimimetic adverse effects (Ignatowska-Jankowska et al., 2015; Slivicki et al., 2018). As such, MAGL inhibitions and CB1 receptor positive allosteric modulation are compelling pharmacological approaches to increasing CB1 receptor-mediated neuromodulation that has not yet been explored in Dravet syndrome, or other paediatric epilepsy preclinical models.

The present chapter aimed to determine if Cnr1 could be a genetic modifier in a Scn1a−/− mouse model of Dravet syndrome. We measured CB1 mRNA and protein expression, along with endocannabinoid concentrations in the Scn1a−/− mouse model of Dravet syndrome, to identify
reduced CB1 receptor expression and reduced 2-AG concentrations in the F1 seizure-susceptible strain of mice. After identifying a potential double deficit in the endocannabinoid system of seizure susceptible mice, we hypothesized that targeting these deficits could be anticonvulsant. Thus, we investigated and demonstrated that pharmacological potentiation of endocannabinoid signalling with a CB1 receptor PAM, or MAGL inhibitor was anticonvulsant against hyperthermia-induced seizures in F1.\textit{Scn1a}^{+/-} mice.

\section*{Materials and methods}

\subsection*{Mice}

All research and animal care procedures were approved by the University of Sydney Animal Ethics Committee in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and by the Northwestern University Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice heterozygous for \textit{Scn1a} (\textit{Scn1a}^{+/-}) were generated by targeted deletion of \textit{Scn1a} exon 1 as previously described (Miller et al., 2014) and obtained from The Jackson Laboratory (MMRRC Stock No: 37107-JAX) for the Australian studies. \textit{Scn1a}^{+/-} mice on the 129S6/SvEvTac (129) background (129.\textit{Scn1a}^{+/-}) were maintained by continuous backcrossing. 129.\textit{Scn1a}^{+/-} mice were crossed with C57BL/6J (B6) mice to generate [129 x B6] F1.\textit{Scn1a}^{+/-} mice and WT mice. Genotyping was performed as previously described (Miller et al., 2014). Gene transcription studies were conducted at Northwestern University, U.S.A, where the mouse facility operated on a 14 h light/10 h dark cycle. Whereas, all other work too place at the University of Sydney, Australia, where the facility was kept on a 12 h light/dark cycle. All animals had \textit{ad libitum} access to food and water and were group housed in individually ventilated cages.
**Digital-drop real-time PCR (ddRT-PCR)**

Transcript expression of target genes were determined using ddRT-PCR and performed as previously described (Hawkins et al., 2019; Hawkins et al., 2016). Briefly, at P24, cortical and hippocampal samples were dissected from WT and Scn1a\(^{+/-}\) mice on both the 129 and F1 (129 x B6) background strains. To achieve sufficient mRNA concentrations for analyses, primary pools of mRNA were created by combining tissue from 3 – 4 mice, with at least one male and female in each group to control for potential sex-dependent differences. Primary mRNA pools were used to assess Cnr1 expression. For analysis of the 2-AG metabolizing enzymes, RNA superpools were created by combining 4-3 primary RNA pools \((n = 12 – 16 \text{ mice/superpool})\). Total RNA was isolated before first-strand cDNA was synthesized and ddRT-PCR was performed using TaqMan Gene Expression Assays (Life Technologies) for mouse Cnr1 (Mm01212171_s1); Mgl1 (Mm00449274_m1); Dagla (Mm00813830_m1); Daglb (Mm00523381_m1); Abhd6 (Mm00481199_m1); Abhd12 (Mm00470489_m1); Tbp Mm00446971_m1. After amplification, a QX200 droplet reader with QuantaSoft v1.6.6.0320 software was used to analyse droplets and transcript levels of target genes were expressed as a ratio relative to Tbp concentrations.

**Western blot**

Western blot analyses were performed as previously described (Hawkins et al., 2019; Hawkins et al., 2016) with modifications. Briefly, animals were euthanized via cervical dislocation and decapitation. Brains were rapidly extracted and snap-frozen in liquid nitrogen. Hippocampal sections were dissected from brains thawed on ice and snap-frozen again in liquid nitrogen. Frozen hippocampal sections were homogenized by hand in ice cold 0.32 M sucrose, 5 mM Tris (pH 7.4) supplemented with protease inhibitor (Merck 4693159001) before being centrifuged for 10 min at
2,000 g, at 4°C. Supernatants were collected and centrifuged for 40 min at 20,000 g, at 4°C. The pellets were re-homogenised in 5 mM Tris (pH 8.2) and 1 mM ethylenediaminetetraacetic acid (EDTA) supplemented with protease inhibitor. Samples were centrifuged again for 40 min at 20,000 g, at 4°C, then the procured pellet was resuspended in a solution of 50 mM Tris (pH 7.5), 10 mM (EDTA) supplemented with protease inhibitor (Roche, 4693159001). Protein lysates (20 µg) were denatured in 2x laemmlie sample buffer (Biorad, 1610737) and 5% beta mercaptoethanol for 15 min at room temperature, before being separated on a 10% SDS-page gel and transferred to nitrocellulose membranes. Membranes were probed for CB1 using a rabbit polyclonal antibody (1:200, Abcam, ab23703) and for β-tubulin using a mouse monoclonal antibody (1:500, Sigma-Aldrich, T5201) for 1 h at room temperature, then with secondary antibodies, anti-Rabbit Alexa Fluor 800 (A32735) and anti-Mouse Alexa Fluor 680 (A28183) at 1:20,000 for 30 min at room temperature. The probed membranes were imaged with an Odyssey imaging system (LI-COR Biosciences). Signal-intensity analysis of western blots was performed using Image Studio Lite software (LI-COR Biosciences) and data was presented as the signal intensity of CB1 as a ratio of β-tubulin.

**Drugs**

To investigate the anticonvulsant efficacy of potentiating endocannabinoid signalling, we elected to use the CB1 positive allosteric modulator (PAM) with GAT229 (Mitjavila et al., 2018) and the irreversible MAGL inhibitor ABX-1431 (Cisar et al., 2018). S-(−)-3-(2-Nitro-1-phenylethyl)-2-phenyl-1H-indole (GAT229) was generously gifted by Assoc. Prof. Ganesh Thakur (Northeastern University, USA), while 1,1,1,3,3,3-hexafluoropropan-2-yl-4-[(2-pyrrolidin-1-yl-4-(trifluoromethyl)phenyl)methyl]-piperazine-1-carboxylate (ABX1431) was purchased from WuXi AppTec (Shanghai, China) and Diazepam was purchased from Sigma
Aldrich (St Louis, USA). We investigated the anticonvulsant potential of a GAT229 at 30 and 100 mg/kg and ABX-1431 at 3, 10 and 30 mg/kg (Cisar et al., 2018; Mitjavila et al., 2018). Our starting dose of 30 mg/kg for GAT229 was based on the structurally-similar but more potent CB1 PAM, ZCZ011, eliciting in vivo effects at 40 mg/kg (Ignatowska-Jankowska et al., 2015). Whereas the ABX-1431 dosing regimen was based off work demonstrating its ability to inhibit Magl at a 4 mg/kg dose when administered orally in mice (Cisar et al., 2018). All drugs were prepared in a vehicle of ethanol:Tween80:0.9% saline (1:1:18) fresh on the day of experimentation and were administered via intraperitoneal (i.p.) injection in an injection volume of 10 mL/kg. Experimental time points of 30 min for GAT229 and 60 min for ABX-1431 administration were based on previously determined time-to-peak plasma and brain concentrations or effect from the literature or our pharmacokinetic studies (Berger et al., 2012; Kaczocha et al., 2014).

**Endocannabinoid and drug analyses using high-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS)**

Analysis of endocannabinoid and structurally-related lipids in brain tissue were determined via LC/MS/MS as previously described (Clarke et al., 2017; Stuart et al., 2013). Briefly, male mice at P24 were euthanized at the same time of day via cervical dislocation. Brains were rapidly extracted and flash frozen with liquid nitrogen. Hippocampal sections were dissected from brains thawed on ice and re-frozen in liquid nitrogen. Samples were stored in the -80C freezer until analysed. Homogenised hippocampal samples underwent solid phase extraction using 500 mg C18 solid-phase extraction columns (Agilent Technologies, Santa Clara, CA) to isolate the lipids, 2-AG), AEA, PEA, OEA, LEA and DHEA. Elutants (20 μL) were separated in a C18 Zorbax reversed-phase analytical column (Agilent Technologies, Santa Clara, CA) and ionized using a Shimadzu 8030 triple quadrupole mass spectrometer via positive electrospray ionization through
a multiple reaction monitoring method. Internal standard arachidonoyl ethanolamide-d₄ (d₄-AEA) was purchased from Tocris Bioscience (St. Louis, MO), whereas standards for 2-AG and AEA, PEA, OEA, LEA and DHEA were purchased from Cayman Chemical (Ann Arbor, MI).

**Hyperthermia-induced seizures**

Hyperthermia-induced seizure experiments were conducted as previously described in both male and female F1.Scn1a^+/− mice from P14 to P16 (Hawkins et al., 2017). Mice received an i.p. injection of vehicle, GAT229 or ABX-1431. Then after a pre-determined experimental time-point, the mice were fitted with a rectal probe that was connected to a temperature controller, which was connected to a heat lamp, and allowed to acclimatize for 5 min. The baseline body temperature of the mice was recorded before their core body temperature was elevated with a heat lamp at a rate of 0.5°C every 2 min until the onset of the first clonic convulsion with loss of posture or until 42.5°C was reached. Mice that reached 42.5°C were held at temperature for 3 min and if no seizure occurred, they were considered seizure-free. Body temperatures of seizure onset were recorded and then the mice were euthanized via isoflurane inhalation before plasma and brain samples collected and stored at -80°C until assayed.

**Analytical methods with HPLC/MS/MS**

Plasma and brain concentrations of GAT229 and ABX-1431 in experimental mice were assayed with HPLC/MS/MS and were performed based on previously described methods with modifications (Anderson et al., 2019a; Anderson et al., 2019b). Briefly, plasma samples (50 μL aliquots) were spiked with 5 μL internal standard (2 μg/mL diazepam) and extraction was achieved by vortex-mixing with 4 X volume of acetonitrile. The organic layer was isolated by centrifugation (4000 g for 10 min at 4°C) and evaporated to dryness under nitrogen at room temperature. For
GAT229, residues were reconstituted in methanol (150 μL) and 0.1% formic acid (250 μL) in water; while for ABX-1431, residues were reconstituted in acetonitrile (90 μL) and 0.1% formic acid in water (300 μL). Then supported-liquid extraction (SLE) was performed and extracts were eluted in methyl tert-butyl ether (MTBE) and dried to completeness under nitrogen at room temperature before GAT229 samples were reconstituted in 50 μL of 0.1% formic acid in water and methanol (2:3 v/v); while ABX-1431 samples were reconstituted in 0.1% formic acid in water and acetonitrile (1:1 v/v) for HPLC/MS/MS analysis.

For brain analyses, brain hemispheres were homogenized in 10 X methanol: water (1:1) and the supernatant layer was isolated by centrifugation (20,000 g for 30 min at 4°C), supplemented with acetonitrile (300 μL), spiked with 15 μL internal standard (2μg/mL diazepam) and vortexed for 10 min. Samples were first centrifuged (10 min at 18,000 g and 4°C) before supernatants were collected and centrifuged again (2 h at 18,000 g and 4°C). Filtrates were collected and dried to completeness under nitrogen, reconstituted for SLE and underwent SLE as described above. For GAT229, SLE eluents were reconstituted in 50 μL of 0.1% formic acid in water and methanol (2:3 v/v); whereas for ABX-1431, SLE eluents were reconstituted in 0.1% formic acid in water and methanol (2:3 v/v) for analysis via HPLC/MS/MS.

GAT229 and ABX-1431 concentrations were analysed using a Shimadzu Nexera ultra-high-performance liquid chromatograph coupled to a Shimadzu 8030 triple quadrupole mass spectrometer (Shimadzu Copr., Kyoto, Japan). For GAT229, mobile phases 0.1% formic acid in water (A) and methanol (B) were delivered with a flow rate of 0.3 L/min as follows: mobile phase B was first held at 40% for 1 min, then increased from 40% to 90% over 2 min, held at 90% for 3.5 min, decreased back to 40% over 0.5 min and equilibrated at 40% for 0.5 min. For ABX-1431, mobile phases 0.1% formic acid in water (A) and acetonitrile (B), were delivered at a flow of 0.6
L/min as follows: mobile phase B was first held at 50% for 0.5 min, increased from 50% to 80% over 2.5 min, increased from 80% to 100% over 0.1 min, held at 100% for 2.9 min, decreased back to 50% over 0.1 min and equilibrated at 40% for 0.5 min. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode using electrospray ionization in positive mode (ESI+), with a capillary voltage of 4.5 kV, a source temperature of 175°C, and a desolvation temperature of 300°C. Mass transitions and collision energy (CE) were optimized for each compound individually and analyte transitions and CEs can be found in Table 1. Quantitative analysis of plasma and brain drug levels were performed by comparing samples to standard curves prepared with known amounts of drug.

Table 1. Analyte mass transitions used for HPLC/MS/MS quantification.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Q1 (V)</th>
<th>CE (V)</th>
<th>Q3 (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAT229</td>
<td>343.1</td>
<td>296.3</td>
<td>-16</td>
<td>-13</td>
<td>-22</td>
</tr>
<tr>
<td></td>
<td>343.1</td>
<td>282.1</td>
<td>-25</td>
<td>-14</td>
<td>-20</td>
</tr>
<tr>
<td></td>
<td>343.1</td>
<td>193.2</td>
<td>-16</td>
<td>-29</td>
<td>-13</td>
</tr>
<tr>
<td>ABX-1431</td>
<td>508.0</td>
<td>228.3</td>
<td>-26</td>
<td>-20</td>
<td>-16</td>
</tr>
<tr>
<td></td>
<td>508.0</td>
<td>166.2</td>
<td>-24</td>
<td>-55</td>
<td>-29</td>
</tr>
<tr>
<td>Diazepam</td>
<td>284.6</td>
<td>257.0</td>
<td>-30</td>
<td>-21</td>
<td>-18</td>
</tr>
<tr>
<td></td>
<td>284.6</td>
<td>220.0</td>
<td>-30</td>
<td>-25</td>
<td>-25</td>
</tr>
</tbody>
</table>

Q1 = Quadrupole 1, precursor ion selection voltage, CE = Collision Energy, Q3 = Quadrupole 3, product ion selection voltage
Statistical analyses

Statistical analyses were performed using SPSS software, version 24 (IBM Corp, Armonk, New York USA). The ddRT-PCR transcript, protein and endocannabinoid and related-lipid data were analysed using two-way ANOVA, which compared main effects of background strain and genotype. If a strain by genotype interaction effect was identified, then planned Bonferroni’s comparisons were employed (129.WT vs 129.Scnn1a+/--; F1.WT vs F1.Scnn1a+/--; 129.WT vs F1.WT; 129.Scnn1a+/-- vs F1.Scnn1a+/--). For hyperthermia-induced seizures, threshold temperatures were compared using the time to event analysis (log-rank Mantel-Cox). For analytical analyses of experimental samples, plasma and brain concentrations for GAT229 were compared with unpaired t-tests; while ABX-1431 samples were compared with a one-way ANOVA, followed by Tukey’s post hoc tests. No significant sex differences were observed across all observable measures, and so groups were collapsed across sexes. Comparisons were statistically significant if \( p < 0.05 \).
Results

Figure 1. CB1 receptor mRNA and protein expression in WT and Scn1a¹⁺⁻ mice on 129 and F1 (129 x B6) background strains. (a) Relative transcript levels of the CB1 receptor gene, Cnr1, in the hippocampus was determined by ddRT-PCR and are expressed as a ratio of Tbp (n = 7 – 11 biological replicates). (b) Hippocampal protein expression of CB1, relative to β-tubulin, was assayed by western blot (n = 7 – 8 mice), with (c) a representative blot. A more elaborate representative blot can be found in the supplementary materials. Blank columns represent WT data while coloured columns represent Scn1a¹⁺⁻ data. Data is presented as the mean ± standard error of the mean (SEM) with squares representing primary mRNA pools and circles represent individual mouse data. Data was analyzed using a two-way ANOVA.
F1 seizure-susceptible background strain mice displayed reduced hippocampal Cnr1 mRNA and CB1 receptor protein than 129 seizure-resistant strain mice.

Seizure susceptible F1.Scn1a<sup>+/−</sup> mice start to develop spontaneous seizures between the 3<sup>rd</sup> and 4<sup>th</sup> post-natal weeks (Miller et al., 2014; Mistry et al., 2014). Thus, we examined whether hippocampal Cnr1 transcript and CB1 protein expression were altered at P24 using ddRT-PCR and western blotting, respectively. Cnr1 mRNA expression was modestly but significantly lower in the F1 seizure-susceptible strain compared to the 129 seizure-resistant strain (two-way ANOVA: strain, F<sub>1, 31</sub> = 7.178, p = 0.012). Heterozygous deletion of Scn1a did not influence Cnr1 transcript expression as there was no effect of genotype (F<sub>1, 31</sub> = 0.007, p = 0.986), nor a strain by genotype interaction (F<sub>1, 31</sub> = 1.495, p = 0.231). We then sought to confirm these transcriptional changes at the functional protein level using western blotting of the CB1 receptor in hippocampal samples (Figure 1b-c). CB1 protein concentrations relative to β-tubulin correlated with transcript expression as there was significantly less protein expression in F1 mice compared to 129 mice (two-way ANOVA: strain, F<sub>1, 27</sub> = 5.513, p = 0.026), with no effect of genotype (F<sub>1, 27</sub> = 3.801, p = 0.062) nor a strain by genotype interaction (F<sub>1, 27</sub> = 1.583, p = 0.219).
Figure 2. Hippocampal concentrations of endocannabinoids in WT and Scn1a+/- mice from 129 and F1 (129 x B6) background strains. Hippocampal concentrations of endocannabinoids (a) 2-AG and (b) AEA as determined by HPLC/MS/MS. Blank columns represent WT data while coloured columns represent Scn1a+/- data. Data is represented as the mean + SEM with individual circles representing individual mouse data (n = 14 – 16). Statistical comparison between groups were made using a two-way ANOVA.

F1 seizure-susceptible background strain mice displayed lower hippocampal 2-AG concentrations than 129 seizure-resistant mice.

Next we evaluated hippocampal concentrations of the two main endocannabinoids, 2-AG and anandamide (Figure 2), along with the structurally-related fatty acid amides, PEA, OEA, LEA and DHEA (Table 2) using HPLC/MS/MS. Hippocampal 2-AG concentrations were significantly lower in F1 seizure-susceptible mice compared to 129 seizure-resistant mice (Figure 2a; two-way ANOVA: strain, F_{1,56} = 4.7, p = 0.035) with no main effect of genotype (F_{1,56} = 0.025, p = 0.875) and no strain by genotype interaction (F_{1,56} = 1.161, p = 0.286). Alternatively, hippocampal
anandamide concentrations were not influenced by strain or genotype (Figure 2b). While there was no effects of strain or genotype on hippocampal concentrations of the other lipids DHEA, LEA, OEA and PEA (Table 2).

Table 2. Hippocampal concentrations of fatty acid amides in WT and Scn1a<sup>+/−</sup> mice from 129 and F1 (129 x B6) background strains, as determined by HPLC/MS/MS.

<table>
<thead>
<tr>
<th></th>
<th>129.WT</th>
<th>129.Scn1a&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>F1.WT</th>
<th>F1.Scn1a&lt;sup&gt;+/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEA</td>
<td>11.94 ± 2.71</td>
<td>12.02 ± 4.10</td>
<td>10.23 ± 3.24</td>
<td>10.34 ± 3.36</td>
</tr>
<tr>
<td>LEA</td>
<td>22.45 ± 6.66</td>
<td>25.10 ± 7.53</td>
<td>20.16 ± 6.55</td>
<td>21.57 ± 7.21</td>
</tr>
<tr>
<td>OEA</td>
<td>59.81 ± 26.24</td>
<td>67.46 ± 26.27</td>
<td>59.51 ± 21.48</td>
<td>60.99 ± 22.63</td>
</tr>
<tr>
<td>PEA</td>
<td>80.70 ± 22.08</td>
<td>91.00 ± 34.48</td>
<td>132.73 ± 124.31</td>
<td>95.17 ± 31.63</td>
</tr>
</tbody>
</table>

Data is represented as the mean ± SEM (pmol/g; n = 14 – 16). Statistical comparisons were made using a two-way ANOVA.
Figure 3. Hippocampal transcript expression of 2-AG’s synthesizing and catabolising enzymes in WT and Scn1a+/− mice from 129 and F1 (129 x B6) background strains. (a) Schematic diagram of 2-AG metabolism highlighting the genes coding the synthesizing and degradation enzymes. Hippocampal transcript expression of 2-AG’s synthesizing enzymes, (b) Dagl-α (Dagla) and (c) Dagl-β (Daglb); and 2-AG’s metabolizing enzymes, (d) Magl (Mgll), (e) Abhd6 (Abhd6) and (f) Abhd12 (Abhd12) as determined with ddRT-PCR and expressed as a ratio of Tbp. Data is presented as the mean + SEM with triangles representing mRNA superpools (n = 3 – 6 biological replicates). Blank columns represent WT data while coloured columns represent Scn1a+/− data. Data were analysed using a two-way ANOVA with planned Bonferroni comparisons, *p < 0.05.
2-AG metabolizing enzymes are less expressed in the hippocampus of seizure-susceptible mice and *Daglb* mRNA expression is selectively increased seizure-susceptible F1.*Scn1a*⁺/⁻ mice

After identifying altered 2-AG concentrations between the seizure-resistant and seizure-susceptible background strains, we measured the hippocampal transcript expression for 2-AG’s synthetic and metabolizing enzymes using ddRT-PCR (Figure 3). We did not detect any differences in the expression of 2-AG’s main synthesizing enzyme *Dagla* (Figure 3b). However, we found that heterozygous *Scn1a* deletion selectively increased *Daglb* mRNA expression, but only in mice on the seizure-susceptible F1 strain (Figure 3c). This was supported by a significant strain by genotype interaction (two-way ANOVA: F₁,₁₁ = 5.198, p = 0.044), with no main effects of strain (F₁,₁₁ = 0.812, p = 0.387) or genotype (F₁,₁₁ = 2.11, p = 0.174). Planned Bonferroni comparisons confirmed F1.*Scn1a*⁺/⁻ mice had significantly more *Daglb* mRNA expression than F1.WT mice (p = 0.016) and 129.*Scn1a*⁺/⁻ mice (p = 0.033). We also identified that heterozygous *Scn1a* deletion significantly reduced the degradative enzyme *Mgll’s* mRNA expression across both background strains (Figure 3d; two-way ANOVA: genotype, F₁,₁₁ = 6.728, p = 0.025; strain, F₁,₁₁ = 0.660, p = 0.434, strain by genotype interaction, F₁,₁₁ = 1.925, p = 0.193). Whereas *Abhd6* mRNA expression was significantly reduced in F1 mice compared to 129 mice (Figure 3e; two-way ANOVA: strain, F₁,₁₁ = 11.02, p = 0.007), with no effect of genotype (F₁,₁₁ = 0.908, p = 0.361) and no strain by genotype interaction (F₁,₁₁ = 0.255, p = 0.624). We did not observe any statistically significant differences in *Abhd12* mRNA expression (Figure 3f).
Figure 4. Efficacy of GAT229, a CB1 receptor PAM, and ABX-1431, a MAGL inhibitor, against hyperthermia-induced seizures in F1.Sc^n1a^/+ mice. Threshold temperature for GTCS induced by hyperthermia following administration of (a) vehicle or GAT229 (30 or 100 mg/kg); and (b) vehicle or ABX-1431 (3, 10 or 30 mg/kg). Data is shown as average temperatures of seizure induction + SEM, with open circles representing individual mouse data (n = 14 – 16 mice). Data was analysed with log-rank Mantel-Cox with mean temperature thresholds that are significantly greater than the vehicle group depicted in green, *p < 0.05 compared to vehicle.

Positive allosteric modulation of the CB1 receptor and inhibition of MAGL were anticonvulsant against hyperthermia-induced seizures.

The anticonvulsant efficacy of GAT229, a CB1 receptor PAM, and ABX-1431, a MAGL inhibitor, were assessed against hyperthermia-induced seizures in F1.Sc^n1a^/+ mice (Figure 4). Administration of 30 and 100 mg/kg of GAT229 equivalently and significantly elevated the temperature threshold for GTCS compared to vehicle control mice (Figure 4a, log-rank Mantel-Cox test: p = 0.035 and p = 0.024, respectively). Analysis of GAT229 concentrations from
experimental animals revealed there was no significant differences in plasma and brain concentration of the drug between the 30 and 100 mg/kg doses (Table 3; unpaired t-test: $t_6 = 0.19$, $p = 0.855$ and $t_{10} = 0.434$, $p = 0.673$, respectively).

Table 3. Plasma and brain concentrations of GAT229 at the time of testing against hyperthermia-induced seizures.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Plasma concentration (μg/mL)</th>
<th>Brain concentration (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>3.1 ± 0.2</td>
<td>7.6 ± 0.4</td>
</tr>
<tr>
<td>100</td>
<td>3.2 ± 0.2</td>
<td>7.9 ± 0.4</td>
</tr>
</tbody>
</table>

Data is represented as mean ± SEM ($n = 3 – 7$). Comparisons were made using unpaired t-tests.

Administration of 10 mg/kg dose of the MAGL inhibitor ABX-1431 significantly raised the body temperature threshold for hyperthermia-induced GTCS compared to vehicle controls (Figure 4b, log-rank Mantel-Cox test: $p = 0.0008$), whilst 3 and 30 mg/kg doses were ineffective. Despite plasma concentrations displaying a dose-dependent increase, with concentrations for the 30 mg/kg dose significantly greater than the 3 mg/kg dose (Table 4, one-way ANOVA: $F_{2,18} = 8.786$, $p = 0.002$; Tukey's post-hoc test, $p = 0.002$), there was no significant difference in brain concentrations between the administered doses (one-way ANOVA: $F_{2,12} = 1.725$, $p = 0.220$).
Table 4. Plasma and brain concentrations of ABX-1431 at the time of testing against hyperthermia-induced seizures.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Plasma concentration (ng/mL)</th>
<th>Brain concentration (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>198 ± 31</td>
<td>275 ± 71</td>
</tr>
<tr>
<td>10</td>
<td>373 ± 53</td>
<td>736 ± 154</td>
</tr>
<tr>
<td>30</td>
<td>883 ± 114***</td>
<td>536 ± 87</td>
</tr>
</tbody>
</table>

Data is represented as mean ± SEM (n = 5 - 7). Comparisons were made one-way ANOVA followed by Tukey’s post hoc tests, ***p < 0.001.

Discussion

There is a dire need for the development of better therapeutics for the treatment of Dravet syndrome and one strategy for drug discovery is the identification of genetic modifiers that contribute to Scn1a mutations’ incomplete phenotype penetrance. As the Dravet syndrome phenotype in the Scn1a<sup>−/−</sup> mouse model is dependent on the background strain of the mice, it provides a platform to identify these potential genetic modifiers by comparing the transcriptome of the seizure-susceptible and seizure-resistant background strains. The endocannabinoid system functions to regulate neuronal excitability and may therefore contain druggable targets for the treatment of Dravet syndrome (Kawamura et al., 2006; Sugaya et al., 2016; Takahashi & Castillo, 2006). When investigating the expression of endocannabinoid system-related genes in RNA-seq analysis, Cnr1 was more highly expressed in the F1 seizure-susceptible strain, highlighting it as a potential genetic modifier of Scn1a (Hawkins et al., 2019).

The current chapter sought for the first time to investigate whether key components of the endocannabinoid system are altered in the Scn1a<sup>−/−</sup> mouse model of Dravet syndrome, with an
emphasis on CB1 receptors. We confirmed that Cnr1 mRNA and functional CB1 protein concentrations were significantly lower in the hippocampus of F1 seizure-susceptible mice compared to seizure-resistant 129 strain mice. Moreover, we found F1 mice had significantly less hippocampal 2-AG concentrations, with no differences in anandamide concentrations between the strains. After detecting a potential deficit in the endocannabinoid system of susceptible F1 mice, we assessed the anticonvulsant potential of augmenting the endocannabinoid system and demonstrated that positive allosteric modulation of CB1 receptors and MAGL inhibition were anticonvulsant against hyperthermia-induced seizures in the phenotypic F1. Scn1a+/− mice. These data provide first evidence in support of Cnr1 being a putative genetic modifier of Scn1a.

We hypothesised that less hippocampal CB1 receptor mRNA and protein expression, along with less hippocampal 2-AG concentrations in the F1 mice may suggest reduced hippocampal endocannabinoid signalling in F1 mice compared to 129 mice. As 2-AG regulates neuronal excitability through CB1 receptors (Gao et al., 2010; Tanimura et al., 2010), deficient endocannabinoid signalling on excitatory neurons may contribute to the heightened neuronal excitability and seizures observed in F1. Scn1a+/− mice. We provide evidence in support of the endocannabinoid system double deficit hypothesis by showing that pharmacological potentiation of endocannabinoid signalling is anticonvulsant in Dravet syndrome mice. That is, both positive allosteric modulation of CB1 receptors with GAT229 and the irreversible MAGL inhibitor ABX-1431 were anticonvulsant against hyperthermia-induced seizures in F1. Scn1a+/− mice. To unequivocally demonstrate that CB1 receptors mediate the anticonvulsant effects of GAT229 and ABX1431, future studies could attempt to reverse the effects of these compounds using CB1 receptor antagonists. However, this may be problematic as CB1 antagonists have themselves
demonstrated proconvulsant actions in numerous conventional seizure models (Hsu et al., 2012; Perescis et al., 2017; Wallace et al., 2003).

As we only studied hippocampal CB1 receptor mRNA and protein expression in homogenate hippocampal tissue samples, we cannot distinguish between CB1 receptor expression on excitatory pyramidal neurons versus inhibitory interneurons (Howlett & Abood, 2017; Kano, 2014; Kawamura et al., 2006). Future studies could use single-cell sequencing or double-labelling immunofluorescence to evaluate the CB1 receptor mRNA or protein expression on specific neuronal populations in Scn1a+/− mice. CB1 receptors appear to be expressed more abundantly on inhibitory interneurons (Katona, 2015), so CB1 receptor activation would be anticipated to disinhibit neuronal excitability leading to proconvulsant effects. However, here the CB1 receptor PAM GAT229 and the MAGL inhibitor ABX-1431 both had anticonvulsant effects. Therefore, it appears more likely that the predominate effects of CB1 receptor activation is to reduce neuronal hyperexcitability through actions on CB1 receptors expressed on excitatory pyramidal neurons. This might be because CB1 receptors are more efficiently coupled to G proteins at excitatory synapses (Katona, 2015; Steindel et al., 2013). Moreover, CB1 receptors are almost exclusively expressed on cholecystokinin-expressing interneurons, which only account for 10-20% of all GABAergic neurons, and are different to the parvalbumin and somatostatin interneurons impacted by partial genetic deletion of Scn1a (Marsicano & Lutz, 1999; Whissell et al., 2015). Future electrophysiological experiments could be employed to disentangle the effects of GAT229 and ABX-1431 on excitatory and inhibitory transmission in Scn1a+/− mice.

This is the first demonstration of a CB1 receptor PAM having anticonvulsant activity. GAT229 displays no intrinsic activity at CB1 receptors, but instead potentiates CB1 receptor signalling in the presence of orthosteric ligands (Laprairie et al., 2017). Thus, the anticonvulsant
efficacy of GAT229 would suggest it potentiates the recruitment of the endocannabinoid system to counter seizure-associated hyperexcitability. Future studies are needed to show that seizures increase endocannabinoid transmission. The efficacy of GAT229 plateaued at the 100 mg/kg dose as its anticonvulsant effect was no greater than the effects of the 30 mg/kg dose. The plateau in efficacy may be due to GAT229 being poorly soluble and having poor absorption, as demonstrated by both doses yielding comparable plasma and brain concentrations. Unfortunately, due to the nature of Dravet syndrome being a paediatric disease, experiments are conducted on juvenile mice and we therefore cannot deliver drugs by central routes of administration. Consistent with the observation that F1 strain mice have less brain 2-AG concentrations, an alternative explanation for the limited anticonvulsant efficacy of GAT229 is that its positive allosteric modulation is limited by the restricted endocannabinoid tone in F1.Scn1a+/− mice. One possible approach to surmount this issue is to observe whether greater anticonvulsant effects can be attained through the co-administration of GAT229 with ABX1431 to increase concentrations of the orthosteric ligand 2-AG.

This Chapter provides novel evidence that a MAGL inhibitor has anticonvulsant effects in a mouse model of intractable, childhood epilepsy. Our results are consistent with MAGL inhibitors displaying anticonvulsant properties in other conventional seizure and epilepsy models (Sugaya et al., 2016; Terrone et al., 2018; von Ruden et al., 2015a; Zareie et al., 2018). ABX-1431 was selected as our test compound because it is a highly selective irreversible MAGL inhibitor that has good oral bioavailability, which is conducive to our future experiments that will deliver drug in mouse chow to assess effects on spontaneous seizures and survival (Cisar et al., 2018). There is also a chance for more rapid translation as ABX1431 has passed Phase 1 safety trials and is currently in Phase II clinical trials for the treatment of Tourette’s syndrome (NCT03625453).
Disappointingly, ABX-1431 demonstrated an inverted-U-shaped dose-response in the present study, with the middle dose level proving anticonvulsant but the lower and higher doses failing to demonstrate efficacy. We predict that ABX-1431 should dose-dependently increase brain 2-AG concentrations, as oral doses of ABX-1431 (> 2 mg/kg) dose-dependently increased 2-AG levels more than 3-fold (Cisar et al., 2018). However, we need to confirm that ABX-1431 increased brain 2-AG concentrations in our experimental mice, which might provide some insight into the inverted U-shaped dose-response.

Assuming a dose-response increase in 2-AG concentrations, one possible explanation for loss of efficacy at the higher ABX-1431 dose is that the excessive 2-AG concentrations impinge upon CB1 receptors expressed on GABA interneurons. This would result in suppression of inhibitions (disinhibition) that would counter and potentially override the anticonvulsant excitatory-suppressing effects of 2-AG observed at the lower dose. This hypothesis is supported by other MAGL inhibitors, including JZL184 and KML29, inhibit excitatory neurotransmission at low doses but inhibiting inhibitory transmission at higher doses (Wang et al., 2017b). Another possible explanation for ABX-1431’s loss of anticonvulsant efficacy at the higher dose is through high 2-AG concentrations exerting actions other excitatory targets. For example, 2-AG can behave as a negative allosteric modulator of adenosine A3 receptors and an agonist at GPR55 and TRPV1 receptors (Lane et al., 2010; Petrosino et al., 2016; Ryberg et al., 2007), which are all mechanism that could increase neuronal excitability (Kaplan et al., 2017; Naziroglu & Ovey, 2015; Shirazi et al., 2014; Swiader, Kotowski & Luszczki, 2014).

The current Chapter also investigated the expression of 2-AG’s synthetic and degradative enzymes at the transcript level. Heterozygous Scn1a deletion downregulated 2-AG’s main degradative enzyme, Mgll, as well as another catabolising enzyme Abhd6 in F1 mice. Together,
these expression patterns imply that F1.Scn1a<sup>+/−</sup> mice may have increased 2-AG concentrations. However, our finding that the F1 background strain displayed less hippocampal 2-AG concentrations mismatched our findings of changes in 2-AG synthetic and metabolizing enzymes. This inconsistency could occur due to tissue samples being collected in different laboratories with different sampling times, as it is well-established that endocannabinoids concentrations fluctuate with circadian rhythms (Liedhegner, Sasman & Hillard, 2014; Valenti et al., 2004). Future studies could assess whether changes in these enzymes occur at a functional protein level, in conjunction with measuring endocannabinoid concentrations in the same animals.

A notable finding from this work was the increase of Daglb mRNA expression selectively in the phenotypic F1.Scn1a<sup>+/−</sup> mice. Although both the α and β isoforms of DAGL synthesise 2-AG, the β isoform is almost exclusively centrally expressed in microglia and doesn’t overly influence global 2-AG levels as demonstrated in Daglb knock-out mice (Tanimura et al., 2010; Viader et al., 2016; Wilkerson et al., 2016). Microglia are the immunocompetent cells of the CNS (Kettenmann et al., 2011), with microglial 2-AG concentrations increasing in response to injury and eliciting a protective role (Hohmann et al., 2019; Kreutz et al., 2009; Kreutz et al., 2007; Stella, 2009; Viader et al., 2016; Walter et al., 2003). Additionally, Dagl-β enzymes function is indirectly responsible for the production of inflammatory cytokines, including PGE2, IL-6, TNFα (Hsu et al., 2012). The increased expression of Daglb in F1.Scn1a<sup>+/−</sup> mice then suggests that these mice may have increased microglial 2-AG concentrations, which may be occurring in response to neuronal damage. While the increased Daglb mRNA expression could be due to an increase in Daglb on microglia, it could also be due to an increase in microglia cell density. Future work could provide a more detailed appraisal of immune function and neuroinflammation in Dravet syndrome mice. Assessment of endocannabinoid concentrations from isolated microglia may not be feasible,
but triple-immunolabelling with probes for microglia (Iba-1), Dagl-β and CB2 receptors would inform on microglial cell count and morphology (Carlisle et al., 2002).

In summary, we present evidence for a potential double deficit in the endocannabinoid system of seizure-susceptible F1 Dravet syndrome mice with reduced expression of hippocampal CB1 receptors and 2-AG concentrations. Moreover, we demonstrated potentiation of endocannabinoid signalling through positive allosteric modulation of CB1 receptors and MAGL inhibition was anticonvulsant against hyperthermia-induced seizures in F1.Scna1a±/− mice, supporting Cnr1 as a putative genetic modifier of Scn1a. Future work extending upon these experiments should investigate the efficacy of augmenting endocannabinoid signalling on the other phenotypes displayed by the Scn1a±/− mice, including spontaneous seizures, premature mortality and behavioural comorbidities.
Supplementary Figure 1. An example of a western blot used to evaluate hippocampal cannabinoid receptor type 1 (CB1) expression in WT and Scn1a\textsuperscript{+/+} mice on 129 and F1 (129 x B6) background strains. A cellulose membrane was imprinted with hippocampal protein fractions that were then probed with both CB1 (1:200, Abcam, ab23703) and β-tubulin (1:500, Sigma-Aldrich, T5201) antibodies labelled with immunofluorescent secondary antibodies (Alexa Fluor 800 (A32735) and 680 (A28183), respectively), before being imaged at (a) 700 nm, (b) 800 nm wavelengths. (c) Both 700 nm and 800 nm images overlayed. F, female; M, male; WT, wildtype; 129 and F1 (129 x B6) represent background strains; white arrow points to CB1 protein (predicted molecular weight: 53 kDa). β-tubulin’s predicted molecular weight is 50 kDa.
Chapter 3

Targeting Gpr55 as a treatment strategy in a $Scn1a^{+/-}$ mouse model of Dravet syndrome
Introduction

Dravet syndrome is a catastrophic and intractable childhood epilepsy with patients experiencing poor quality of life due to unmanaged seizures, associated behavioural comorbidities and significant risk of SUDEP (Cooper et al., 2016; Dravet et al., 2005; Dravet & Oguni, 2013). Dravet syndrome patients are refractory to current treatments, emphasizing the need to develop novel therapeutics (Knupp & Wirrell, 2018). Over 80% of patients with Dravet syndrome express loss-of-function mutations in SCN1A, which encodes the α1-subunit of voltage gated sodium channel (Na\textsubscript{V}1.1) that is predominantly expressed on inhibitory neurons (De Jonghe, 2011; Depienne et al., 2009; Marini et al., 2011); thereby resulting in hyperexcitability through disinhibition. However, carrying a mutation in SCN1A does not guarantee development of a severe epilepsy phenotype (Kearney, 2011; Meisler & Kearney, 2005), suggesting additional factors influence disease severity.

Incomplete penetrance of the Scn1a phenotype is also observed in the Scn1a\textsuperscript{+/−} mouse model of Dravet syndrome. Heterozygous deletion of Scn1a on a 129 background strain shows no overt phenotype but gene deletion on a mixed F1 (129 x C57BL/6) background yields a severe epilepsy phenotype corresponding to what is observed in patients, with febrile seizures, spontaneous seizures, premature mortality, along with behavioural and cognitive impairments (Miller et al., 2014; Mistry et al., 2014; Yu et al., 2006; Chapter 4). Phenotypic Scn1a\textsuperscript{+/−} mice also respond to pharmacological agents in a way that is similar to patients, further validating the reliable use of this model for drug development (Anderson et al., 2019a; Hawkins et al., 2017; Kaplan et al., 2017). The sensitivity of the Dravet syndrome phenotype to background strain in mice indicates that non-Scn1a genes are influencing the severity of the phenotype, which are termed ‘genetic modifier’ genes and may serve as novel drug targets. G protein-couple receptor 55 (Gpr55) was
identified as a genetic modifier candidate when its mRNA was shown to be more highly expressed in the hippocampus of seizure-susceptible F1 (129S6/SvEvTac x C57BL/6J) mice than the seizure-resistant 129S6/SvEvTac mice in an RNA-seq analysis of a Scn1a+/− mouse model (Hawkins et al., 2019).

GPR55 is being investigated as a target for pathologies including cancer, metabolism inflammation and pain (Alhouayek, Masquelier & Muccioli, 2018; Simcocks et al., 2018), but our understanding of its function in the CNS is still developing. We know that Gpr55 receptors are expressed in brain regions including the hippocampus and cortex (Henstridge et al., 2011; Ryberg et al., 2007; Wu et al., 2013). Moreover, they couple to Ga12/13 and/or Gaq proteins in a ligand-dependent manner with their activation leading to an increase in cell excitability, predominantly through increased intracellular Ca2+ concentrations (Henstridge et al., 2009; Lauckner et al., 2008; Oka et al., 2010; Ryberg et al., 2007). GPR55’s endogenous ligands include L-α-lysophosphatidylinositol (LPI) analogues, which are specific for GPR55 (Kapur et al., 2009; Oka et al., 2007; Oka et al., 2009), along with the endocannabinoids anandamide and 2-AG, their congener palmitoylethanolamide (PEA), and by various peptides including pituitary adenylate cyclase activating peptide-27 (PACAP-27) (Foster et al., 2019; Ryberg et al., 2007).

Supporting Gpr55’s role as a genetic modifier and its potential to influence the Dravet syndrome phenotype is its ability to modulate neuronal excitability. Within the hippocampus, Gpr55 receptors have been shown to be distributed presynaptically on glutamatergic neurons (Sylantyev et al., 2013; Wu et al., 2013), with their activation stimulating glutamatergic release and subsequently promoting excitatory transmission (Deliu et al., 2015; Hurst et al., 2017; Kramar et al., 2017; Lauckner et al., 2008; Sylantyev et al., 2013). Moreover, inhibition of Gpr55 has been attributed as the anticonvulsant mechanism of cannabidiol (CBD), which has proven anticonvulsant
in three phase III trials for patients with Dravet syndrome (Devinsky et al., 2017; Devinsky et al., 2019; Devinsky et al., 2018a) and in two Scn1a+/− mouse models (Anderson et al., 2019a; Kaplan et al., 2017).

In this study we aim to utilise a Scn1a+/− mouse model of Dravet syndrome to measure key constituents of the Gpr55-LPI system with quantitative PCR and mass spectrometry. Then we aim to investigate the contribution of Gpr55 to the severe epilepsy phenotype by examine the impact of heterozygous Gpr55 deletion on key Scn1a+/− phenotype features. We hypothesise that Gpr55 mRNA expression will be greater in F1 seizure-susceptible mice and that genetic downregulation of Gpr55 expression will attenuate features of the Dravet syndrome phenotype.

Materials and Methods

Animals

All research and animal care procedures were approved by the University of Sydney Animal Ethics Committee in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and by the Northwestern University Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Scn1a+/− mice were generated by targeted deletion of Scn1a exon 1 as previously described (Miller et al., 2014) and were maintained by continuous backcrossing to the 129S6/SvEvTac (129) background strain (129.Scn1a+/−). For the Australian studies, 129.Scn1a+/− mice were obtained from The Jackson Laboratory (MMRRC Stock No: 37107-JAX). To generate mice for experiments, 129.Scn1a+/− mice were crossed with C57BL/6J (B6) resulting in [129 x B6] F1.Scn1a+/− mice and wildtype mice (WT). Mice with homozygous Gpr55 deletion on a F1 (129 x B6, 50:50) strain background (F1.Gpr55−/−) were received as a gift from Prof Ken Mackie, Indiana
University, and were rederived (Australian BioResources) using B6 mice to generate mice heterozygous for Gpr55 on a N2 (129 x B6, 75:25) background strain (N2.Gpr55+/−). The gene transcription studies were performed at Northwestern University, U.S.A, where the mouse facility operated on a 14 h light/10 h dark cycle. All other work was performed at the University of Sydney, Australia, where the facility was kept on a 12 h light/dark cycle. All animals were group housed in IVC cages and had ad libitum access to food and water. For Scn1a and Gpr55 genotyping, primers and PCR conditions were performed as previously described (Miller et al., 2014; Wu et al., 2013, respectively).

**Quantitative digital droplet PCR (ddRT-PCR)**

Confirmation of transcript expression with ddRT-PCR for our genes of interest was performed as previously described (Hawkins et al., 2019; Hawkins et al., 2016). Briefly, hippocampal and cortical samples were dissected from male and female WT and Scn1a+/− littermates from both 129 and F1 background strains at P24. In order to sufficiently increase RNA yield for gene analysis, tissue from 3 – 4 mice, with at least one male and female in each group to control for potential sex-dependent differences, were combined to generate primary RNA pools. Primary pools were used to evaluate Gpr55 mRNA expression. Primary RNA pools (3 – 4) were combined into super RNA pools (n = 12 – 16 mice/superpool) to evaluate Pla2g4a, Mboat7, Ddhd1 and Lclat1 mRNA expression. First-strand cDNA was synthesized then ddRT-PCR was performed using TaqMan Gene Expression Assays (Life Technologies) for mouse Ddhd1 (Mm00616337_m1); Gpr55 (Mm02621622_s1); Lclat1 (Mm01237235_m1); Mboat7 (Mm00513220_m1); Pla2g4a (Mm00447040_m1); Tbp (Mm00446971_m1). After amplification, droplets were analysed and relative transcript levels of target genes were expressed as a ratio of the gene to Tbp concentrations.
Cell culture

HEK wildtype cells and those expressing human GPR55 (hGPR55) were obtained from the Medical University of Graz (Kargl et al., 2012). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum (Sigma-Aldrich) and 100 U penicillin plus 100 μg/mL streptomycin (Gibco) and grown at 37°C and 5% CO₂. Additionally, hGPR55 cells were grown under selection (800 μg/mL G418; Invitrogen). When confluent, cells were lysed in RIPA plus complete protease inhibitor (Roche) with constant agitation for 35 min at 4°C. Cells were centrifuged at 18,000 g for 30 min (4°C) and the supernatant was stored at -20°C until analysed.

LPI analysis

Quantification of 1-stearoyl (18:0) and 2-arachidonoyl (20:4) LPI were determined via high-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS). Male WT and Scn1a+/− littermates (P24) from both 129 and F1 backgrounds were euthanized via cervical dislocation between 12:00 and 12:15. Brains were rapidly extracted and flash frozen with liquid nitrogen. Hippocampal sections were dissected on ice then snap-frozen in liquid nitrogen and stored at -80°C until analysed. Hippocampal dissections were homogenized in methanol (3000 µL) and spiked with 17:1 LPI as an internal standard. Homogenates were centrifuged at 20,000 g for 20 min at 4°C. Supernatants underwent solid phase extraction using 500 mg C18 solid-phase extraction columns (Agilent Technologies, Santa Clara, CA) to collect lipids in an elution of 65%, 85% and 100% methanol. Elutants (20 µL) were separated using a C18 Zorbax reversed-phase analytical column (Agilent Technologies, Santa Clara, CA) coupled to a Shimadzu 8030 triple quadrupole mass spectrometer. The mass spectrometer was operated in positive electrospray
ionization mode with multiple reaction monitoring. LPI analogues, 1-stearoyl (18:0) and 2-arachidonoyl (20:4) were quantified by comparing samples to standards prepared with known amounts of each analogue. Total LPI content was determined by combining concentrations from each of the elutions. Internal standards, 17:1 Ly.so PI, 18:0 Lyso PI and 20:4 Lyso PI, were purchased from Avanti Polar Lipids (USA).

Genetic deletion of Gpr55 in Scn1a+/− mice

To investigate the contribution of Gpr55 to the Scn1a+/− phenotype, Scn1a+/− mice with or without heterozygous deletion of Gpr55 were generated. N2.Gpr55+/− mice were crossed with 129.Scnn1a+/− mice to generate Scn1a+/− mice on a mixed 129:B6 (62.5:37.5) background strain. Scn1a+/−; Gpr55+/+ and Scn1a+/−; Gpr55+/− mice were used for experiments. Different cohorts of mice were used for hyperthermia-induced seizure testing to those used to assess spontaneous seizures and survival.

Hyperthermia-induced seizures

Hyperthermia-induced seizures were performed in mice aged P14 – 16 as previously described (Hawkins et al., 2017) using a rodent temperature regulator (TCAT-2DF, Physitemp Instruments, Inc, Clifton, NJ) reconfigured with a Partlow 1160 + controller (West Control Solutions, Brighton, UK) that was connected to a heat lamp and RET-3 rectal temperature probe. Briefly, core body temperature was elevated using an overhead heatlamp by 0.5°C every 2 min until a generalized tonic-clonic seizure (GTCS) occurred or 42.5°C was reached. If body temperature reached 42.5°C, then it was maintained for 3 min and if no GTCS occurred within this time then the mouse was considered seizure-free.

Spontaneous seizure frequency and survival monitoring
Spontaneous seizure frequency monitoring was performed as previously described (Hawkins et al., 2017). At P18, a single, brief GTCS was induced by hyperthermia as described above then immediately terminated by rapidly cooling body temperature back down to 37°C with the use of a cooling pad. Spontaneous GTCS were then quantified over 60 h of continuous video recording from 12:00 on P19 through to 00:00 on P22. The number of spontaneous GTCS were quantified from the video recordings by a researcher blind to the conditions. On P22 the mice were returned to their home-cages and survival was monitored until P30.

**Statistical analysis**

SPSS software was used to perform statistical analyses, version 24 (IBM Corp, Armonk, New York USA). For ddRT-PCR transcript and LPI analyses, statistical comparisons were made using two-way ANOVA. If a strain by genotype interaction effect was identified, then Bonferroni’s planned comparisons were employed (129.WT vs 129.Scnn1a+/--; F1.WT vs F1.Scnn1a+/--; 129.WT vs F1.WT; 129.Scnn1a+/--; vs F1.Scnn1a+/--). Log-rank Mantel-Cox was used to compare data from hyperthermia-induced seizure and survival experiments. For spontaneous seizures, statistical comparisons were conducted using Fisher’s exact test (proportion seizure-free and proportion of hindlimb seizures) and Mann-Whitney U test (seizure frequency). No significant sex differences were observed across all observable measures, so groups were collapsed across sexes. Comparisons were deemed statistically significant if \( p < 0.05 \).
Results

Figure 1. Hippocampal expression of Gpr55 transcript was elevated in F1 seizure-susceptible mice. Relative hippocampal Gpr55 transcript expression of WT (blank columns) and Scn1a+/− (coloured columns) mice on 129 and F1 genetic background strains. Expression was determined by ddRT-PCR and expressed as a ratio to Tbp (n = 7 – 11 biological replicates). Data is represented as the mean + SEM, with squares representing primary RNA pools (3 – 4 mice/primary RNA pool). Statistical comparisons between groups were made using a two-way ANOVA.

Hippocampal Gpr55 transcript expression is elevated in the F1 seizure-susceptible mouse strain

In order to confirm RNA-seq findings reported by Hawkins et al. (2019), we conducted ddRT-PCR to measure Gpr55 mRNA expression in the hippocampus of WT and Scn1a+/− mice on both 129 and F1 genetic backgrounds. Hippocampal Gpr55 mRNA expression was significantly higher in mice on the F1 seizure-susceptible strain compared to mice on the 129 seizure-resistant
strain (Figure 1; two-way ANOVA: strain, $F_{1,31} = 63.102, p < 0.0001$). There was no effect of genotype ($F_{1,31} = 0.811, p = 0.375$) nor a strain by genotype interaction ($F_{1,31} = 0.000, p = 0.986$).

We then aimed to evaluate whether this difference in mRNA expression would correlate with a change in Gpr55 protein expression. Unfortunately, we were unable to address this question as three commercially-available GPR55 antibodies lacked specificity for Gpr55 (see Supplementary material).

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**Figure 2. Hippocampal concentrations of LPIs in WT and Scn1a$^{+/−}$ mice on 129 and F1 background strains.** Hippocampal concentrations of (a) 2-arachidonoyl LPI and (b) 1-stearoyl LPI, the main endogenous ligands for Gpr55, in WT (blank columns) and Scn1a$^{+/−}$ (coloured columns) mice on 129 and F1 genetic background strains were determined with HPLC/MS/MS ($n = 7–9$ mice). Data is shown as the mean + SEM, with circles representing individual mice. Statistical comparisons between groups were made using a two-way ANOVA, followed planned Bonferroni’s comparisons with $*p < 0.05$. 
Hippocampal concentrations of 2-arachidonoyl LPI and 1-stearoyl LPI were lower in F1 mice and heterozygous Scn1a deletion selectively reduced 2-arachidonoyl LPI on the 129 strain.

While there are 6 LPI analogues (1-palmitoyl (16:0), 1-stearoyl (18:0), 1- or 2-oleoyl (18:1), 2-linoleoyl (18:2) and 2-arachidonoyl (20:04) LPI) known to activate Gpr55 (Oka et al., 2009), 2-arachidonoyl LPI and 1-stearoyl LPI are the most abundant in rodent brains (Inloes, Jing & Cravatt, 2018; Oka et al., 2009). Additionally, they are the most potent and efficacious ligands for human GPR55-expressing cells (Oka et al., 2009). Therefore, we measured the hippocampal concentrations of these two main LPI analogues, 2-arachidonyl and 1-stearoyl LPI, using HPLC-MS/MS. We determined that heterozygous Scn1a deletion selectively reduced hippocampal 2-arachidonoyl LPI concentrations but only in mice on the seizure-resistant 129 background strain (Figure 2a). This was supported by a significant strain by genotype interaction (two-way ANOVA: F1, 28 = 5.675, p = 0.024), with no main effects of strain (F1, 28 = 0.618, p = 0.438) or genotype (F1, 28 = 1.954, p = 0.173). Planned Bonferroni comparisons confirmed that 2-arachidonoyl LPI concentrations were significantly greater in 129.WT mice compared to 129.Scn1a+/− mice (p = 0.015), and compared to F1.WT mice (p = 0.038). In comparison, a strain-dependent effect was observed for 1-stearoyl LPI, with significantly lower concentrations in the F1 mice compared to the 129 mice (Figure 2b: two-way ANOVA: main effect of strain, F1, 29 = 5.902, p = 0.022; no main effect of genotype, F1, 29 = 1.635, p = 0.211; and no strain by genotype interaction, F1, 29 = 0.853, p = 0.184).
Figure 3. Hippocampal mRNA expression of the best characterized LPI synthetic and catabolic enzymes in the Scn1a+/− mouse model. (a) A schematic diagram depicting components of 1-stearoyl and 2-arachidonoyl LPI’s metabolism. Genes are shown in parenthesis. Phosphatidylinositol (PI) is converted to 2-arachidonoyl LPI and 1-stearoyl LPI by phosphatidic
acid-preferring phospholipase A1 (PA-PLA$_1$; Ddhd1) and phospholipase A$_2$ (cPLA$_2$; Pla2g4a), respectively. While 2-arachidonoyl LPI and 1-stearoyl LPIs are converted back to PI through 1-acylglycerol-3-phosphate acyltransferase 8 (AGPAT8; Lclat1) and membrane-bound O-acyltransferase 7 (MBOAT7; Mboat7), which is also known as LPI-acyltransferase-1 (LPIAT1). Transcript expression of (b) Pla2g4a, (c) Mboat7, (d) Ddhd1 and (e) Lclat1 in WT (blank columns) and Scn1a$^{+/−}$ (coloured columns) mice on 129 and F1 genetic background strains. Expression was determined using ddRT-PCR and expressed as a ratio of Tbp ($n = 3 − 5$ biological replicates). Data represents the mean + SEM, with triangles representing mRNA superpools ($n = 12 − 16$ mice/superpool). Statistical comparisons between groups were made using a two-way ANOVA.

**Heterozygous Scn1a deletion decreased hippocampal expression of the LPI metabolising enzyme Mboat7.**

Since significant differences in LPI levels were observed across strains, we aimed to determine whether this resulted from differences in the synthesis or degradation. We evaluated the mRNA expression of the best-characterised enzymes involved in the synthesis and metabolism of these LPI analogues using ddRT-PCR (**Figure 3a**). Briefly, 2-arachidonoyl LPI is synthesized by PA-PLA$_1$, which is encoded by Ddhd1, while 1-stearoyl LPI in synthesised by cPLA$_2$, encoded by Pla2g4a (Alhouayek, Masquelier & Muccioli, 2018). Then, 2-arachidonoyl LPI and 1-stearoyl LPIs are converted back to their precursor by AGPAT8, encoded by Lclat1, and MBOAT7, encoded by Mboat7, respectively (Alhouayek, Masquelier & Muccioli, 2018). We found no effects of strain or genotype in the mRNA expression of Pla2g4a or Lclat1 (**Figures 3b and e**, respectively). Although Ddhd1 mRNA expression appears to be greater in the F1 seizure-susceptible strain compared the 129 seizure-resistant strain, the difference was not statistically significant (**Figure 3d**: two-way ANOVA, strain: $F_{1, 11} = 3.826, p = 0.076$). However,
heterozygous deletion of Scn1a significantly reduced Mboat7 mRNA expression (Figure 3c; two-way ANOVA: genotype, $F_{1, 10} = 6.932, p = 0.025$), with no effect of strain ($F_{1, 10} = 0.28, p = 0.609$) nor a strain by genotype interaction ($F_{1, 10} = 0.123, p = 0.733$, respectively).

Figure 4. Heterozygous Gpr55 deletion was anticonvulsant and restored the survival deficit in Scn1a+/- mice. (a) Threshold temperatures for hyperthermia-induced GTCS of individual Scn1a+/- mice with WT expression of Gpr55 (Scn1a+/-; Gpr55+/-) or heterozygous deletion of Gpr55 (Scn1a+/-; Gpr55+/-). Heterozygous deletion of Gpr55 significantly increased the temperature threshold for GTCS of Scn1a+/- mice (green, open symbols). Data is shown as average temperatures of GTCS ± SEM, with open circles representing individual mice ($n = 14–16$ mice, log-rank Mantel-Cox, *$p < 0.05$). (b) Spontaneous GTCS frequency of individual animals (open symbols) quantified over a 60 h period ($n = 16–18$ mice, Fisher’s exact test). Expression of Gpr55
had no effect on spontaneous seizure frequency or the proportion of mice that had spontaneous seizures. (c) Total number of spontaneous GTCS with or without tonic hindlimb extension. Heterozygous Gpr55 expression significantly the proportion of seizures that progressed to tonic hindlimb extension \((n = 16 – 18\) mice, Fisher’s exact test, \(*p < 0.05\)). (d) Survival curves comparing Scn1a\(^{+/−}\); Gpr55\(^{+/+}\) and Scn1a\(^{+/−}\); Gpr55\(^{+/−}\) mice. Survival of Scn1a\(^{+/−}\) mice was significantly improved with heterozygous Gpr55 deletion (green line) \((n = 16 – 18\) mice, log-rank Mantel-Cox, \(p < 0.05\)).

**Heterozygous Gpr55 deletion has anticonvulsant effects against hyperthermia-induced seizures and restores survival in Scn1a\(^{+/−}\) mice.**

We utilized Gpr55\(^{+/−}\) mice to evaluate whether the increased Gpr55 transcript expression of seizure-susceptible F1 mice contributed to the severe epilepsy phenotype of F1. Scn1a\(^{+/−}\) mice. We generated Scn1a\(^{+/−}\) mice that had WT expression of Gpr55 (Scn1a\(^{+/−}\); Gpr55\(^{+/+}\)) or heterozygous deletion of Gpr55 (Scn1a\(^{+/−}\); Gpr55\(^{+/−}\)) and then assessed the effect on hyperthermia-induced seizures, spontaneous seizures and survival. We observed that heterozygous Gpr55 deletion elicited an anticonvulsant effect against hyperthermia-induced seizures in Scn1a\(^{+/−}\) mice. Scn1a\(^{+/−}\); Gpr55\(^{+/−}\) mice had a significantly increased temperature thresholds for GTCS compared to Scn1a\(^{+/−}\); Gpr55\(^{+/+}\) mice (Figure 4a; log-rank Mantel-Cox test: \(p = 0.038\)).

We then examined whether heterozygous Gpr55 deletion would be anticonvulsant against spontaneous seizures (Figure 4b). Spontaneous seizures were observed in 39% \((7/18)\) of Scn1a\(^{+/−}\); Gpr55\(^{+/+}\) mice with an average frequency of 0.08 GTCS per hour, compared to 19% \((3/16)\) of Scn1a\(^{+/−}\); Gpr55\(^{+/−}\) mice with an average frequency of 0.02 GTCS per hour (Figure 4b). While it appeared as though heterozygous Gpr55 deletion reduced the number of Scn1a\(^{+/−}\) mice that exhibited spontaneous seizures, it was not statistically significant (Fisher’s Exact test: \(\chi^2_1 = 1.655,\)
Nor was there a difference in overall seizure frequency (Mann-Whitney U test: $U = 115, p = 0.217$). In addition to quantifying seizure frequency, we also monitored the effect of heterozygous $Gpr55$ deletion on seizure severity. Each GTCS seizure was scored for progression to the most severe stage of full tonic hindlimb extension (hindlimbs at 180 to torso). Interestingly, despite $Scn1a^{+/—}; Gpr55^{+/—}$ mice exhibiting a smaller number of total seizures, a significantly larger proportion of the total seizures advanced to hindlimb extension ($10/23 = 0.435$) compared to $Scn1a^{+/—}; Gpr55^{++/—}$ mice ($18/83 = 0.217$) (Figure 4c; Fisher's exact test: $\chi^2 = 4.4, p = 0.036$).

Finally, we assessed the effect of heterozygous $Gpr55$ deletion on survival of $Scn1a^{+/—}$ mice. Survival of $Scn1a^{+/—}$ mice was significantly improved with heterozygous deletion of $Gpr55$ (Figure 4d; Mantel-Cox log-rank test: $p = 0.025$). All $Scn1a^{+/—}; Gpr55^{++/—}$ mice ($n = 16$) survived to P30 compared to only 72% (13/18) of the $Scn1a^{+/—}; Gpr55^{++/—}$ mice.

**Discussion**

$Gpr55$ was highlighted as a potential genetic modifier when an RNA-seq analysis reported more than double the mRNA expression in the seizure-susceptible F1 background strain compared to the seizure-resistant 129 background strain in a $Scn1a^{+/—}$ mouse model (Hawkins et al., 2019). In support of $Gpr55$’s potential to influence the Dravet syndrome phenotype is $Gpr55$ receptor activation promoting hippocampal excitatory transmission (Hurst et al., 2017; Kramar et al., 2017; Sylantyev et al., 2013), and CBD’s anticonvulsant efficacy being attributed to $Gpr55$ inhibition (Kaplan et al., 2017). We hypothesised that the increased $Gpr55$ mRNA expression observed in the F1 may be increasing excitatory neurotransmission, thereby exacerbating the neuronal hyperexcitability and contributing to the severe seizure phenotype observed in F1.$Scn1a^{+/—}$ mice. Therefore, this chapter further investigated the potential of $Gpr55$ as a genetic modifier in the $Scn1a^{+/—}$ mouse model of Dravet syndrome.
We determined that mice on the mixed F1 seizure-susceptible background strain express significantly more hippocampal Gpr55 transcript than mice on the 129 seizure-resistant strain, reaffirming that Gpr55 may be a genetic modifier of Scn1a. Additionally, we demonstrated that hippocampal concentrations of 2-arachidonoyl and 1-stearoyl LPI, the major endogenous ligands of Gpr55, were altered in distinct strain- and genotype-dependent manners. Moreover, we also demonstrated that heterozygous deletion of Gpr55 was anticonvulsant against hyperthermia-induced seizures and ameliorated the survival deficit normally observed in the F1. Scn1a+/− mice.

We found significantly less 1-stearoyl LPI levels in mice on the F1 genetic background compared to those on a 129 background strain. This strain-dependent reduction was only apparent in WT mice for 2-arachidonoyl LPI. Intriguingly, on the 129 background strain specifically, significantly less 2-arachidonoyl LPI was measured in Scn1a+/− mice than in WT mice. We hypothesise that the observed reduction of LPI concentrations in Scn1a+/− mice on the 129 background may be an adaptive mechanism to counter the neuronal hyperexcitability caused by Nav1.1 haploinsufficiency. Reduced concentrations of 2-arachidonoyl LPI would likely lead to less Gpr55 activation and consequently less network excitability. F1. Scn1a+/− mice did not exhibit this same reduction in 2-arachidonoyl LPI levels compared to F1.WT mice, which in the context of the increased Gpr55 expression of F1 mice could contribute to the severe epilepsy phenotype observed in F1. Scn1a+/− mice. Future studies might investigate whether administration of 2-arachidonoyl LPI or a brain-penetrant GPR55 agonist would triggers seizures in 129. Scn1a+/− mice.

We wanted to determine whether the differences that we observed in LPI levels were the result of inherent differences in expression of the synthesizing or metabolizing enzymes. Therefore, this chapter also assessed transcript expression of the best-characterised LPI-modulating enzymes. We report that heterozygous deletion of Scn1a reduced expression of
Mboat7, an enzyme that metabolizes 1-stearoyl LPI, irrespective of background strain. However, this does not correlate with the differences observed with 1-stearoyl LPI, which showed a strain-dependent effect with reduced levels in F1 mice. The other enzymes examined showed no significant differences in gene expression.

Although differences in mRNA expression of these enzymes does not account for the observed differences in LPI levels, it does not discount that there could be differences in protein expression. Future experiments could determine whether mRNA expression of these enzymes correlates with a functional change in protein expression. In this chapter we chose to investigate the expression of the major synthesizing and metabolizing enzymes for LPI but the complexity of the pathways involved in LPI metabolism may mean we missed additional key enzymes. For example, LPI analogues can also degraded by lysophospholipase A isoforms 1 and 2 (Wepy et al., 2019), an isoform of phospholipase C specific for LPI for which the gene has not yet been identified (Murase & Okuyama, 1985; Tsutsumi et al., 1994; Ueda et al., 1993), autotaxin, a lysophospholipase D isoform (Aoki et al., 2002; Xie & Meier, 2004), and a/b hydrolase domain 6 (Thomas et al., 2013).

One noteworthy finding from the work in this chapter was that heterozygous Scn1a deletion downregulated Mboat7 transcript expression irrespective of background strain. As one of 1-stearoyl LPI’s degradative enzymes, the reduction in Mboat7 transcript expression did not correspond with the reduced 1-stearoyl LPI concentrations observed. Mboat7 is an LPI-specific acyltransferase involved in phospholipid remodelling of membranes (Gijon et al., 2008). Specifically, Mboat7 transfers arachidonic acid to 1-stearoyl LPI to form 1-stearoyl-2-arachidonoyl PI, an important membrane constituent (Anderson et al., 2013; Lee et al., 2012). Mboat7 has also been implicated in the synthesis and metabolism of additional lipid mediators,
including other species of LPI, PIs, and phosphatidylinethanolamines (PEs) (Anderson et al., 2013; Lee et al., 2012). This suggests that the hippocampal concentrations of these other lipid mediators may be altered by heterozygous Scn1a deletion and calls for a more comprehensive lipidomic investigation of the Scn1a+/− mouse model that future work could address.

Interestingly, loss-of-function gene variants of MBOAT7 have been associated with a form of intellectual disability that is accompanied by autism spectrum disorder and epilepsy in humans (Jacher et al., 2019; Johansen et al., 2016; Yalnizoglu et al., 2019). Moreover, mice with homozygous Mboat7 deletion exhibited cortical and hippocampal atrophy due to disordered cortical lamination and premature death (Anderson et al., 2013; Lee et al., 2012). Dravet syndrome patients and Scn1a+/− mouse models have also reported associated behavioural comorbidities including cognitive impairments, along with cortical atrophy (particularly white matter) and decreased lifespan (Brunklaus et al., 2012; Dravet, 2011; Genton, Velizarova & Dravet, 2011; Han et al., 2012; Ito et al., 2013; Perez et al., 2014; Striano et al., 2007; Chapter 4).

The reduced Mboat7 expression resulting from heterozygous deletion of Scn1a might contribute to the epilepsy phenotype observed in Scn1a+/− mice. However, the reduction in Mboat7 was observed in both the seizure-resistant 129 and the seizure-susceptible F1 strains, suggesting that reduced Mboat7 is less likely to contribute to seizure phenotypes since the overt seizure phenotype is absent in 129.Scn1a+/− mice. However, the behavioural phenotype of 129.Scn1a+/− mice has not been assessed and it is possible that 129.Scn1a+/− mice could exhibit behavioural impairments in the absence of a seizure phenotype. This theory is supported by evidence demonstrating that the Dravet syndrome seizure and behavioural-deficit phenotypes arise from NaV1.1 haploinsufficiency in differing regions of the brain (Bender et al., 2013; Cheah et al.,
2012). Future work could directly examine the impact of Mboat7 expression on the behavioural phenotypes of Scn1a+/− mice.

Finally, we sought to examine the impact of heterozygous Gpr55 deletion on the epilepsy phenotype observed in the seizure-susceptible Scn1a+/− mice. Indeed, we found that heterozygous deletion of Gpr55 in Scn1a+/− mice on a mixed 129:B6 (62.5:37.5) background was anticonvulsant against hyperthermia-induced seizures and significantly improved survival. Heterozygous deletion of Gpr55, however, had no significant effect on spontaneous seizures. These findings align with CBD, a Gpr55 antagonist, demonstrating anticonvulsant efficacy against hyperthermia-induced seizures (Anderson et al., 2019a; Kaplan et al., 2017). Our findings are also consistent with inhibition of Gpr55 reducing the frequency of action potential outputs and increasing the frequency of spontaneous inhibitory postsynaptic currents in granule cells from Scn1a+/− hippocampal slices (Kaplan et al., 2017).

Although we found that heterozygous deletion of Gpr55 was anticonvulsant against hyperthermia-induced seizures and improved survival of Scn1a+/− mice, there was a major limitation to these studies. Unfortunately, the Gpr55+/− mice we acquired were not congenic on a B6 background but rather were on a mixed 129:B6 (25:75) genetic background. As such, when these mice were crossed with 129.Scn1a+/− mice for seizure experiments, the genetic background of the progeny would have varied ranging from 50 to 75% of 129. Since the background strain directly influences the severity of the Scn1a+/− phenotype and congenic 129.Scn1a+/− mice exhibit no overt phenotype, the anticonvulsant effect of heterozygous Gpr55 deletion may have been overestimated and the increased presence of modifier loci from the 129 background strain could contribute to the observed anticonvulsant effect. This would also explain why we observed a lower rate of spontaneous seizures and reduced mortality in the Scn1a+/−; Gpr55+/+ mice compared to
those previously reported for Scn1a+/− mice (Hawkins et al., 2017; Miller et al., 2014; Mistry et al., 2014). In order to unambiguously determine the whether Gpr55 is a genetic modifier of Scn1a+/− mice, the genetic-cross experiments would need to be repeated using Gpr55+/− mice on a congenic B6 background.

In summary, we present evidence that suggests Gpr55 may be a genetic modifier of the Scn1a+/− phenotype. We confirmed that seizure-susceptible F1 mice express significantly more hippocampal Gpr55 mRNA and report reduced hippocampal concentrations of 1-stearoyl LPI. We also report that 2-arachidonoyl LPI was reduced by heterozygous deletion of Scn1a but only on the seizure-resistant 129 background strain. The work in this chapter also demonstrated that heterozygous deletion of Gpr55 was anticonvulsant against hyperthermia-induced seizures and rescued survival in seizure-susceptible Scn1a+/−. Although there are limitations to the research in this chapter, our findings further reinforce the view that Gpr55 may be a novel therapeutic target in the management of Dravet syndrome.
Supplementary materials

Unsuccessful evaluation of Gpr55 protein with western blot

Supplementary Figure 1. Unsuccessful validation of three commercially-available primary antibodies for Gpr55 using wildtype (WT) and Gpr55−/− (KO) tissue, and human GPR55-expressing cells. The predicted protein fraction for Gpr55 was 37 kDa but signals above 37 kDa can occur due to post-translational modifications. (a) Representative genotyping bands confirming tissue hosts as Gpr55 WT and KO mice. Representative blots probed with ThermoFisher 720285 antibody (1:200) for (b.i) hippocampal and (b.ii) whole-brain lysates from WT and KO tissue. (c) Representative blot probed with Abcam Ab203663 antibody (1:200) on whole-brain lysates from WT and KO animals. Representative blots probed with Cayman Chemical 10224 antibody (1:200) on (d.i-d.iii) whole-brain lysates; with (d.iv) signals blocked by Gpr55 blocking peptide. (d.v) Blot for lysates from wildtype and human GPR55 receptor-expressing HEK cells probed with Cayman Chemical 10224 antibody (1:200); with (d.vi) all signals blocked by Gpr55 blocking peptide.
Evaluation of Gpr55 protein expression was unsuccessful

We aimed to examine whether the strain-dependent difference in Gpr55 mRNA expression translated to a functional difference in protein expression. Unfortunately, assessing Gpr55 protein expression was unsuccessful due to commercially-available Gpr55 primary antibodies proving inadequate (Supplementary figure 1). We tested three commercially-available antibodies (ThermoFisher, Abcam and Cayman Chemical), none of which exhibited specificity or selectivity for Gpr55. The predicted protein fraction for Gpr55 was 37 kDa with possibly heavier fractions occurring due to post-translational modifications. To validate the antibodies, we used tissue from wildtype and Gpr55-null mice, with genotypes confirmed using PCR (Supplementary figure 1a). The first antibody that we used was from ThermoFisher (720285) and it produced strong signals at \(~80\) kDa in the hippocampus (Supplementary figure 1b.i, 5-40 \(\mu\)g) and at \(~30\) kDa in whole-brain lysate (Supplementary figure 1b.ii, 100 \(\mu\)g); however, the signal was equally present in both WT and Gpr55\(^{-/-}\) tissue.

The second antibody tested was from Abcam (Ab203663), which produced a strong signal at \(~35\) kDa in whole-brain lysates (Supplementary figure 1c, 100 \(\mu\)g), but again was present in the Gpr55\(^{-/-}\) lysates. The third antibody tested was from Cayman Chemical (10224) and it produced a signal at \(~42\) kDa in whole-brain lysate (30-100 \(\mu\)g) but was again present in Gpr55\(^{-/-}\) tissue (Supplementary figure 1d.i-ii). A Gpr55 blocking peptide effectively blocked all the signals that were produced by the Cayman antibody (Supplementary figure 1d.iii). For further validation, we also tested the Cayman antibody on WT HEK cells and HEK cells expressing human GPR55 receptors (Supplementary figure 1d.v, 100 \(\mu\)g). There was a weak signal around the 75 kDa mark that was specific to the GPR55-positive cells that could potentially be a GPR55 dimer. We attempted to denature the potential GPR55 dimer but saw no change after incubation at 60°C for
15 min; and all signals were blocked by the Gpr55 blocking peptide (Supplementary figure 1d.vi).

**Western blot materials and methods**

Western blot analyses were performed as previously described (Hawkins et al., 2019; Hawkins et al., 2016) with modifications. Briefly, protein from hippocampal and whole-brain sections were isolated from Gpr55\(^{+/+}\) and Gpr55\(^{-/-}\) adult mice. Tissue samples were homogenised by hand in ice cold 0.32 M sucrose, 5 mM Tris (pH 7.4) supplemented with protease inhibitor (Roche) before being centrifuged for 10 min at 2,000G, at 4°C. Supernatants were collected and centrifuged for 40 min at 20,000 G, at 4°C. The pellets were re-homogenised in radioimmunoprecipitation assay (RIPA) solution (5 mM Tris (pH 8.2) and 1 mM Ethylenediaminetetraacetic acid (EDTA)) supplemented with protease inhibitor (+). Samples were centrifuged again for 40 min at 20,000 G, at 4°C, then the procured pellet was resuspended in a solution of 50 mM Tris (pH 7.5), 10 mM EDTA supplemented with protease inhibitor. For both cells and tissue, protein levels were quantified using Bradford reagent (Bio-Rad) on a NanoDrop One. 5-100 μg of protein lysate was denatured with laemmli sample buffer (Bio-Rad) and 5% beta mercaptoethanol for 15 min at room-temperature, or 15 min at 60°C where specified. Proteins were separated on a 10% SDS-page gel and transferred to nitrocellulose membranes. Membranes were probed for GPR55/Gpr55 using rabbit polyclonal antibodies (1:200; ThermoFisher Scientific, 720285; Abcam, Ab203663; Cayman chemical, 10224) and for β-tubulin using a mouse monoclonal antibody (1:500, Sigma-Aldrich, T5201) for 1 h at room temperature. Thereafter, membranes were incubated with secondary antibodies, anti-Rabbit Alexa Fluor 800 (Thermo Fisher Scientific, A32735) and anti-Mouse Alexa Fluor 680 (Thermo Fisher Scientific, A28183) at 1:20,000 for 30 min at room temperature. The probed membranes were imaged with an Odyssey
imaging system (Licor) and relative protein levels were determined by densitometry using ImageStudio software (Licor).
Chapter 4

Adolescent behavioral abnormalities in a Scn1a\(^{+/−}\) mouse model of Dravet syndrome
Adolescent behavioral abnormalities in a Scn1a\textsuperscript{+/-} mouse model of Dravet syndrome

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Abstract

Dravet syndrome is an intractable pediatric epilepsy associated with SCN1A mutations. In addition to having a large seizure burden and reduced lifespan, patients with Dravet syndrome also exhibit delays in reaching normal developmental milestones in attentional, emotional, and cognitive function. These developmental delays manifest in autistic-like social withdrawal and compulsive behavior. Additionally, cognitive impairments including deficits in sensorimotor processing and memory function are present. Several mouse models utilizing heterozygous deletion of Scn1a (Scn1a\textsuperscript{+/-} mice) have been generated that recapitulate many aspects of Dravet syndrome. Studies in these mouse models of Dravet syndrome have characterized behavioral phenotypes in adult mice. In the present study, we characterized the behavioral phenotype of Scn1a\textsuperscript{+/-} mice generated by targeted deletion of Scn1a exon 1 (Scn1a\textsuperscript{+/-} mice) during adolescence. Identifying behavioral deficits in adolescent mice would more closely model the early onset of attentional, emotional, and cognitive delays observed in patients with Dravet syndrome. The behaviors of adolescent Scn1a\textsuperscript{+/-} and wildtype (WT) mice were compared across several behavioral domains. We assessed motor function (open-field test), sociability and social recognition memory (three-chambered social preference and social interaction tests), memory function (novel object recognition, Barnes maze, fear conditioning paradigm), anxiety-related behavior (elevated plus maze and open-field thigmotaxis), startle reflex and sensorimotor gating (prepulse inhibition of startle (PPS) test), and repetitive compulsive behavior (marble burying test). Adolescent Scn1a\textsuperscript{+/-} mice exhibited normal locomotor activity, marble burying behavior, sociability, and sensorimotor gating. However, adolescent Scn1a\textsuperscript{+/-} mice displayed increased anxiety-related thigmotactic behavior, atypical fear expression, blunted acoustic startle responses, and impaired social recognition and spatial memory. Our results show that Scn1a\textsuperscript{+/-} mice display various behavioral impairments during adolescence, which provides a foundation for testing early intervention therapies targeting developmental delays modeled in Dravet syndrome mice.

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Abbreviations: DI, discrimination index; Na\textsubscript{v}1.1, voltage-gated neuronal sodium channel; P, postnatal day; PPI, prepulse inhibition of startle; SEM, standard error mean; WT, wildtype.

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1. Introduction

Dravet syndrome is a rare and catastrophic form of intractable childhood epilepsy with an incidence of approximately 1 in 15,000 to 40,000 [1–5] and a high premature mortality rate [6,7]. Dravet syndrome typically presents with febrile seizures within the first year of life in previously healthy infants, which progress to both febrile and afebrile seizures and the development of psychomotor and cognitive impairments [8]. Commonly reported behavioral comorbidities that are associated with Dravet syndrome include hyperactivity and attention disorders, motor disorders, intellectual disabilities, along with autism spectrum disorder-associated asocial and compulsive behaviors [9–16]. Patients with Dravet syndrome also exhibit deficits in auditory sensorimotor processing [13,17], which may precede and contribute to the associated attention, language, and cognitive impairments [9–11,16,18]. Behavioral comorbidities are stronger predictors than seizure frequency of a lower quality of life [15,16], highlighting a dire need for treatments that specifically target behavioral deficits during early development.

Approximately 80% of patients with Dravet syndrome have a mutation in SCN1A, the gene encoding the $\alpha_1$-subunit of the voltage-gated neuronal sodium channel (Nav1.1) [19]. SCN1A mutations associated with Dravet syndrome are typically severe missense and nonsense mutations that result in haploinsufficiency [20]. Mice with heterozygous deletion of Scn1a (Scn1a$^{+/-}$ mice) recapitulate many aspects of Dravet syndrome, such as susceptibility to hyperthermia-induced seizures, the development of spontaneous seizures, and reduced lifespan [21–23].

The present study sought to phenotype the behavior of the Scn1a$^{+/-}$ mouse line developed by targeted deletion of exon 1 (Scn1a$^{tm1Kea}$), which has not yet been characterized [22]. Previous studies have phenotyped the behavior of adult mice with heterozygous loss-of-function alleles, developed by targeted deletion of Scn1a exon 26 (Scn1a$^{tm1Wac}$) or knock-in of the Scn1a-R1407X mutation (Scn1a$^{tm1Kzy}$), and described hyperactivity, stereotyped behaviors, lowered sociability, poor spatial learning, and memory deficits [24,25]. In the current study, we examined the aforementioned behaviors, as well as repetitive, compulsive behaviors, acoustic startle responses, and sensorimotor gating, which have not been investigated
in Dravet syndrome mice. Furthermore, we assessed whether any behavioral abnormalities in Scn1a<sup>+</sub>− mice were present during adolescence to more closely model the onset of developmental delays in attention, emotional, and cognitive function observed in patients with Dravet syndrome. Identifying behavioral impairments in adolescence would provide a platform to test early intervention therapeutics aiming to correct the behavioral disturbances.

2. Material and methods

2.1. Mice

All research and animal care procedures were approved by the University of Sydney Animal Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Scn1a<sup>tm1Kea</sup> mice were purchased from the Jackson Laboratory (stock 37107-JAX; Bar Harbor, MA, USA) and maintained by continuous backcrossing to 129S6/SvEvTac (129.Scn1a<sup>+</sub>−). To generate mice for experiments, 129.Scn1a<sup>+</sub>− mice were crossed with wildtype (WT) C57BL/6J, resulting in F1 mice on a mixed 129S6/SvEvTac × C57BL/6J background (Scn1a<sup>+</sub>). Mice used for experiments were male littermates with n = 11 for WT mice and n = 10 for Scn1a<sup>+</sub>− mice, unless otherwise specified, with the same mice being used for all experiments. Mice were genotyped as previously described [22] and were group housed (3–4 mice per cage), unless otherwise specified, in individually ventilated cages in a specific pathogen-free facility with food and water available ad libitum. All experiments were conducted during the light phase of a 12-hour light/dark cycle.

2.2. Behavioral testing paradigm

A schematic depiction of the sequence of behavioral tests can be seen in Fig. 1. The test mice were all individually handled for 3 min for three consecutive days before testing began. Behavioral testing of age-matched WT and Scn1a<sup>+</sub>− mice began at postnatal days (P) 34–37 during adolescence and after the vulnerable mortality period (50% of F1.Scn1a<sup>+</sub>− mice to P30) [22]. The behavioral tests were performed in the following test order: 1) three-chambered social preference, 2) open-field, 3) social interaction, 4) novel object recognition, 5) Barnes maze, 6) elevated plus maze, 7) acoustic startle, 8) prepulse inhibition of startle (PPI), 9) marble burying, and 10) contextual fear conditioning. Four days prior to behavioral testing, all mice were individually housed to enhance social
motivation in the three-chambered social preference and the social interaction tests [26]. Mice remained individually housed until after the novel object recognition test at which point they were returned to group housing with their former cage mates. Additionally, mice were habituated to the testing room for at least 60 min prior to each experiment. All arenas and equipment were cleaned with 80% ethanol in between trials. None of the mice exhibited overt seizure behavior during any of the video-recorded behavioral tests.

2.3. Three-chambered social preference

The three-chambered social preference test was performed as previously described with modifications [27]. The apparatus consisted of a long rectangular blue Perspex box (60 × 42 × 22 cm) divided into three identical 20 cm long chambers separated by two transparent partitions, each with a square opening (10 × 10 cm), with the light intensity in the center of the arena at 10 lx. The two chambers on either end of the apparatus contained a wire cage (5 × 5 × 5 cm) that was used to hold a mouse. The wire cage allowed exposure to visual, auditory, olfactory, and some tactile stimuli while preventing aggressive or sexual interactions. Each trial was conducted for 10 min. During the habituation phase (trial 1), the test mouse was placed in the empty arena. Social preference was tested during trial 2, where an unfamiliar mouse was caged in one chamber and the other cage was left empty. Preference for social novelty was assessed during trial 3, with one chamber containing the mouse from trial 2 (familiar mouse) and the other a stranger mouse (novel mouse). All trials were captured with a video recording system and tracking software (TopScan; Clever Sys Inc., U.S.A.) that measured distance traveled and time spent in a 5-centimeter radius of the cages. Social mice used for this experiment were age-matched male C57BL/6J mice habituated to testing conditions by being contained in experimental wire cages for 20 min on three consecutive days prior to testing. One Scn1a+/− mouse escaped from the arena during trial 3 (social novelty) so was excluded from trial 3 analyses.

2.4. Open-field test

The open-field test was conducted in a blue, Perspex, 40 × 40 × 40 cm arena (10 lx). Mice were placed into the arena for 10 min and video-recorded
by an overhead camera. TopScan was used to measure total distance traveled in the whole arena and differentiate activity that occurred in the inner (70%) and outer (30%) regions. Time spent in the center of the arena was used to assess anxiety-like behavior [28].

2.5. Social interaction

The social interaction test was performed as previously described with modifications [29,30]. Immediately following the open-field test, a stranger mouse was introduced into the arena for 10 min. The stranger mice were male A/JArc mice (Australian Resources Centre, Australia), aged 5–7 weeks old. An overhead camera was used to record each session.

Total duration and frequency of social contact, including sniffing, adjacent lying, following, crawling under/over, and mutual grooming [29,31], were manually recorded by a researcher blinded to genotype using scoring software (ODLog; Macropod Software Pty Ltd., Australia).

2.6. Novel object recognition test

The novel object test assesses recognition memory and was performed as previously described with modifications in the open-field arena [32]. During the familiarization phase, two identical objects were adhered to the floor of the arena on opposite corners, and mice were placed in the arena for 10 min. Mice were returned to their home cages for 60 min before a second 10-minute trial. Prior evaluation of object recognition performance in a Scn1a<sup>om1Wac</sup> mouse model utilized a 24-hour intertrial interval [24]. Here, we chose a 60-minute intertrial interval based on research demonstrating improved object recognition using a 60-minute delay compared with a 24-hour delay [32].

In the second trial, mice were presented with the familiar object from the previous trial and a novel object. The objects used were polypropylene Falcon®

![Fig 1: Sequence of behavioral tests](image)
tubes (50 mL, 30 × 115 mm), filled with clean bedding and Corning® U-shaped polystyrene cell culture flasks (75 cm²) covered in red electrical tape. The objects and their locations varied among animals and were counterbalanced across genotypes. Sessions were recorded by an overhead camera, and TopScan (Clever Sys Inc., U.S.A.) was used to measure total distance traveled and time spent within a 5-centimeter radius of each object. Additionally, for the test trial, time spent directly interacting with the objects, defined as nosing, sniffing, circling, rearing on, and climbing on the objects, were manually recorded by a researcher blinded to group using ODLog scoring software. The discrimination index (DI) was calculated as \[ \frac{\text{(novel object interaction time} - \text{familiar object interaction time)}}{\text{total interaction time}} \times 100 \], with DI ≥ 25 indicating preference for the novel object [33].

2.7. Barnes maze test

Spatial learning and memory were assessed using the Barnes maze (San Diego Instruments, U.S.A.) [34], which consisted of a white circular platform (91 cm diameter) that had 20 circular holes (5 cm diameter) around its perimeter. An escape box (17 × 13 × 7 cm) was located under one of the holes, while the other holes led to false escape boxes (6 × 6 × 3 cm). The maze was located 70 cm off the ground and was placed under bright lights (1350 lx) with spatial cues placed on the walls and an overhead video camera recording each session. The paradigm employed here was based on the paradigm utilized by Ito et al. [25] demonstrating impairment in spatial learning and memory in \( Scn1a^{m1Kzy} \) mice. Mice were habituated to the Barnes maze for 5 min, and to the escape box for 1 min, the day before training. Mice received three training sessions per day (5 min), with an intertrial period of 20 min, for four consecutive days. During training, mice started in a tube (15 × 15 × 20 cm) in the center of the maze then had 5 min to search for the escape box. The location of the escape box remained consistent for the same animal but was varied among animals with the location being counterbalanced across conditions. If a mouse failed to find the target box within the 5-minute trial, it was guided to the hole by the researcher. When the mouse entered the escape box, it remained there for 1 min before being returned to its homecage. On day 5, a probe test was performed for 3 min in the absence of the escape box. Any-MAZE tracking software (Stoelting Co., U.S.A.) measured distance traveled,
latency to find the target zone, time in the target zone, and number of entries into the target zone.

2.8. The elevated plus maze

The elevated plus maze was used to assess anxiety-like behavior [35] and was performed in low-light conditions (150 lx). The arena consisted of four arms (4.5 × 30 cm) made of blue Perspex and arranged in a “plus shape”. Two of the opposing arms were enclosed by walls (30 × 14.5 cm), and the arena was elevated 41 cm off the floor. At the commencement of the test session, mice were placed in the center of the arena and video-recorded from above for 10 min. TopScan (Clever Sys Inc., U.S.A.) was used to measure total distance traveled, the number of entries, and the time spent in open and closed arms.

2.9. Acoustic startle response and prepulse inhibition of startle

The PPI test was used to assess the startle reflex and auditory sensorimotor gating. Tests were conducted in SR lab chambers (San Diego Instruments, U.S.A.) (50 lx) as previously described with modifications [36]. First, the acoustic startle response was independently assessed by exposing mice to 30 presentations of the startle stimulus (120 dB, 40 ms) with a randomized intertrial interval of 12–30 s. Immediately following the acoustic startle test, PPI was assessed. Prepulse inhibition of startle consisted of 50 trials comprised of 10 repetitions each of the following: no stimuli, startle stimulus alone (120 dB for 40 ms), and PPI trials with 3 different prepulse intensities presented in a pseudorandomized order with randomized intertrial intervals of 12–30 s. Prepulse inhibition of startle trials consisted of a 20-millisecond prepulse stimulus (74, 78, and 82 dB) that were presented 100 ms before a 40-millisecond startle stimulus (120 dB). The mean startle amplitude during the 100 ms following the startle stimulus was recorded as the startle response. The percentage of PPI was calculated as \[
\frac{(\text{startle response } 120 \text{ dB}) - (\text{prepulse + pulse response})}{\text{startle response } 120 \text{ dB} } \times 100\%.
\]

2.10. Marble burying

Marble burying was used to assess repetitive, compulsive-like behavior [37]. The test was conducted in opaque Perspex cages (38 cm × 21 cm × 13 cm) containing normal bedding (4.5 cm deep) overlaid with 20 glass marbles (15 mm diameter) equidistant in a 4 × 5 arrangement. Cages were set in a room with the light
intensity set to 15 lx. For testing, a single mouse was placed in the cage with the lid closed for 60 min. At the end of the trial, the mouse was removed from the cage, and a researcher blinded to genotype counted the number of marbles buried. Marbles with more than 2/3 of its surface covered was counted as buried. Bedding was replaced, and marbles were cleaned with 80% ethanol, rinsed with water, and dried between trials.

2.11. Contextual fear conditioning

Fear conditioning was conducted in shock chambers (Clever Sys Inc., U.S.A.) (5 lx) as previously described with modification [38]. The behavioral paradigm employed was based on prior demonstration of impairments in fear memory in adult Scn1a<sup>tm1Wac</sup> mice [24]. Briefly, a mouse was placed in the chamber for 198 s before receiving a single, footshock (2 s, 0.5 mA). The mouse remained in the chamber for another 60 s before it was returned to its homecage. The first 3 min of the habituation period was used as a measure of baseline freezing, whereas the 60 s immediately following the footshock was used to assess immediate fear response. Thirty minutes after conditioning, the mice were reexposed to the shock-paired context in the absence of shock for 3 min to assess short-term memory. This was repeated 24 h after to assess long-term memory. All trials were video-recorded, and FreezeScan (Clever Sys Inc., U.S.A.) software was used to measure freezing behavior.

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, La Jolla California USA) and SPSS version 24 (IBM Corp, Armonk, New York USA). Normality of distribution and equality of variance were assessed using the D’Agostino & Pearson normality test and the F test, respectively. If these parameters were violated, then data were analyzed using nonparametric tests. The performance of WT and Scn1a<sup>+/−</sup> mice was generally compared using unpaired parametric t-tests or nonparametric Mann–Whitney U tests. For the three-chambered social preference tests, paired parametric t-tests or nonparametric Wilcoxon matched-pairs signed rank tests were employed to compare preferences. The data for the training phase of the Barnes maze and fear conditioning were analyzed using repeated two-way analysis of variance (ANOVA), followed by Sidak's post hoc tests. Prepulse inhibition of startle data were analyzed using nonparametric aligned ranks
transformation (ART) ANOVA by first aligning and ranking data for each variable using ARTool (version 1.6.2, Washington USA) then analyzing differences between variables with a factorial ANOVA. The Log-rank Mantel–Cox test was used to analyze the ability to locate the target zone during the probe test of the Barnes maze because one animal failed to enter the target zone. Differences were deemed statistically significant when \( p < 0.05 \).

3. Results

3.1. Scn1a\(^{+/−}\) mice displayed normal locomotor activity but anxiogenic-like thigmotactic behavior in the open-field test

Heterozygous Scn1a deletion did not influence locomotor activity as both WT and Scn1a\(^{+/−}\) mice traveled comparable distances during the open-field test (Fig. 2a, unpaired t-test: \( t_{19} = 0.054, p = 0.957 \)). Assessing the time course of distance traveled per minute showed that both groups of mice similarly habituated to the open field with a reduction in locomotor activity over time (Fig. 2b, repeated two-way ANOVA: minutes, \( F_{9,171} = 33.54, p < 0.0001 \); genotype, \( F_{1,19} = 0.003, p = 0.957 \); minute by genotype interaction, \( F_{9,171} = 1.737, p = 0.0841 \)). The impact of Scn1a\(^{+/−}\) genotype on anxiety-like behavior was also evaluated in the open-field test by comparing the time spent in the inner 70% of the arena (Fig. 2c and d, representative traces). Overall, Scn1a\(^{+/−}\) mice spent significantly less time in the center region of the arena than WT mice, and they engaged in more “wall-hugging” thigmotactic behavior (Fig. 2e, unpaired t-test: \( t_{19} = 2.115, p = 0.044 \)). Time-course analysis showed that while both genotypes of mice increased time
spent in the center region over time, Scn1a<sup>+/−</sup> mice spent significantly less time in the center over the entire course of the test, suggesting greater anxiety-related behavior (Fig. 2f, repeated two-way ANOVA:

- minutes, $F_{9, 171} = 3.593, p = 0.0004$
- genotype, $F_{1, 19} = 4.645, p = 0.044$
- minute by genotype interaction, $F_{9, 171} = 0.275, p = 0.098$.

Fig. 2. Scn1a<sup>+/−</sup> mice displayed anxiogenic-like behavior in the open-field test. (a) Total distance traveled during a 10-minute open-field test by wildtype (WT) and Scn1a<sup>+/−</sup> mice and (b) distance traveled per minute in the test. Representative locomotor traces from a (c) WT and (d) Scn1a<sup>+/−</sup> mouse. (e) Total time spent in the center region of the open-field arena, defined as the inner 70%, and (f) time spent in center during each minute of the test. Data represent means± standard error mean (SEM) with open circles representing individual mice, $n = 10–11$ and were analyzed using unpaired t-tests and repeated two-way ANOVA. *$p < 0.05$. 

*
3.2. *Scn1a*+/− mice did not exhibit altered anxiety-like behavior in the elevated plus maze or repetitive, compulsive behavior in the marble burying test

Despite *Scn1a*+/− mice displaying anxiogenic-like behavior in the open-field test, this finding was not reproduced in the elevated plus maze. Total time spent on the open arms (Fig. 3a), the time spent on the open arms as a ratio of the time spent on the closed arms (Fig. 3b), and the entries to open arms (Fig. 3c) appeared larger in the *Scn1a*+/− mice than the WT mice, but this effect did not reach statistical significance for any of the measures (unpaired t-test: \( t_{19} = 1.877, p = 0.076 \), Mann–Whitney test: \( U = 35, p = 0.173 \) and unpaired t-test: unpaired t-test: \( t_{19} = 1.917, p = 0.071 \), respectively). We also measured repetitive, compulsive behavior in the marble burying test. There was no significant difference between WT and *Scn1a*+/− mice on the number of marbles buried (Fig. 3c, unpaired t-test: \( t_{19} = 0.712, p = 0.485 \)).
3.3. *Scn1a*<sup>+/−</sup> mice displayed impaired preference for social novelty compared with WT mice

We conducted the social interaction test to examine whether the *Scn1a*<sup>+/−</sup> genotype affects social behavior. There was no difference between WT and *Scn1a*<sup>+/−</sup> mice in the total time (Fig. 4a, unpaired t-test: t<sub>19</sub> = 1.216, p = 0.239) or frequency of social interactions with a novel mouse (Fig. 4b, unpaired t-test: t<sub>19</sub> = 0.308, p = 0.761). We also sought to examine whether heterozygous *Scn1a* deletion affects more subtle measures of social behavior using the three-chambered social preference test. We first measured preference for a cage holding a stranger mouse over an empty cage. Interestingly, we found that both WT and *Scn1a*<sup>+/−</sup> mice lacked preference for the stranger mouse; as both groups spent an equivalent time in proximity to the stranger mouse compared with the empty cage (Fig. 4c, WT, Wilcoxon matched-pairs signed rank test: Z = 10, p = 0.700; paired t-test: t<sub>10</sub> = 0.17, p = 0.868; *Scn1a*<sup>+/−</sup>, paired t-test: t<sub>9</sub> = 1.4, p = 0.195, respectively). We then investigated social recognition, by measuring the preference for a novel mouse over a familiar mouse (Fig. 4d). As expected, WT mice displayed a preference...
for the novel mouse, spending significantly more time in proximity to the novel mouse than the familiar mouse (paired t-test: $t_{10} = 3.074, p = 0.012$). $Scn1a^{+/-}$ mice, however, showed no preference and spent an equivalent time exploring the novel mouse to that of the familiar mouse (paired t-test: $t_{8} = 1.032, p = 0.332$).

### 3.4. $Scn1a^{+/-}$ mice displayed better novel object recognition and subtle impairments in spatial memory compared with WT mice

We utilized the novel object recognition test to evaluate recognition memory, in which a DI score of $>25$ indicates a preference for the novel object [33]. Interestingly, $Scn1a^{+/-}$ mice showed preference for the novel object with a mean DI score of $26.4 \pm 7.4$, which was significantly greater than the mean DI score of WT mice.
mice who tended to prefer the familiar object (−17.5 ± 13.9) (Fig. 5a; unpaired t-test: t_{19} = 2.74, p = 0.013). Total time spent interacting with both objects was equivalent between groups (WT = 83.5 ± 17.3 s; Scn1a^{+/−} = 90.2 ± 26.4 s; unpaired t-test: t_{19} = 0.218, p = 0.83).

Spatial learning and memory were assessed using the Barnes maze. During the training phase, both groups showed similar learning of the task evidenced by reduced latencies to locate the escape box across training days (Fig. 5b; repeated two-way ANOVA: trial day, F_{3, 54} = 21.12, p < 0.001; genotype: F_{1, 18} = 0.742, p = 0.4; trial day × genotype: F_{3, 54} = 0.54, p = 0.657). While it appeared that Scn1a^{+/−} mice displayed longer latencies to first enter the target zone, this did not reach statistical significance (Fig. 5c; Mantel–Cox Log-rank test: $\chi^2_1 = 3.196$, p = 0.074). However, Scn1a^{+/−} mice covered a significantly greater distance than WT mice to this first entry into the target zone, with one Scn1a^{+/−} mouse failing to enter the target zone (Fig. 5d; Mantel–Cox Log-rank test: $\chi^2_1 = 5.381$, p = 0.02). This could not be explained by increased locomotor activity, as there was no differences between WT and Scn1a^{+/−} mice in the total distance traveled during the probe test (Fig. 5e; Mann–Whitney test: U = 41, p = 0.529). This suggests that Scn1a^{+/−} mice exhibited a subtle spatial memory deficit during the probe test. No significant differences, however, were found between genotypes in the percentage of time spent in the target zone (Fig. 5f; Mann–Whitney test: U = 39, p = 0.436) or correct entries made into the target zone (Fig. 5g unpaired t-test: t_{17} = 0.77, p = 0.452).

3.5. Scn1a^{+/−} mice exhibited an acoustic startle deficit with intact sensorimotor gating, and an atypical fear response compared with WT mice

Acoustic startle, PPI, and contextual fear conditioning paradigms were utilized to evaluate startle response, auditory sensorimotor gating, and fear memory of WT and Scn1a^{+/−} mice, respectively. During the acoustic startle test, startle amplitude was measured across 30 repeated presentations of a 120 decibel stimulus. Scn1a^{+/−} mice displayed significantly reduced acoustic startle responses compared with WT mice across all trials (Fig. 6a, repeated two-way ANOVA: trials, F_{29, 551} = 1.537, p = 0.038; genotype, F_{1, 19} = 8.936, p = 0.007; trials by
Fig. 6. *Scn1a*+/− mice exhibited an acoustic startle deficit and an atypical fear response. (a) Mean startle responses to 30 repeated presentations of 120 dB acoustic stimuli, with n = 10–11, repeated-measures ANOVA. (b) The % of inhibition of the startle response when preceded by prepulse stimuli during the PPI of acoustic startle test, with n = 10–11, ART ANOVA. (c) Freezing behavior during contextual fear conditioning paradigm was measured as a percentage of total test time. Baseline freezing before footshock administration (baseline), and freezing behavior immediately, 30 min, and 24 h after footshock administration are displayed, n = 10–11, repeated-measures ANOVA, with Sidak's post hoc test. ***p < 0.001. Box-and-whiskers plots represent the median (horizontal line) and the mean (+ symbol) with box boundaries representing the 25th to 75th percentile and the whiskers representing the minimum and maximum values. Otherwise, data represent means ± SEM with open circles represent individual mice.

Genotype interaction, F(29, 551) = 0.95, p = 0.542). Despite the startle reflex deficit, *Scn1a*+/− displayed similar PPI to WT mice (Fig. 6b). Both WT and *Scn1a*+/− mice showed an equivalent prepulse stimulus intensity-dependent increase in PPI (ART ANOVA, prepulse stimulus intensity: F(2, 57) = 16.131, p < 0.0001; genotype: F(1, 19) = 0.000, p = 0.986; genotype by prepulse intensity interaction: F(2, 38) = 1.843, p = 0.172), suggesting that heterozygous *Scn1a* deletion does not affect sensorimotor gating function.

In the contextual fear conditioning paradigm (Fig. 6c), WT mice typically display significantly increased freezing behavior in response to footshock. Whereas this behavior was observed in the WT mice, it was not consistently observed across all trials for the *Scn1a*+/− mice (repeated two-way ANOVA: main effect of trials, p < 0.0001; main effect of genotype, p = 0.002;
and a trial by genotype interaction, \( p = 0.002 \). Baseline freezing behavior prior to footshock was not different between WT and \( Scn1a^{+/−} \) mice (Sidak's post hoc test: \( p = 0.992 \)). Immediately following the footshock, WT mice exhibited the expected increase in freezing behavior compared with baseline (Sidak's post hoc test: \( p < 0.001 \)). However, this fear response was absent in the \( Scn1a^{+/−} \) mice (Sidak's post hoc test: \( p = 0.996 \)). Accordingly, the WT mice showed significantly higher freezing responses than \( Scn1a^{+/−} \) mice immediately postshock (Sidak's post hoc test: \( p = 0.002 \)).

During the reexposure trial that took place 30 min and 24 h after footshock, both WT and \( Scn1a^{+/−} \) mice displayed significantly elevated freezing behavior compared with baseline (Sidak's post hoc test: \( p < 0.001 \) for all comparisons), which did not differ significantly between genotypes (Sidak's post hoc test: \( p = 0.128 \) and \( p = 0.595 \), respectively).

4. Discussion

Identifying behavioral abnormalities present in adolescent \( Scn1a^{+/−} \) mice would better model the early onset of attentional, emotional, and cognitive impairments that are observed in patients with Dravet syndrome. Studies in \( Scn1d^{tm1Wac} \) and \( Scn1d^{tm1Kzy} \) mouse models of Dravet syndrome have demonstrated various behavioral abnormalities, but these studies were restricted to adult mice [24,25]. Here, we provide data demonstrating behavioral abnormalities in \( Scn1d^{tm1Kzy} \) mice during adolescence. Adolescent \( Scn1a^{+/−} \) mice exhibited altered behavior across various domains, including increased anxiety-like behavior, subtle impairments in spatial memory, blunted acoustic startle reflexes, and atypical fear responses. In addition, our study demonstrates that heterozygous \( Scn1a \) deletion modulated novelty preference depending on the nature of the stimuli. \( Scn1a^{+/−} \) mice showed an enhanced preference for novel, inanimate stimuli but an impaired preference for novel, social stimuli.

Adolescent \( Scn1a^{+/−} \) mice did not exhibit a hyperactive phenotype in the open-field test, a phenotype reported in adult mice [24,25]. However, \( Scn1a^{+/−} \) mice displayed increased anxiety-like behavior as gauged by increased time being spent in the peripheral regions of the open-field test arena [28,39]. Interestingly, the anxiogenic phenotype of the \( Scn1a^{+/−} \) mice was not reproduced in the elevated plus maze. Consistent with this model-dependent effect on anxiety-like behavior, the \( Scn1d^{tm1Kzy} \) mouse model of
Dravet syndrome also exhibited increased anxiety in the open-field test but decreased anxiety in the elevated plus maze [25]. Collectively, these results imply that anxiety-related behavior observed in the open-field test may have distinct qualities to that measured in the elevated plus maze. Alternatively, the increased exploration of the open arms in the elevated plus maze might reflect increased fear-motivated escape behavior, which has been observed following stress exposure in C57BL/6J mice [40]. Future studies might expand on the characterization of anxiety-related behaviors in Scn1a<sup>tm1Kzy</sup> mice using other models such as the light–dark test or zero maze that may help clarify the nature of these behaviors in these mice.

Our results show that heterozygous Scn1a deletion selectively impairs preference for social novelty without affecting general social behavior. Scn1a<sup>+/−</sup> mice displayed normal general social behavior, as there were no differences between WT and Scn1a<sup>+/−</sup> mice in the total time of social interaction or frequency of social behaviors. Whereas in the three-chambered social preference test, WT mice displayed a clear preference for a novel mouse over a familiar mouse, when the Scn1a<sup>+/−</sup> mice did not show such a preference. This indicates that Scn1a<sup>+/−</sup> mice may display social recognition memory deficits. Other mouse models of Dravet syndrome displayed social recognition deficits, along with sociability deficits [24,25]. In the current study, neither the WT nor Scn1a<sup>+/−</sup> mice showed a preference for a novel mouse over a novel inanimate object. The mixed genetic (129S6/SvEvTac × C57BL/6J) background of the Scn1a<sup>+/−</sup> mice may contribute to this unexpected performance. C57BL/6 mice display clear preferences for social stimuli [41]. The social preference of 129S6/SvEvTac mice remains unreported; however, a different 129 substrain (129S1/SvJ) lacks social preference [41].

Unexpectedly, WT mice demonstrated poor object discrimination in the novel object recognition task with a tendency to favor exploration of the familiar object. In another study, C57BL/6 mice displayed robust recognition of novel objects [32], while 129S6/SvEvTac mice failed to exhibit novel object recognition [42]. Therefore, as the Scn1a<sup>+/−</sup> mice used in this study were on a mixed background (129S6/SvEvTac × C57BL/6J), the introduction of 129S6/SvEvTac genetics into the F1 mixed background...
strain may have countered the usual performance of C57BL/6J mice on this task. Surprisingly, the Scn1a+/− mice displayed a significant preference for the novel object during the novel object recognition test. While the Scn1a+/− mice displayed intact spatial learning on the Barnes maze during acquisition, a subtle spatial memory impairment was observed during the probe test as Scn1a+/− mice covered a significantly greater distance to locate the target zone than WT mice. In adult mouse models of Dravet syndrome, more pronounced impairments in learning and memory were reported. Scn1atm1Kzy and Scn1atm1Wac mice both made more errors in locating the target, while Scn1atm1Wac mice also displayed an increased latency to find the target during both the learning phase and probe test of the Barnes maze [24,25].

Repetitive, compulsive behavior and sensorimotor processing deficits occur in patients with autism and Dravet syndrome [11,13,43,44]. However, object-oriented compulsive behavior and sensorimotor gating have never been examined in any mouse model of Dravet syndrome. Adolescent Scn1a+/− mice exhibited normal marble burying behavior, a measure of repetitive, compulsive behavior. Sensorimotor gating, as measured in the PPI paradigm, was normal. However, it is possible that if the PPI test was repeated in adult Scn1a+/− mice, an impairment in sensorimotor gating may be observed since deficits in PPI have been identified in adult patients with temporal lobe epilepsy and autism spectrum disorder [45,46].

Here, we show that adolescent Scn1a+/− mice display a remarkably blunted acoustic startle response. Impaired acoustic startle reflexes may indicate defects in hearing [47]. As all the prepulse stimuli promoted a prepulse intensity-dependent attenuation of the startle response to a loud acoustic stimulus, it appears that Scn1a+/− mice do not have any grossly impaired hearing function. This is consistent with patients with Dravet syndrome having normal auditory function [48]. It is possible that the reduced startle response in the Scn1a+/− mice is due to a brainstem dysfunction since this brain region mediates the startle reflex [49]. Indeed, patients with Dravet syndrome have reduced white matter volumes in the brainstem, which could suggest impaired brainstem function [50]. Furthermore, conditional knockout of Scn1a in inhibitory neurons promoted hyperexcitability of neurons in the brainstem [51].
Whether such effects of heterozygous Scn1a deletion similarly affect the brainstem regions mediating startle reflex is unknown and requires further investigation.

In the contextual fear conditioning paradigm, adolescent Scn1a+/− mice displayed severely reduced freezing behavior immediately following shock exposure, which was not observed in other Scn1a+/− mouse models [24,52]. One possible explanation for the reduced freezing behavior is that Scn1a+/− mice have impaired pain perception. Pain sensitivity has not been assessed in any of the mouse models of Dravet syndrome, although NaV1.1 channels contribute to sensory perception of mechanical pain [53,54], and SCN1A gain-of-function mutations have been linked to familial hemiplegic migraine. However, impaired pain perception would not explain expression of fear memory 30 min and 24 h postshock, suggesting successful acquisition of long-term fear memory. One explanation for the long-term fear expression is that contextual fear may have been acquired vicariously through ‘social transfer’ when mice were returned to group-housed cages between trials. This is supported by contextual fear being transferred or enhanced through observation or social interaction [55,56].

Our findings of impaired social recognition memory, intact novel object recognition, and increased anxiety-like behavior in adolescent Scn1a+/− mice are congruent with other behavioral studies conducted in adult mouse models of Dravet syndrome, aged 10–28 weeks and 6–8 months [24,25]. By examining the adult Scn1a+/− phenotype, these studies have neglected the early onset of the behavioral abnormalities observed in patients with Dravet syndrome. The present study demonstrates that behavioral deficits are present in adolescent mice, thus more closely modeling the early onset of developmental delays observed in young patients with Dravet syndrome. While clinical studies have reported that behavioral comorbidities associated with Dravet syndrome are present in young patients, these behavioral deficits appear to worsen with age [9,10,14,57]. A future study is required to characterize the behavioral phenotypes of Scn1aem1Kea mice in adulthood, which would allow us to track the developmental progression of impaired cognitive, social, emotional, and motor function. If the impact of NaV1.1 haploinsufficiency on behavioral impairments
is progressive, then early intervention treatments may be required to attain the best outcomes in improving quality of life for patients with Dravet syndrome.

Declaration of competing interest

Associate Professor Jonathon Arnold is Deputy Academic Director of the Lambert Initiative for Cannabinoid Therapeutics, a philanthropically funded research center at the University of Sydney. He has served as an expert witness in various medicolegal cases involving cannabis and in 2018, was a temporary advisor to the World Health Organization (WHO) on their review of cannabis and the cannabinoids. His research is funded by the Lambert Initiative and the Australian National Health and Medical Research Council (NHMRC). A/Prof Arnold and Dr. Lyndsey Anderson hold patents on cannabinoid therapies (PCT/AU2018/05089 and PCT/AU2019/050554). The remaining authors have no conflicts of interest. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Chapter 5

General discussion
The research conducted in this thesis advances our understanding of the endocannabinoid and GPR55-LPI systems and their influence on the phenotype of the $\text{Scn1a}^{+/-}$ mouse model of Dravet syndrome. Results within the thesis identifies and validates CB1 and Gpr55 receptors as potential therapeutic targets for the treatment of Dravet syndrome. The research presented in Chapter 2 demonstrated that mice with the F1 seizure-susceptible background strain had reduced hippocampal CB1 expression and 2-AG concentrations, and that potentiation of endocannabinoid signalling with a MAGL inhibitor and CB1 receptor PAM was anticonvulsant against hyperthermia-induced seizures. The work conducted in Chapter 3 confirmed that hippocampal Gpr55 mRNA expression was increased and that LPI concentrations were reduced in the F1 seizure-susceptible strain compared to the 129 seizure-resistant strain of mice. We also reported in Chapter 3 that heterozygous deletion of $\text{Gpr55}$ in $\text{Scn1a}^{+/-}$ mice improved seizure susceptibility and rescued premature mortality. Additionally, the work in Chapter 4 expanded the current knowledge of the behavioural impacts of heterozygous $\text{Scn1a}$ deletion during adolescence and showed that $\text{Scn1a}^{+/-}$ mice had multiple deficits in measures of social, attentional and cognitive function. This provides a strong platform for future investigations into new therapies aimed at correcting the developmental delays in cognitive and emotional functioning observed in Dravet syndrome.

The endocannabinoid system plays an integral role in regulating neuronal excitability, and there is a plethora of preclinical research that has investigated endocannabinoid system modulation in various animal models of seizures and epilepsy (Gaston & Szaflarski, 2018; Katona, 2015; Rosenberg, Patra & Whalley, 2017; Soltesz et al., 2015). Moreover, a preeminent mechanism for the anticonvulsant effects of CBD is antagonism of the GPR55 receptor, a receptor once thought to be the 3rd cannabinoid receptor but now known to be activated by the lipid mediator LPI (Oka
et al., 2009). Yet, specific targeting of endocannabinoid and GPR55-LPI system components as a therapeutic strategy for Dravet syndrome or other paediatric epilepsies had not yet been investigated. As discussed in Chapter 1, epilepsy disorders may all produce seizures, but their aetiology and underlying pathophysiology can vary. This knowledge suggests that anticonvulsant efficacy of endocannabinoid or GPR55-LPI system modulation in one preclinical seizure or epilepsy model may not necessarily transfer to another. Therefore, the work in this thesis aimed to investigate the endocannabinoid and GPR55-LPI systems in a Dravet syndrome mouse model by utilising the ‘genetic modifier’ approach. As the Dravet syndrome phenotype in Scn1a+/− mice is critically-dependent on background strain genetics, the ‘genetic modifier’ approach compares the transcriptome of seizure-susceptible and seizure-resistant mice to detect divergences in gene expression that may influence the epilepsy phenotype, and ultimately identify druggable targets. Thus, by comparing the expression of genes involved in the endocannabinoid and GPR55-LPI systems between the two background strains we identified a number of gene candidates and selected Cnr1 and Gpr55 as the genes to be investigated in Chapters 2 and 3 of this thesis, respectively.

The work in Chapter 2 identified a potential double deficit in the F1 seizure-susceptible mice with reduced expression of CB1 receptor mRNA and protein, along with less 2-AG levels in the hippocampus. As the endocannabinoid system is as a regulator of excess neuronal activity (Zou & Kumar, 2018), we hypothesised that our observed strain-dependent variability may be contributing to the hyperexcitability observed in the Scn1a+/− mice on the seizure-susceptible background strain. We therefore investigated and demonstrated that pharmacological potentiation of endocannabinoid signalling with a CB1 receptor PAM and MAGL inhibitor were anticonvulsant against hyperthermia-induced seizures. One way to strengthen the role of the endocannabinoid
system as a modifier of \textit{Scn1a} disease would be to assess the impact of \textit{Cnr1} or \textit{Dagl} deletion on the phenotype of the \textit{Scn1a}+/− mice. However, this method of validation is ethically undesirable as we predict this would exacerbate the seizure phenotype. Although the work conducted in Chapter 2 supports the anticonvulsant potential of potentiating the endocannabinoid system, putatively through activation of CB1 receptors, future research will need confirm that CB1 receptor activation was indeed the mechanism of action. This may prove difficult as antagonism of CB1 receptors has previously been shown to increase seizure activity (Rosenberg, Patra & Whalley, 2017). However, if a CB1 receptor antagonist was shown to have no intrinsic activity in the \textit{Scn1a}+/− mouse model, then it could be co-administered with a CB1 receptor PAM or MAGL inhibitor to determine its ability to disrupt these agents’ anticonvulsant effects and thus infer if their effects are indeed mediated by the CB1 receptor.

The work in Chapter 2 was limited in that it only evaluated the efficacy of a CB1 receptor PAM and MAGL inhibitor against the hyperthermia induced seizures. It is important to assess the robustness of the anticonvulsant efficacy of these compounds against other epilepsy-relevant endpoints available in the model, such as the spontaneous seizure and survival phenotypes, as these phenotypes might be subserved by different neurochemical mechanisms (Hawkins et al., 2017). Drugs that enhance endocannabinoid transmission being effective against hyperthermia-induced seizures is not surprising given prior research showing that CB1 receptor blockade exacerbated acute febrile seizures in rats (Feng et al., 2016). However, whether endocannabinoid stimulating agents also have anticonvulsant efficacy against spontaneous seizures may not be so straightforward.

In Dravet syndrome, children usually first present with febrile seizures that progress to afebrile spontaneous seizures (Dravet, 2011). Therefore, to replicate this in our model, we induce
a febrile seizure before examining spontaneous seizures (Hawkins et al., 2017). Interestingly, febrile seizures upregulate CB1 receptor expression, mostly at inhibitory synapses, resulting in disinhibition of neuronal excitability (Chen et al., 2003). Moreover, febrile seizures caused CB1 receptor-mediated adulthood seizure-susceptibility, as demonstrated by SR141716, a CB1 receptor antagonist, preventing the development of adult seizure susceptibility (Feng & Chen, 2016). Thus, endocannabinoid system stimulating agents might actually have proconvulsant effects against spontaneous seizures preceded by a febrile seizure. Future research could then investigate the effects of CB1 receptor PAMs or MAGL inhibitors against spontaneous seizures that have or have not been preceded by an induced febrile seizure. Moreover, it would be useful to explore how the endocannabinoid system responds to febrile seizures in the short and long-term, by examining alterations of endocannabinoid system components at both excitatory and inhibitory synapses.

It would also be important for future studies to address whether the compounds maintain their efficacy with repeated administration. Repeated stimulation of CB1 receptors has been demonstrated to cause downregulation and/or desensitisation of CB1 receptors, which has led to the development of tolerance of efficacy. This was seen with the repeated administration of CB1 receptor agonist WIN55,212 losing efficacy against seizures (Blair et al., 2009) and the irreversible MAGL inhibitor JZL184 losing its analgesic effect (Schlosburg et al., 2010). Further support for the need to investigate chronic administration is demonstrated by the chronic use of high doses of JZL184 proving proconvulsant in a mouse model of pilocarpine induced temporal lobe epilepsy (Ma et al., 2014).

Future work extending on Chapter 2’s results could also investigate how endocannabinoid concentrations change in response to seizures. An improvement on measuring endocannabinoid concentrations from tissue homogenates would be to assess endocannabinoids in vivo with either
microextraction or microdialysis techniques that both utilise probes to extract analytes from the surrounding interstitial space. With solid-phase microextraction, the probe is coated with an “extraction medium” that directly absorbs surrounding organic compounds, before being removed then desorbed to collect analytes. Whereas, with microdialysis, the probe is comprised of a semipermeable surface that has a constant flow of perfusate moving through it that can absorb molecules of desired size and solubility from the surrounding space before dialysate being discharged for collection (Chefer et al., 2009). As endocannabinoids are lipophilic, they exhibit poor solubility in standard perfusate solutions. However, the addition of the oligosaccharide β-cyclodextrin has improved the solubility of lipids in the perfusate, improving sampling efficiency of endocannabinoids by tenfold (Walker et al., 1999). β-cyclodextrin is a cylindrically shaped molecule with a hydrophobic core that binds endocannabinoids, while the hydrophilic outer surface of the complex promoting solubility. For both microdialysis and microextraction, the collected analytes can then be analysed with any analytical technique. These techniques have been successfully utilised to analyse endocannabinoid concentrations and could be applied to future research to further illuminate the role of these mediators in childhood epilepsy models (Aslam et al., 2019; Serrano et al., 2018; Sustkova-Fiserova et al., 2017; Zestos & Kennedy, 2017).

These in vivo sampling techniques are advantageous because they allow repeated sampling from live animals and gaining a better temporal understanding of endocannabinoid concentrations would better direct therapeutic developments. Improved temporal resolution of endocannabinoid levels is important as the fluctuating concentrations of endocannabinoid concentrations throughout the day could have differing functional implications for regulation of excitability. For example, 2-AG concentrations diminish during the dark phase of the light/dark cycle in multiple brain region in rats (Valenti et al., 2004), while Dravet syndrome mice are more likely to exhibit spontaneous
seizures and experience SUDEP during their dark phase (Teran et al., 2019). This suggests that MAGL inhibition may be a better served through administration in the dark phase. Moreover, these techniques have the ethical advantage of requiring less animals, which would also provide cleaner data by reducing inter-animal variability while avoiding any post-mortem accumulation of endocannabinoids (Sugiura, Yoshinaga & Waku, 2001). Furthermore, these techniques could be modified to extend to the quantification of additional lipid mediators, such as LPVs.

A notable finding from the work conducted in Chapter 2 was the increase of Daglb mRNA expression selectively in the phenotypic F1.Scnn1a+/− mice. DAGL-β is expressed almost exclusively in microglia (Viader et al., 2016), therefore, if transcriptional expression translates to protein, our findings suggest that F1.Scnn1a+/− mice may have increased microglial cell 2-AG activity. Research indicates that microglial cell 2-AG levels increase in response to cell damage to elicit neuroprotection via CB2 receptors, which exhibit increased expression on activated microglia (Hohmann et al., 2019; Kreutz et al., 2009; Kreutz et al., 2007; Stella, 2009; Viader et al., 2016; Walter et al., 2003). Future research could use multi-probe immunofluorescence staining of Dravet syndrome mouse brain tissue with antibody probes for Dagl-β and CB2 receptors to quantify expression of these proteins, in conjunction with a probe for Iba-1 to detect microglia number and morphology. If protein expression correlates with transcription, then the impact of Daglb on the Dravet syndrome phenotype could be assessed genetically by crossing Daglb knockout mice with Dravet syndrome mice (Tanimura et al., 2010; Viader et al., 2016; Wilkerson et al., 2016) or pharmacologically with specific Dagl-β inhibitors (Luk et al., 2018; Wilkerson et al., 2016).

Dagl-β is a compelling target as the activation of microglia has been implicated in the process of epileptogenesis (Devinsky et al., 2013; Eyo, Murugan & Wu, 2017). For example,
selective activation of microglia resulted in mice developing severe early-onset spontaneous seizures and premature death during post-natal weeks 3 and 4 (Zhao et al., 2018). This is noteworthy because Dravet syndrome mice develop spontaneous seizures and premature death during this exact time-frame (Miller et al., 2014; Mistry et al., 2014). Moreover, although preliminary, this finding does suggest that F1.\textit{Scn1a}^{+/−} mice may be exhibiting neuroinflammation in the hippocampus. Increased inflammatory response to seizure insults facilitates epileptogenesis, as demonstrated by the pharmacological or genetic inhibition of interleukin 1β receptor preventing the development of seizure susceptibility (Feng et al., 2016). Additionally, many anticonvulsants exert therapeutic efficacy via anti-inflammatory actions in various seizure models (Aronica et al., 2017), while many cannabinoids, including CBD, exhibit anti-inflammatory activity (Nagarkatti et al., 2009; van Niekerk, Mabin & Engelbrecht, 2019). Therefore, whether neuroinflammation occurs in the Dravet syndrome mouse model remains an open question and its investigation might provide useful traction on understanding potential anticonvulsant mechanisms of cannabinoids.

Another useful investigation that would greatly advance our understanding of the pathophysiology of the Dravet syndrome phenotype would be comparing the transcriptome of different neuronal cell types using single-cell sequencing (Cadwell et al., 2017; Ho et al., 2018). This technique can investigate the transcriptome from a single cell and can therefore be utilised to compare differences between the WT and \textit{Scn1a}^{+/−} mice from both seizure-susceptible and seizure-resistant background strains of different cell types, including different neuronal populations, astrocytes and microglia. For instance, Gpr55 receptors are also expressed on microglia with Gpr55 activation reducing the number of activated microglia and demonstrating neuroprotective effects in a model of excitotoxicity (Kallendrusch et al., 2013; Pietr et al., 2009). Therefore, this
technique could be useful for investigating the expression of multiple targets of interest in a cell-specific manner to better inform on their possible contribution to the Dravet syndrome phenotype.

Chapter 3 of the thesis investigated the role of the GPR55-LPI system in the Scn1a<sup>+/−</sup> mouse model. It showed that the F1 seizure-susceptible mice had increased hippocampal Gpr55 transcript expression and overall reduced LPI concentrations. Interestingly, heterozygous deletion of Scn1a only reduced hippocampal LPI concentrations in mice on the seizure-resistant 129 background strain and not in mice on the seizure-susceptible F1 strain. This suggests that the GPR55-LPI system in the seizure-susceptible strain may not be able to adapt to the impact of heterozygous deletion of Scn1a. We also demonstrated that heterozygous deletion of Gpr55 was anticonvulsant against hyperthermia-induced seizures and ameliorated the survival deficit normally observed in the phenotypic Scn1a<sup>+/−</sup> mice, albeit with a technical limitation requiring these experiments to be repeated with Gpr55 knockout mice that are congenic to the C57BL/6 background strain.

A background strain can be made congenic with continuous backcrossing, that can be accelerated with the assistance of background strain sequencing (Wong, 2002). However, a potential limitation with this method is the conservation of the original 129 genetics on loci that influence phenotype. This has previously been observed by our collaborator Dr Jennifer Kearney (Northwestern University, Chicago) who backcrossed a background strain to 99.9% congenic C57BL/6 with the remaining 0.1% of 129 genetic material segregating to survival-influencing loci (unpublished data). A better approach would be to generate a new line of Gpr55 knockout mice directly on the C57BL/6 background using CRISPR-Cas9 technology (Wefers et al., 2017). Nevertheless, there is research demonstrating that the selective Gpr55 antagonist, CID16020046, reduced the frequency of action potential outputs and increased the frequency of spontaneous inhibitory postsynaptic currents in granule cells from Scn1a<sup>+/−</sup> hippocampal slices (Kaplan et al.,
Therefore, despite the limitations of the work conducted in Chapter 3, when taken in conjunction of prior work showing GPR55 antagonism reduced neuronal hyperexcitability, the data presented here putatively supports Gpr55 as a new anticonvulsant drug target for Dravet syndrome.

A novel interesting finding from Chapter 3 that requires further investigation is that heterozygous Scn1a deletion downregulated the mRNA expression of Mboat7. This enzyme is involved in phospholipid remodelling and is known to degrade 1-stearoyl LPI (Anderson et al., 2013; Lee et al., 2012), while also being involved in the remodelling of other lipid mediators such as other species of LPI, PIs, and phosphatidylinolenolamines (PEs) (Anderson et al., 2013; Lee et al., 2012). Gene variants of MBOAT7 in humans has been associated with intellectual disability, autism spectrum disorder and epilepsy (Jacher et al., 2019; Johansen et al., 2016; Yalnizoglu et al., 2019), while Mboat7 gene deletion in rodents causes brain atrophy and premature death (Anderson et al., 2013; Lee et al., 2012). It is therefore possible that diminished MBOAT7 expression in Scn1a+/- mice might be linked to brain atrophy, especially in white matter, and cognitive impairments observed in Dravet syndrome patients (Brunklaus et al., 2012; Dravet, 2011; Genton, Velizarova & Dravet, 2011; Han et al., 2012; Ito et al., 2013; Perez et al., 2014; Striano et al., 2007).

Downregulation of Mboat7 mRNA expression in both seizure-susceptible and seizure-resistant mouse strains suggests it is not critically important to the seizure phenotype. However, as the impact of heterozygous deletion of Scn1a on cognitive and emotional function has not been examined in the 129 strain, Mboat7 may still be implicated in the behavioural comorbidities. Future work would first need to confirm alterations at the functional protein level and if confirmed, investigate any potential implications. Unfortunately, there are currently no pharmacological tools
available to directly potentiate the activity of MBOAT7 and observe its effect on behavioural phenotypes. However, a comprehensive lipidomic analysis of the effects of Mboat7 downregulation might reveal other druggable targets to revert the metabolic impact of Mboat7 deficiency.

The work in Chapter 2 and 3 assessed the endocannabinoid and Gpr55-LPI systems at a single age and in a single brain region. We chose P24 as this is when susceptible mice exhibit spontaneous seizures, however the Dravet syndrome phenotype changes through development (Miller et al., 2014; Mistry et al., 2014). As detailed in Chapter 1, susceptibility to febrile seizures is prominent in the earlier years of life before afebrile spontaneous seizures develop. Seizures can resolve or lessen with age, while the behavioural deficits that begin in childhood worsen with age. The endocannabinoid system also changes throughout development, as demonstrated by age-related changes in receptor expression and endocannabinoid concentrations (Kwok et al., 2017; Lu & Mackie, 2016; Meyer, Lee & Gee, 2018). Therefore, a temporal understanding of dynamic changes in the endocannabinoid system in the Dravet syndrome mouse model may better inform how the endocannabinoid system contributes to the different staged of pathophysiology of this condition and might better inform therapeutic strategies that aim to target endocannabinoid signalling.

The work in this thesis solely focused on the hippocampus because it is a region that is integral to the Dravet syndrome phenotype. Nav1.1 haploinsufficiency significantly impairs neuronal excitability the hippocampus (Liautard et al., 2013; Rubinstein et al., 2015; Tsai et al., 2015), and deletion of Scn1a in mice on a C57Bl6J background selectively in the hippocampus was sufficient for the development of thermal seizures and spatial memory deficits during adulthood (Stein et al., 2019). However, the study conducted by Stein et al. (2019) could not
reproduce other behavioural deficits such as hyperactivity or social deficits, signifying that other brain regions must be important to the phenotype. Future research could therefore expand investigation to additional brain regions including the cortex, which is also implicated in the aetiology of the epilepsy phenotype as demonstrated by impaired cortical inhibitory transmission and through forebrain-specific Scn1a deletion studies (Cheah et al., 2012; Han et al., 2012; Kalume et al., 2007; Kaplan et al., 2017; Ogiwara et al., 2013; Rubinstein et al., 2015; Tai et al., 2014; Tsai et al., 2015; Yu et al., 2006). Supporting the need to expand investigation of the endocannabinoid and GPR55-LPI system to additional brain regions is research demonstrating that the CB1 and GPR55 receptor function can vary between brain regions. For example, Gpr55 activation increased excitatory transmission in the hippocampus but promoted inhibitory transmission in the striatum (Hurst et al., 2017; Kramar et al., 2017; Musella et al., 2017; Sylantyev et al., 2013). Moreover, as we saw improved survival in the Scn1a mice with heterozygous deletion of Gpr55, it would be informative to assess expression and function of Gpr55 in brain regions that may be implicated in SUDEP, such as the breathing centres in the brainstem (Kuo et al., 2019).

Gaining a better understanding of CB1 and Gpr55 receptor expression and function throughout development and in response to seizures would provide vital information on how the broader endocannabinoid system could be contributing to and responding to Na\(_{\text{V}}\)1.1 impairment-induced hyperexcitability. Transcript expression of candidate receptors can be measured via quantitative PCR or in situ hybridisation, and protein expression can be measured with western blotting or immunohistochemical techniques. These methods require the use of many mice as they provide only single time-points of information per sample. Moreover, we couldn’t successfully measure Gpr55 protein expression with western blotting due to commercially-available antibodies
proving nonspecific, thus there is a need to develop and validate improved Gpr55-specific antibodies.

Another technique that could be utilised to measure receptor expression in vivo that also allows sampling across multiple time-points is a system that utilises fibre photometry optimised for assessment of transcription. This technique combines fibre photometry, which utilises the implantation of optical fibres in select brain regions with the viral delivery of fluorescence or bioluminescence reporters that are targeted to genes of interest and stimulated by the transcription process. This technique was demonstrated to reliably measure the expression of genes that are markers of circadian rhythms across multiple days (Mei et al., 2018). Therefore, the transcription of our candidate genes, for example Cnr1 or Gpr55, can be directly measured in a regionally-specific manner, which can be utilised to measure expression throughout development, as well as in response to seizures and epileptogenesis. Although this technique is limited in only measuring gross regional expression, it would inform on the temporal response to seizures and epileptogenesis. Then further investigation with double- or triple-fluorescent imaging of candidate receptors (e.g. CB1 and Gpr55) and markers for specific neuronal populations, such as glutamate decarboxylate (GAD65/67) for GABAergic neurons and vesicular glutamate transporter 1 (VGLUT1) for glutamatergic neurons, could further provide information on cell-specific expression of the candidate molecular targets.

More detailed functional mechanisms of our candidate molecular targets could be explored in future studies utilising electrophysiological recordings from ex vivo brain slices of Dravet syndrome mice. As activation of CB1 receptors inhibits neuronal cell activity, CB1 receptor function can be directly assessed using whole-cell patch-clamp recordings from either excitatory or inhibitory post-synaptic neurons (Farrell & Soltesz, 2019; Soltesz et al., 2015). As
endocannabinoid signalling is stimulated by depolarisation, the evaluation of excitatory or inhibitory post-synaptic potentials in response to depolarising stimuli in Scn1a+/− mice blain slices will inform on efficacy of depolarisation-induced suppression of neuronal activity, and therefore cannabinoid receptor-mediated suppression of excitatory activity. Confirmation of CB1 receptor-mediated activity can be determined by repeating the experiment in the presence of a CB1 receptor antagonist such as AM251. The function of Gpr55 receptors, on the other hand, can be determined from whole-cell patch-clamp recordings from excitatory or inhibitory postsynaptic neurons conducted in the presence of applied Gpr55 endogenous ligand (LPI) or synthetic ligands (e.g. O-1602) and confirmed with Gpr55 antagonists (e.g. CID16020046) (Sylantyev et al., 2013).

In addition to seizures, Dravet syndrome patients exhibit developmental delays in attentional, emotional and cognitive functions. These behavioural comorbidities are stronger predictors of a lower quality-of-life than seizure frequency (Lagae et al., 2018; Sinoo et al., 2019), highlighting a dire need for treatments that specifically target behavioural deficits. The adult behavioural phenotype of Scn1a+/− mice recapitulates many of the associated behavioural comorbidities reported in humans (Han et al., 2012; Ito et al., 2013). The work conducted in Chapter 4 aimed to characterise for the first time the behavioural phenotype of a Scn1a+/− mouse model during adolescence. This characterisation more closely models the behavioural impairments observed in paediatric Dravet syndrome patients and establishes a platform to test early-intervention therapeutics. We assessed the impact of heterozygous deletion of Scn1a in mice on an array of behavioural measures and report that adolescent Scn1a+/− mice exhibited normal locomotor activity, marble burying behaviour, sociability and sensorimotor gating, but increased anxiety-related thigmotactic behaviour, atypical fear expression, blunted acoustic startle responses, and impaired social recognition and spatial memory.
Cannabis-based therapies may prove to have only modest effects on seizure control but may have profound effects on the non-seizure symptoms associated with Dravet syndrome, ultimately improving the overall quality of life of these patients. CBD’s impact on non-seizure symptoms in Dravet syndrome patients has not been directly assessed in clinical trials. However, there are multiple observational studies suggesting that CBD treatment improved quality of life measures, such as sleep, alertness and mood in paediatric epilepsy patients (McCoy et al., 2018; Porter & Jacobson, 2013; Stockings et al., 2018). Although these are just observational studies, they are supported by preclinical research showing that CBD reversed social deficits in adult Scn1a<sup>++</sup> mice in the three-chambered social preference test and in the standard social interaction test (Kaplan et al., 2017). Future work extending on this thesis could utilise the behavioural assessment platform established here to assess the behavioural impact of early-intervention treatment with CBD and other cannabinoids.

This behavioural platform could also be utilised to examine the impact of Cnr1, Gpr55 or Mboat7, and any other potential genetic modifiers, on the behavioural phenotypes of Scn1a<sup>++</sup> mice. The endocannabinoid system has been well-established to be involved in many behavioural modalities, including learning and memory (Kruk-Slomka et al., 2017; Mechoulam & Parker, 2013; Riedel & Davies, 2005), suggesting that CB1 receptor-mediated endocannabinoid signal potentiation could be therapeutically beneficial. Whereas, pharmacological and genetic studies in rodents have shown Gpr55 influences various behavioural functions including locomotor activity, sociability, as well as fear, recognition and spatial memory (Alavi et al., 2016; Hurst et al., 2017; Kramar et al., 2017; Marichal-Cancino et al., 2018; Meadows et al., 2016; Rahimi, Hajizadeh Moghaddam & Roohbakhsh, 2015). Collectively these reports suggest that Gpr55 activation may have detrimental effects on behaviour and that Gpr55 inhibition may mitigate behavioural...
impairments, as observed by CBD ameliorating social deficits in \textit{Scn1a}^{+/−} mice (Kaplan, Isom & Petrou, 2016). Pharmacological and genetic knockdown studies could be implemented to assess the influence of novel target genes on the \textit{Scn1a}^{+/−} behavioural phenotype to validate new drug targets that extend beyond seizure control.

An interesting observation from this work was that the behavioural deficits reported in adult mouse models of Dravet syndrome, which include hyperactivity, impaired sociability and significant fear and spatial memory deficits were not replicated in our study (Han et al., 2012; Ito et al., 2013). This may be due to the age of the mice at testing, as the behavioural impairments might progressively worsen with age, which has been reported in Dravet syndrome patients (Berkvens et al., 2015; Brunklaus, Dorris & Zuberi, 2011; Genton, Velizarova & Dravet, 2011; Olivieri et al., 2016). The adult behavioural phenotype of these mice could be evaluated to determine if impairments are progressive which would reaffirm the need to explore early intervention therapeutics. However, another possibility for the inability to replicate prior behavioural characterisations of \textit{Scn1a}^{+/−} mice could be the genetic background strain of the mice that were investigated. The \textit{Scn1a}^{+/−} mice tested here were on a mixed F1 (129S6/SvEvTac x C57BL/6J) background, whereas studies in other lines were conducted in mice on a congenic C57BL/6 background (Han et al., 2012; Ito et al., 2013). Severity of the Dravet syndrome seizure phenotype is catastrophically sensitive to C57BL/6 background-strain genetics, with increasing the proportion of C57BL/6 genetics increasing the severity of the seizure phenotype (Hawkins et al., 2016; Miller et al., 2014; Mistry et al., 2014; Rubinstein et al., 2015). Therefore, it is possible that the partial C57BL/6 background genetics used here might explain the less pronounced behavioural impairments that were observed in Chapter 4.
Future work could extend upon the characterisation of the non-seizure behavioural phenotypes of $Scn1a^{+/-}$ mice reported in Chapter 4. For example, characterising the sleep phenotype in our $Scn1a^{+/-}$ mice could be explored as patients report high prevalence of sleep disturbances (~75%) that negatively impact on quality of life (Dhamija et al., 2014; Licheni et al., 2018). We hypothesise that we may observe impaired sleep behaviour in our mouse model as impaired sleep quality and homeostasis has already been reported in other $Scn1a^{+/-}$ mice (Kalume et al., 2015). This is another area where cannabis-based therapies may prove beneficial as there is evidence to suggest that CBD may improve sleep quality (Babson, Sottile & Morabito, 2017; Fleury-Teixeira et al., 2019; Pane & Sacca, 2019; Porter & Jacobson, 2013), and because the therapeutic use of cannabinoids for sleep is a growing area of research (Babson, Sottile & Morabito, 2017).

A limitation of the work in Chapter 4 was that we did not assess behaviour in female mice. During our investigation, the female $Scn1a^{+/-}$ mice displayed increased mortality compared to males that was not statistically significant. Despite most work on $Scn1a^{+/-}$ mouse models reporting no sex-differences (Anderson et al., 2019a; Hawkins et al., 2017), a recent study reported a sex-dependent effect on mortality with female mice displaying a significantly higher mortality rate than male mice (Niibori et al., 2019). Moreover, in support of sex differences was a recent study reporting social impairment in male but not female $Scn1a^{+/-}$ mice (Williams et al., 2019). This suggests that there may be subtle differences in rearing conditions that may influence sex-dependent effects. However, the translational significance of this finding is difficult to determine as there have been no sex-dependent differences reported in the prevalence of Dravet syndrome patients (Bayat, Hjalgrim & Moller, 2015; Brunklaus et al., 2012; Ishii et al., 2017; Krueger & Berg, 2015; Wu et al., 2015), nor for the rate of mortality observed in Dravet syndrome patients.
(Cooper et al., 2016). There has been, however, one study reporting female Dravet syndrome patients display more severe symptoms (Ohmori et al., 2003). Future work could investigate whether our model does indeed display sex-dependent effects on survival and behavioural impairments. The identification of sex-dependent effects may be a consideration that needs to be addressed in the development of therapeutics that are effective for both sexes.

The ‘genetic modifier’ approach has identified additional molecular targets associated with the endocannabinoid system to those explored in this thesis that may hold therapeutic potential. For example, we have identified that mRNA expression of transient receptor potential cation channel subfamily V member 1 (TRPV1) receptors, was significantly increased in F1 seizure-susceptible mice (data not shown). TRPV1 is a non-selective cation channel that is activated by endogenous and exogenous vanilloids, extracellular acidification, heat and anandamide (Bevan, Quallo & Andersson, 2014; Zygmunt et al., 1999). TRPV1 activation enhances neuronal excitability by increasing intracellular concentrations of cations, which increase membrane conductance and consequently neurotransmitter release (Fawley, Hofmann & Andresen, 2014; Naziroglu, 2015). Interestingly, Trpv1 knock-out mice and Trpv1 antagonists, such as capsazepine and AMG-9810, have proven anticonvulsant in pentylenetetrazol-induced seizure models (Jia et al., 2015; Kong et al., 2014; Naderi et al., 2015; Shirazi et al., 2014; Suemaru et al., 2018). Therefore, the influence of Trpv1 on the Dravet syndrome phenotype is currently being investigated. Moreover, the identification of numerous molecular targets that can impact on the Scn1a+/− phenotype supports and further validates the use of the ‘genetic modifier’ approach in novel drug development.
Conclusion

The research presented in this thesis expands our understanding of the Dravet syndrome mouse model by providing insight into how the broader endocannabinoid system could be influencing phenotype severity as well as providing a better understanding of the behavioural impairments that present during adolescence. This research highlights CB1 and Gpr55 receptors as potential modifiers of the Dravet syndrome phenotype. We demonstrated less CB1 and more Gpr55 receptor mRNA expression in mice on the F1 seizure-susceptible background strain compared to mice on the 129 seizure-resistant strain. Moreover, we validated the anticonvulsant potential of these targets by pharmacologically potentiating CB1 receptor activity and with heterozygous deletion of Gpr55. The work in this thesis has also identified additional molecular targets, Dagl-β and Mboat7, that may influence the Scn1a+/− phenotype and warrant further investigation. Lastly, we characterised the behavioural impact of heterozygous Scn1a deletion in adolescent mice to establish a platform that better models the behavioural impairments observed in paediatric Dravet syndrome patients. We identified numerous deviations in measures of social, cognitive and attentional behaviours but did not reproduce all impairments previously reported for adult Scn1a+/− mice. This suggests that developmental delays may be progressive and there may be benefit to early therapeutic intervention. By using the Scn1a+/− mouse model to deepen our understanding of the Dravet syndrome condition, the work in this thesis will assist in the development of novel therapeutics for a more comprehensive treatment of Dravet syndrome.
Chapter 6

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