Cannabidiol improves survivability, seizures and associated behavioural comorbidities in a range of animal models of epilepsy

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By

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Declaration

The work described in this thesis was carried out between October 2015 and December 2018 in the School of Pharmacy at the University of Reading. I confirm that this is my own work and materials from other sources have been properly acknowledged.

Date 26/01/2019 

Pabitra Hriday Patra
Dedicated to the Animals who sacrificed their life for this work
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I am very much grateful to my parents, brother and grandparents and other family members for all your support, and for the sacrifices that you have made on my behalf. You have been my strength and your prayers for me are what sustained me thus far. Also, special thoughts for all my well-wishers and finally, I thank god for everything.
Publications

Part of this thesis


Not part of this thesis


Conference presentations

2018: American Epilepsy Society Annual Meeting, New Orleans, USA *(Poster)*.

2017: American Epilepsy Society Annual Meeting, Washington DC, USA *(Poster)*.

2017: 8th European Workshop on Cannabinoid Research, British Pharmacological Society, University of Roehampton, UK *(Poster)*.
Abstract

Epilepsy is a chronic neurological disease characterised by recurrent seizures, premature mortality and several associated comorbidities such as motor disorder, anxiety, depression, social deficits and cognitive impairment.

Here, the effect of cannabidiol (CBD) in three different preclinical models of epilepsy was investigated. First, the effect of 9-weeks oral CBD administration (200 mg/kg/day) on seizures, motor function, gait and cognition in a reduced intensity status epilepticus induced spontaneous recurrent seizures (RISE-SRS) rat model of temporal lobe epilepsy (TLE) was evaluated. Subsequently, the effect of long-term CBD administration (100 mg/kg, twice daily s.c. injections from postnatal day 8 (P8) to 25/death) on survivability and a number of welfare parameters such as natural activity, reflex/response to touch, total neonatal welfare, orbital tightening and body condition in the Scn1a−/− mouse model was investigated. Finally, the effect of chronic CBD administration (100 mg/kg, twice daily s.c. injections from P8 to 52/death) on premature mortality and comorbidities such as motor dysfunction, social deficits, anxiety, depression and cognitive impairment in the Scn1a+− mouse model of Dravet syndrome was assessed.

I demonstrated for the first time that chronic CBD treatment improved seizures, motor function and cognition without producing any adverse effect on gait in RISE-SRS rat model of TLE. Further, I showed that CBD treatment extended survivability and improved the neonatal welfare parameters in the Scn1a−/− mouse model. Moreover, I established the novel finding that chronic CBD-treatment prevented premature mortality and improved the comorbidities associated with the Scn1a+/− mouse model of Dravet syndrome.

Although the anticonvulsant property of CBD has been shown in animal models of seizures, I am the first to demonstrate that CBD has disease modifying potential, and improves seizures, survivability and comorbidities associated with preclinical models of epilepsy. Notably, this project formed a core part in the development and US-FDA approval of Epidiolex® (GW Pharmaceuticals) in 2018.
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List of abbreviation

AA  Arachidonic acid
ABHD6  Alpha/beta-hydrolase domain containing 6
ACEA  Arachidonoyl-2’-chloroethylamide
ADHD  Attention deficit hyperactivity disorder
AEA  N-Arachidonoyl ethanolamide
AED  Antiepileptic drug
2-AG  2-Arachidonoylglycerol
AIDS  Acquired immune deficiency syndrome
AK  Adenosine kinase
AMPA  Ionotropic α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid
ANOVA  Analysis of variance
4-AP  4-Aminopyridine
BC  Body condition
BDNF  Brain-derived neurotrophic factor
bp  Base pairs
CB1  Cannabinoid receptor 1
CB2  Cannabinoid receptor 2
CB1  Cannabichromene
CBD  Cannabidiol
CBDV  Cannabidivarin
CBG  Cannabigerol
CBN  Cannabinol
cm  Centimetre
DAGL  Diacylglycerol lipase
DNA  Deoxyribonucleic acid
DSE  Depolarised-induced suppression of excitation
DSI  Depolarised-induced suppression of inhibition
EEG  Electroencephalography
ENT  Equilibrative nucleoside transporter (ENT)
EPM  Elevated plus maze
EtNH2  Ethanolamine
FAAH  Fatty acid amide hydrolase
GABA  γ-aminobutyric acid
GAT1  GABA transporter 1
GLAST  Glutamate aspartate transporter
GLT1  Glutamate transporter 1
GPR55  G-protein coupled receptor, GPR55
h  Hour
5-HT  5-Hydroxytryptamine
Hz  Hertz
i.m.  Intramuscular
i.p.  Intraperitoneal
i.v.  Intravenous
ILAE  International League Against Epilepsy
IQ  Intelligence quotient
IQR  Interquartile range
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>KA</td>
<td>Kainic acid</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Inhibitory constant</td>
</tr>
<tr>
<td>LSL</td>
<td>Left stride length</td>
</tr>
<tr>
<td>LTLE</td>
<td>Lateral temporal lobe epilepsy</td>
</tr>
<tr>
<td>mA</td>
<td>Milliampere</td>
</tr>
<tr>
<td>MAGL</td>
<td>Monoacylglycerol lipase</td>
</tr>
<tr>
<td>MES</td>
<td>Maximal electroshock seizure</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic G protein coupled receptor</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>MPEP</td>
<td>2-Methyl-6-(phenylethynyl)pyridine</td>
</tr>
<tr>
<td>MTLE</td>
<td>Mesial temporal lobe epilepsy</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>NA</td>
<td>Natural activity</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>N-Acylphosphatidylethanolamine-hydrolysing phospholipase D</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomole</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>OT</td>
<td>Orbital tightening</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PPAR$\gamma$</td>
<td>Peroxisome proliferator-activated receptor $\gamma$</td>
</tr>
<tr>
<td>PSBB</td>
<td>Post seizure behavioural battery tests</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazole</td>
</tr>
<tr>
<td>QOLCE</td>
<td>Quality of life in childhood epilepsy</td>
</tr>
<tr>
<td>RAM</td>
<td>Radial arm maze</td>
</tr>
<tr>
<td>RISE-SRS</td>
<td>Reduced intensity status epilepticus induced spontaneous recurrent seizures</td>
</tr>
<tr>
<td>RM</td>
<td>Reference memory</td>
</tr>
<tr>
<td>RME</td>
<td>Reference memory error</td>
</tr>
<tr>
<td>RSL</td>
<td>Right stride length</td>
</tr>
<tr>
<td>RT</td>
<td>Reflex/response to touch</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Status epilepticus</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SMEI</td>
<td>Severe myoclonic epilepsy of infancy</td>
</tr>
<tr>
<td>SRS</td>
<td>Spontaneous seizures</td>
</tr>
<tr>
<td>ST</td>
<td>Surface temperature</td>
</tr>
<tr>
<td>SW</td>
<td>Stride width</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TGF$\beta$</td>
<td>Transforming growth factor-$\beta$</td>
</tr>
<tr>
<td>TLE</td>
<td>Temporal lobe epilepsy</td>
</tr>
<tr>
<td>TNW</td>
<td>Total neonatal welfare</td>
</tr>
<tr>
<td>TRPA1</td>
<td>Transient receptor potential of ankyrin type 1</td>
</tr>
<tr>
<td>TRPA2</td>
<td>Transient receptor potential of ankyrin type 2</td>
</tr>
<tr>
<td>TRPM8</td>
<td>Transient receptor potential of melastatin type 8</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid receptor type 1</td>
</tr>
<tr>
<td>TRPV2</td>
<td>Transient receptor potential vanilloid receptor type 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>US-FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>WM</td>
<td>Working memory</td>
</tr>
<tr>
<td>WME</td>
<td>Working memory error</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Δ⁹-THC</td>
<td>Δ⁹-Tetrahydrocannabinol</td>
</tr>
<tr>
<td>Δ⁹-THCV</td>
<td>Δ⁹-Tetrahydrocannabivarin</td>
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Chapter 1: General introduction
1.1 Epilepsy

Epilepsy is a progressive, chronic neurological disorder characterized by recurrent seizures (Blume et al., 2001). An epileptic seizure can be defined as “transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain” (Fisher et al., 2005). However, a more severe form of seizure termed ‘status epilepticus’ (SE) can also occur in which an individual exhibits seizure continuously for more than five minutes or repetitive seizures within this time frame without gaining consciousness (Trinka et al., 2015). If SE persists for more than half an hour it might lead to long-term after effects such as neuronal injury, neuronal death and shift of neuronal networks (Trinka et al., 2015). Epilepsy remains a significant health concern as almost more than 65 million people worldwide are diagnosed with this neurological disease (Thurman et al., 2011). Further, the morbidity associated with epilepsy is so high that individuals with the disease are often stigmatised and become isolated from the society (Dilorio et al., 2003; Tekle-Haimanot et al., 1991; Atadzhanov et al., 2010; Baker et al., 2000; Baker et al., 2005). Epilepsy is more prevalent in low- and middle-income countries; almost 80% people with epilepsy live in these countries (WHO, 2018). The higher prevalence in these countries is mainly attributed to inadequate treatment facilities and only about 20% epileptic patients receive treatment (Mbuba et al., 2008). It is notable that >50% of brain diseases show seizure as a secondary symptom so, while not primary epilepsy, carry many of the same problems (WHO, 2018).

There has always been a matter of debate and confusion among the clinicians and researchers on the diagnosis of epilepsy. According to the International League Against Epilepsy (ILAE), a person can be considered as epileptic if he/she had two unprovoked seizure in more than 24 hours apart, or one unprovoked seizure together with a probability of minimum 60% general recurrence risk for the next 10 years, or diagnosed with an epilepsy
syndrome (Fisher et al., 2014). According to the authors this definition would be helpful for an early diagnosis and intervention to avoid unnecessary injuries associated with accidents during epileptic attacks or prevent the disease progression. However, issues have been raised on the inclusion of the percentage value (60%) in the second part of the definition by Dr. Hauser, which according to him ‘represents an expert consensus and has limited substantive data to support the recommendations’ (Hauser, 2014). The agreed definition of epilepsy therefore remains open to debate.

Next, I will provide a brief historical background of epilepsy before discussing its aetiology, pathophysiology, therapy, classification and animal models. I will also briefly illustrate temporal lobe epilepsy (TLE) and Dravet syndrome due to their importance in this project.

1.1.1 Historical background

The history of epilepsy is closely linked to the history of human civilization, with the first account of epileptic seizures traced back to ~2000 BC in a Mesopotamian text written in Akkadian language (Temkin, 1945). The text described a person having seizures and termed the condition as ‘antasubbû’ (the hand of sin) (Labat, 1951). It was believed that the goddess of the moon punished him for breaching religious/social laws (Labat, 1951). The description of epilepsy can also be found in ancient Egyptian, Babylonian and Greek texts (Daras et al., 1994; Longrigg, 2000). However, during that time epilepsy was not considered as a disease but the outcome of divine possession or punishment. Interestingly, Atreya, an Indian medical author (~600 BC) attributed epilepsy to a brain disorder and rejected the age-old concept of divine possession in his famous text ‘Caraka Samhitā Sutra’ (Pirkner, 1929). Nevertheless, epilepsy was not well understood until the famous Greek author Hippocrates suggested a possible aetiology and therapy (Hippocrates, 1950). In his book ‘On Sacred Disease’ (~400 BC) he mentioned epilepsy as a great disease from where the term ‘grand mal’ originated.
During the post-Hippocratic era a notable contribution was made by Aretaeus (~100 AD) who first described the premonitory symptoms of seizures such as hallucinations, smelling odours, tremors or sensations in the limbs (Aretaeus, 1856). Unfortunately, research on epilepsy became stagnant during the medieval period (~500-1500 BC) where religious superstitions overshadowed the scientific merit and as a result, epilepsy was attributed to demonic possessions or outcome of sins (Beyerstein, 1988).

Epilepsy research regained its scientific importance at the beginning of 16th century and during this time the Swiss-German physician Paracelsus highlighted that epilepsy had natural origins such as hereditary, malnutrition or weakness of semen rather than any divine possessions (Paracelsus, 1933). Further progress in understanding the pathophysiology and therapy was made during the 18th century. For example, Herman Boerhaave, a Dutch doctor tried to define epilepsy as the sudden elimination of vital body functions with increased muscle movement and convulsion (Boerhaave, 1761). Swiss physician Simmon Tissot compiled a large set of previous works in his ‘Traite de l’ epilepsie’ which set up a milestone for epilepsy research (Tissot, 1770). He accepted epilepsy as a hereditary disease and suggested epileptic individual not to get married. Then in 19th century, the French psychiatrist J.D. Esquirol and M. Flourens made significant contribution in understanding different forms of epileptic seizures and their possible origins (Flourens, 1823; Esquirol, 1838). Further, British physician J.C. Prichard described the typical symptoms of an epileptic seizure and he was the first to coin the term ‘partial epilepsy’ (Prichard, 1828). In addition to understanding the disease, researches were carried on to develop therapies for epilepsy and it gained the success with the introduction of potassium bromide by Edward Sieveking as the first antiepileptic drug (Sieveking, 1857). Among other notable works during this time, the famous book ‘Epilepsy: Its Symptoms, Treatment and Relation to Other Chronic Convulsive Diseases’ written by British neurologist J.R. Reynold was important where he differentiated
‘epilepsy proper’ from that of ‘idiopathic epilepsy’ (Reynolds, 1861). Further, J.H. Jackson (1835-1911), who was also known as the father of modern epileptology, suggested that epilepsy is caused due to sudden, rapid and excessive local discharges from grey matter in the brain (Jackson, 1863; Jackson, 1864). Almost a decade later, Richard Caton first recorded electrical activities in the cerebral hemispheres of rabbits and monkeys using a galvanometer which paved the way for using electrophysiology in epilepsy research (Caton, 1875).

Epilepsy research received a fast pace thereafter in 20th century. For instance, In 1912, Kaufman and Pravdicz-Neminsky induced experimental seizures in dogs and suggested a link between the abnormal electrical discharge and epilepsy (Kaufman, 1912; Pravdicz-Neminski, 1912). In the same year, Alfred Hauptmann discovered the anticonvulsant action of phenobarbital which is considered as a major landmark in the history of antiepileptic drug discovery (Hauptmann, 1912). A decade later, the German psychiatrist Hans Berger first performed electroencephalography (EEG) on a human patient during generalised tonic-clonic seizures (Berger, 1929). Almost at the same time, Merritt and Putnam made a serendipitous discovery of the anticonvulsant efficacy of phenytoin (Merritt and Putnam, 1938). In the middle of the 20th century, several other drugs such as carbamazepine (Schindler and Häfliger, 1954), ethosuximide (Vossen, 1958) and sodium valproate (Meunier et al., 1963) were subsequently introduced. Among other prominent figures in mid to late 20th century, Henri Gastaut made significant contribution in the field of epilepsy. He first defined five major EEG patterns (lambda waves, mu rhythm, pi rhythm, rolandic spikes and posterior theta rhythm) in human and also characterised a number of epilepsy types including Lennox-Gastaut syndrome (Dravet and Roger, 1996). Several notable discoveries have been made in the field of epilepsy research since the late 20th centuries till date which will be highlighted in the coming sections.
1.1.2 Aetiology

Epilepsy is a complex neurological disease with multiple aetiological factors that may influence the risk of developing the disease (Thomas and Berkovic, 2014). For instance, brain tumours including melanoma, multiple metastases, haemorrhagic lesions, slowly growing primary tumours, gangliogliomas, low grade astrocytomas, dyssembryoplastic neuroepithelial tumours and oligodendrogliomas are common causes of epilepsy (Liigant et al., 2001; Brahimaj et al., 2014). Further, brain injury is considered as an important factor as around 20% cases of all symptomatic epilepsy are associated with prior brain injury (Lowenstein, 2009). Stroke can also promote the development of epilepsy as it has been reported that about 2-4% of stroke patients experience epilepsy in their lifetime (Olsen, 2001). Moreover, malformations of cortical development significantly contributed to the origin of symptomatic focal epilepsy in children (Fujiwara and Shigematsu, 2004). Interestingly, age serves an important aetiological factor, a meta-analysis study showed that the incidence rate of epilepsy among the children and the elderly are the highest compared to those in between the age of 20-40 years (Cloyd et al., 2006). Among others, cerebral and metabolic anoxia, infection and developmental abnormalities are pertinent risk factors in people of early age, whereas vascular malformations, trauma and hippocampal sclerosis are the predominant causative factors in adolescents, and trauma, tumour and stroke are significant risk factors in elderly people (Pomeroy et al., 1990; Fujiwara and Shigematsu, 2004; Panayiotopoulos, 2005). An important association between Alzheimer’s disease progression and epilepsy has also been documented as around 16% of Alzheimer’s disease patients experience at least one unprovoked generalised tonic-clonic seizure (Romanelli et al., 1990). Further, a higher risk of developing epilepsy is noticed in several autoimmune encephalopathies such as Rasmussen encephalitis, systemic lupus erythematosus and Behcet’s disease (Bien et al., 2005; Liou et al., 1996; Aykutlu et al., 2002). Moreover, chronic heavy alcohol users are reported to be at
higher risk of developing epilepsy and the risk is often dose dependent (Samokhvalov et al., 2010).

Besides these physiological impacts, certain genes play important role in various idiopathic epilepsy syndromes such as Juvenile myoclonic epilepsy (GABRA1 and EFHC1), absence epilepsy (CACNA1A), Dravet syndrome (SCN1A), autosomal dominant frontal lobe epilepsy (CHRNA4), benign familial neonatal convulsion (KCNQ2) and benign familial neonatal-infantile seizures (SCN2A) (Scheffer et al., 1995; Ronen et al., 1993; Claes et al., 2003; Lopes-Cendes, 2008). Of all these genetic epilepsies, the incidence rates of juvenile myoclonic epilepsy and absence epilepsy are the highest which contribute to 5-11% and 2-8% of all epilepsies, respectively (Lopes-Cendes, 2008).

1.1.3 Pathophysiology

The pathogenesis of epilepsy is intricate where several factors interplay to create an imbalance between brain’s inhibitory and excitatory circuitry that ultimately leads to manifestation of spontaneous seizures (Scharfman, 2007). The process through which the neuronal network inside brain shifts from normal to an excitatory one and produces unprovoked seizures is known as epileptogenesis (Engel, 2001; Pitkanen and Lukasiuk, 2011). During the epileptogenesis process a widespread molecular and structural changes along with genetic and epigenetic alterations take place in both neuronal and non-neuronal cells in the brain which ultimately makes the neuronal circuits dysfunctional (Devinsky et al., 2018c).

Glutamate and γ-aminobutyric acid (GABA) are the two main neurotransmitters which play crucial roles in epilepsy (Rowley et al., 1995). Glutamate, the main excitatory neurotransmitter is released from the presynaptic vesicles with the help of calcium ions (Takumi et al., 1998). Glutamate acts through several receptors such as ionotropic α-amino-3-
hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), N-methyl-D-aspartic acid (NMDA) and kainate, as well as metabotropic G protein coupled receptors (mGluRs) to produce excitatory postsynaptic depolarisation (Meldrum et al., 1999; Kew and Kemp, 2005). AMPA receptors are important for fast excitatory neurotransmission via the sodium-potassium channels whereas NMDA receptors are responsible for slow but sustained phase of calcium mediated excitatory neurotransmission (Benke et al., 1998). Further, the mGluRs play important part in modulating glutamatergic signalling in epilepsy (Ure et al., 2006).

Following its action, glutamate is transported back into the presynaptic vesicles by glutamate aspartate transporter (GLAST) and glutamate transporter 1 (GLT1) (Lehre et al., 1995). An excessive glutamatergic activity due to upregulation of glutamatergic receptors, increased glutamate concentration in the synaptic cleft or dysfunction of glutamate transporters produces hyperexcitability manifested by seizures (Seifert et al., 2002; Aronica et al., 2000; Yi and Hazell, 2006).

In contrast to glutamate, GABA is an inhibitory neurotransmitter which hyperpolarises neurons and produces inhibitory postsynaptic potentials (Treiman, 2001). GABA acts through mainly two receptors namely GABA_A and GABA_B (Takeuchi and Onodera, 1972; Kaupmann et al., 1997). The GABA_A are ligand-gated ion channel receptors that regulate fast inhibitory presynaptic potentials by mediating chloride influx, whereas GABA_B are G-protein coupled receptors that regulate slow inhibitory presynaptic potentials by enhancing the conductance of potassium and decreasing the entry of calcium (Meldrum, 1989; Takeuchi and Onodera, 1972; Kaupmann et al., 1997). A reduction in GABAergic inhibition due to an impairment of GABA synthesis or release, alteration of GABA receptors and loss of GABAergic neurons increases the probability of generation of excitatory postsynaptic potentials and development of epilepsy (Schwarzer et al., 1997; Brooks-Kayal et al., 1998; Knopp et al., 2008).
Apart from GABA and glutamate other neurotransmitters such as serotonin, adrenaline and dopamine have also been found to play important roles in the pathogenesis of epilepsy (compiled and reviewed in (Svob Strac et al., 2016)). It has been demonstrated in animal studies that a lower level of serotonin or a lacking in serotonin receptors 5-HT$_{1A}$ or 5-HT$_{2C}$ (5-hydroxytryptamine 1A or 2C) are associated with the generation of seizures (Dailey et al., 1992; Applegate and Tecott, 1998; Sarnyai et al., 2000). Serotonin also indirectly modulates the glutamatergic or GABAergic neurotransmission (Schmitz et al., 1995; Katsurabayashi et al., 2003; Ciranna, 2006). Further, the protective role of noradrenaline against seizures has also been reported where a depletion of endogenous noradrenaline increased the neuronal damage and seizure susceptibility (Szot et al., 1999; Weinshenker et al., 2001). On the contrary, dopamine plays an complex role in the pathology of epilepsy and often based on the type of receptor it acts on (Barone et al., 1991). Studies have suggested that an activation of dopamine D$_1$ receptor exerts proconvulsant effect, whereas the D$_2$ receptor activation exerts anticonvulsant effect (Clinckers et al., 2004; Barone et al., 1991). Furthermore, a reduced binding potential of dopamine to its transporters has also been shown to be involved in juvenile myoclonic epilepsy (Bagdy et al., 2007).

In addition to the neurotransmitters discussed above, ion channels play crucial roles in the maintenance of neuronal network homeostasis. A dysfunction of ion channels or channelopathy has been reported to be associated with epilepsy (Poolos and Johnston, 2012; Lerche et al., 2013). For example, mutations in the genes that code the voltage gated ion channels such as potassium, sodium, calcium and chloride, and ligand gated ion channel receptors for GABA and acetylcholine have been documented in various genetic epilepsy syndromes (Claes et al., 2001; Baulac et al., 2001; Charlier et al., 1998; Dedek et al., 2001; Niemeyer et al., 2004; Bertrand et al., 2002). Moreover, transcriptional and post-translational
modifications of the ion channels might also occur in acquired epilepsies (Scheffer and Berkovic, 2003; Helbig et al., 2008).

Apart from the above mentioned pathophysiological process, several other intracellular signalling and gene expression pathways; including mechanistic target of rapamycin (mTOR), brain-derived neurotrophic factor (BDNF), transforming growth factor-β (TGFβ) and adenosine kinase (AK) are also reported to be involved in epileptogenesis (for review see (Staley, 2015)). However, a detailed discussion of these are beyond the scope of this thesis and are not discussed here.

1.1.4 Antiepileptic drugs (AEDs) and their mechanism of action

The antiepileptic drugs (AEDs) may be divided into three classes based on their development i.e. i) first generation (1857-1958; e.g. potassium bromide, phenobarbital, mephobarbital, ethosuximide etc.) ii) second generation (1960-1975; carbamazepine, valproate, and various benzodiazepines) and iii) third generation (post 1975; e.g. felbamate, lamotrigine, levetiracetam, gabapentin, pregabalin, oxcarbazepine, tiagabine, vigabatrin, zonisamide, topiramate, lacosamide, rufinamide, eslicarbazepine, perampanel, ezogabine, and stiripentol) (Scharfman, 2015; Kanner et al., 2018b; Kanner et al., 2018a).

Most of the AEDs act through one or more of these five mechanisms- i) blocking voltage-gated sodium channels, ii) blocking voltage-gated calcium channels, iii) antagonising glutamatergic neurotransmission, iv) enhancing GABAergic neurotransmission and v) modulating potassium channels. Sodium channels are responsible for initiation and transmission of action potentials and thus play important role in epilepsy (Wallace et al., 1998). Drugs such as phenytoin, carbamazepine, oxcarbazepine, lacosamide and lamotrigine exert their anticonvulsant activity mainly by blocking the voltage gated sodium channels (Brodie, 2017). The T-type calcium channels regulates the intrinsic thalamocortical
oscillations which trigger the spike-wave discharges in generalised absence seizures (Huguenard, 2002). Ethosuximide acts by inhibiting these subtypes of calcium channels in the thalamocortical network thus demonstrates high efficacy in the treatment of absence seizures (Coulter et al., 1989). Additionally, the effectiveness of zonisamide and valproate in absence epilepsy may also be attributed to their action on T-type calcium channels (Broicher et al., 2007). Further, AEDs such as phenobarbital, primidone and benzodiazepines (e.g. diazepam, lorazepam, clonazepam) demonstrate their anticonvulsant action by modulating the GABA<sub>A</sub> receptor and enhancing the GABAergic neurotransmission. Again, tiagabine and vigabatrin act indirectly by enhancing the GABAergic tone which are mediated by inhibition of GABA transporter GAT1 and antagonisation of the GABA transaminase, respectively (Thompson and Gahwiler, 1992; Loscher et al., 1989). The mechanism of action of valproate is although not clear, it has been suggested that valproate mainly acts through enhancing the GABA concentration in the brain (Meldrum, 1980). As previously mentioned that glutamate is the main excitatory neurotransmitter in the brain, thereby blockade of fast ionotropic AMPA glutamate receptor by perampanel also demonstrates antiseizure activity (Hanada et al., 2011). Topiramate has a diverse mode of actions which includes inhibition of voltage-gated sodium channels, enhancement of GABAergic neurotransmission via modulating the GABA<sub>A</sub>-receptor, antagonising the AMPA glutamate receptor, and inhibition of the carbonic anhydrase enzyme (McMillin and Krasowski, 2016). Voltage-gated potassium channels help to maintain the ionic balance in the neurons by permitting the efflux of potassium ions in response to the depolarisation caused due to sodium ion influx, thus a positive modulation of potassium ion channels help in the control of seizures (Rundfeldt, 1997). AEDs such as Ezogabine and retigabine are acting as agonist of Kv7 potassium channels (K<sub>v</sub>7.2–K<sub>v</sub>7.5), present in the nervous system and have been demonstrated to be efficacious in the treatment of focal seizures (Tatulian and Brown, 2003; Rundfeldt, 1997).
Despite the availability of a large number of AEDs, refractoriness or drug-resistance remain a major concern as approximately 30% of people with epilepsy are resistant to one or more of these AEDs (WHO, 2018). Additionally, drug related adverse effects contribute to poor quality of life (discussed in Section 1.2). All these highlight the need for discovery of new safe but efficacious AEDs.

### 1.1.5 Classification of seizures

The ILAE has recently categorised seizures into three types (Figure 1.1) such as focal, generalised and unknown types depending on their origin (Fisher et al., 2017). Focal seizures originate in a small area of one cerebral hemisphere, whereas generalised seizures begin in both hemispheres of the brain and involve the whole brain neuronal network. Further, the type of seizures whose origin/onset are unknown are considered as ‘unknown-onset’ type (Fisher et al., 2017).

State of awareness has been optionally included in further classifying focal seizures into ‘aware’ (formerly known as simple) and ‘impaired awareness’ (formerly known as complex) seizures which however can be ignored if the awareness level is unknown (Fisher et al., 2017). The focal seizures are further subclassified into motor and nonmotor types. Motor seizures may be of different types such as atonic (loss of body tone), tonic (sustained stiffening of limbs and neck), clonic (rhythmic jerking of limbs), myoclonic (irregular, brief jerking of limbs) and epileptic spasm (flexion or extension of arms and flexion of trunk) (Fisher et al., 2017; Blume et al., 2001). An example of nonmotor seizure is behavioural arrest which is characterised by cessation of movement and unresponsiveness. Seizures which after focal onset become generalised tonic-clonic (previously known as grand mal) are called as ‘focal to bilateral tonic-clonic seizures’ (Fisher et al., 2017). Tonic-clonic seizures present
with bilateral symmetric convulsive movements (stiffening followed by jerking) of all limbs (Blume et al., 2001).

In case of classifying generalised onset seizures, awareness level is not considered because in most of the cases awareness is impaired, thus these types of seizures are classified into motor and nonmotor/absence (sudden cessation of activity and awareness) categories. Motor generalised seizures include tonic-clonic or other motor such as atonic, tonic, clonic and myoclonic seizures (Fisher et al., 2017).

Similar to generalised onset seizures, the unknown type seizures are also subclassified as motor and nonmotor types. Additionally, an ‘unclassified’ section has been kept in the ‘unknown onset’ type to represent the seizures that do not fit into other categories or the available information are inadequate to categorise them. ILAE cited an example to explain the unclassified seizures “A 75-year-old man known to have epilepsy reports an internal sense of body trembling and a sense of confusion. No other information is available. EEG and MRI are normal. This event is unclassified.” (Fisher et al., 2017)
Figure 1.1. ILAE 2017 classification of seizure types (basic version). Adapted from (Fisher et al., 2017). Seizures are primarily classified into focal, generalised and unknown types based on their origin/onset. Awareness level is optionally added for classification of focal seizures. Focal, generalised and unknown types are further subclassified into motor and nonmotor types. Focal seizures if become generalised tonic-clonic then they are termed as ‘focal to bilateral tonic-clonic seizures’. Seizures with insufficient information or that do not fit into other categories are termed as ‘unclassified’.

1.1.6 Classification of epilepsy

In 2017, the ILAE classified epilepsy into four major categories i) focal ii) generalised, iii) combined generalised and focal, and iv) unknown (Scheffer et al., 2017) (Figure 1.2). This classification provides the clinicians a well-defined therapeutic approach to treat epilepsies. In the new epilepsy classification framework, the first step for clinicians is to identify the seizure type which is followed by the classification of the epilepsy type (Scheffer et al., 2017). This classification has the provision for diagnosis of more specific epilepsy
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syndromes such as West syndrome, childhood absence epilepsy and Dravet syndrome. An epilepsy syndrome is a cluster of clinical, electrical and age-dependent features which together define a specific epileptic disorder (ILAE, 1989). It is to be noted that a person must be diagnosed as epileptic according to the definition of epilepsy given by the ILAE in 2014 before his/her epilepsy type can be classified (Fisher et al., 2014). In this classification, a major focus has been given on aetiologies and comorbidities in every step of the diagnostic process (Scheffer et al., 2017).

Figure 1.2. ILAE classification of epilepsies. Adapted from (Scheffer et al., 2017). Based on different types of seizures, epilepsy has been classified into four major types i.e. focal, generalised, combined generalised and focal, and unknown. The third level in this classification system is to identify the specific epilepsy syndrome where possible. Further, aetiologies and comorbidities need to be considered for a precise diagnosis of the epilepsy type.
Currently a large number of epilepsy types are recognised, all of which are mentioned on the website of the Epilepsy Foundation (Scharfman, 2015), but in the interest of this project I will only highlight two of them i.e. temporal lobe epilepsy and Dravet syndrome in the next two sections.

1.1.7 Temporal lobe epilepsy (TLE)

Temporal lobe epilepsy (TLE) is one of the most common forms of epilepsy characterised by recurrent, unprovoked focal seizures which originate in the temporal lobe of the brain (Manford et al., 1992; Fisher et al., 2017). Inside the temporal lobe, the seizure foci of TLE usually are located in the hippocampus, amygdala, subiculum and entorhinal cortex (Bertram, 2009). A focal seizure in TLE may spread across other brain areas to become a focal to bilateral seizure (Bone et al., 2012). Several etiologies have been attributed to TLE which includes head injury, cerebral stroke, structural lesion, brain tumour, brain infection or idiopathic (Blair, 2012). TLE may be of two types i.e. mesial temporal lobe epilepsy (MTLE) and lateral temporal lobe epilepsy (LTLE) (Engel, 2001). The MTLE is the most common type where seizure foci are located at the medial or inner aspect of the temporal lobe such as hippocampus, parahippocampal gyrus and amygdala (Engel, 2001). The LTLE is in contrast a rarer type where seizure foci are located at the lateral or outer surface of the temporal lobe (Engel, 2001). TLE, though rare may be of hereditary e.g. autosomal dominant LTLE which is associated with LGII gene (Nobile et al., 2009). In addition to clinical manifestation of spontaneous seizures, TLE is often accompanied by several pathological features like hippocampal sclerosis, mossy fiber sprouting, dispersion in the granule cell, astrogliosis and blood brain barrier damage (Pitkanen and Lukasiuk, 2011; Vrinda et al., 2018). Along with seizures several other diseases/conditions such as anxiety, depression, suicidality, motor deficit and memory impairment are often associated with TLE which significantly reduced the patient’s quality of life (England et al., 2012) (See section 1.2). Of importance, TLE is
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often refractory in nature and none of the presently available drugs can provide satisfactory control over seizures and associated conditions (comorbidities) (Asadi-Pooya et al., 2017).

1.1.8 Dravet syndrome

Dravet syndrome is a severe form of myoclonic epilepsy in children that usually begins at six to nine months of age with the first presentation of seizures (Dravet et al., 2005). In 1978, Dr Charlotte Dravet first characterised this disease as severe myoclonic epilepsy of infancy (SMEI) and this early onset, distinguishes it from other similar paediatric epilepsies such as Lennox-Gastaut syndrome, which typically manifests after the age of one (Dravet et al., 2005; Kalume, 2013). Dravet syndrome is a rare (orphan) disease affecting ~1 in 20,000-40,000 children (Shmuely et al., 2016). It is typically triggered by a fever which initiates the first generalised or partial seizure (Dravet et al., 2005). During the first year of life, seizures are relatively infrequent but, over time, can lead to status epilepticus. In subsequent years the symptoms become more severe with generalised tonic-clonic, myoclonic, absence and focal seizures being common, status epilepticus rarely occurs after 10 years of age (Akiyama et al., 2010). A premature mortality rate of 21% has been reported in Dravet syndrome patients (Dravet et al., 2005; Genton et al., 2011). Apart from seizures, several additional comorbidities are associated with Dravet syndrome (e.g. psychomotor developmental delay, gait abnormality (crouch gait), hyperactivity, attention deficit, autism, sleep disorder, anxiety-like behaviours, language impairment and severe cognitive deficits) which exert a profound adverse effect upon patient quality of life (Black and Gaebler-Spira, 2016; Brunklaus et al., 2011; Genton et al., 2011; Li et al., 2011).

The SCN1A gene which encodes Na\(_{\text{v}}\)1.1, a voltage-gated sodium channel, plays a pivotal role in Dravet Syndrome. It has been reported that 70-80% of patients exhibit a deletion or mutation (truncating, missense or splice site mutations leading to loss of function)
of this gene on chromosome 2q, where 85% are de novo mutations (Harkin et al., 2007; Marini et al., 2011). Although the SCN1A gene is expressed in neuronal cell membranes of the central and peripheral nervous systems (Black and Gaebler-Spira, 2016), higher expression has been observed in hippocampal GABAergic inhibitory interneurons such that a loss-of-function mutation leads to abnormal interneuron firing and subsequent disinhibition to promote the severe myoclonic epilepsy observed in Dravet Syndrome (Cheah et al., 2012; Yu et al., 2006). Mutations in some other epilepsy-associated genes e.g. SCN1B, STXBP1, GABRA1, GABRA2, CHD2 and PCDH19 have also been reported to play a role in Dravet syndrome (Carvill et al., 2014; Depienne and LeGuern, 2012). The genetic mutation often contributes to the manifestation of the disease independently of the seizures, which is why Dravet syndrome is also referred as a ‘channelopathy’ (Brunklaus and Zuberi, 2014).

Presently the major therapeutic strategies include a conventional AED (e.g. valproate, clonazepam etc.) in combination with a newer drug (e.g. stiripentol, topiramate etc.) (Ceulemans et al., 2004; Chiron, 2005). Since conventional sodium channel blocking AEDs (e.g. carbamazepine and lamotrigine) affect the NaV1.1 channel, their use can actually worsen seizures in Dravet syndrome and has been associated with premature death (Arzimanoglou, 2009; Guerrini et al., 1998). Seizures in Dravet syndrome are largely refractory to treatment and none of the presently available medications offer satisfactory control of symptoms (Devinsky et al., 2017; Devinsky et al., 2018b).

1.1.9 Animal models of seizures and epilepsy

Animal models of seizures and epilepsy provide valuable information on disease pathogenesis and help in the discovery of new AEDs. In the scope of this thesis, here I will provide a general overview of animal models that are currently in use for drug screening and then specifically describe the models of TLE and Dravet syndrome.
Animal models that are presently used for the screening of novel AEDs are mainly the models of epileptic seizures such as maximal electroshock seizure (MES), 6 Hz psychomotor seizure and subcutaneous pentylenetetrazole (s.c. PTZ) tests (White et al., 1995; Patra et al., 2018). The MES test is predictive of generalised tonic-clonic seizures where a suprathreshold electrical stimulus is applied to a rat (150 mA) or mouse (50 mA) transcorneally for a brief period of time (0.2 seconds) for seizure induction (White et al., 1995; Patra et al., 2018). The 6-Hz psychomotor seizure model in mice is also a useful model for drug screening of refractory partial epilepsy (White et al., 1995; Patra et al., 2018). In this model, a 6 Hz corneal stimulation is applied at two currents (22 mA and 44 mA) for 3 seconds (White et al., 1995; Patra et al., 2018). Notably, 22 mA current intensity is able to evoke seizures in 97% of test mice (Barton et al., 2001). The typical characteristics of this type of seizures are exhibited by a minimal clonic phase followed by stereotyped behaviours that are considered similar to the aura of human with partial seizures (Toman et al., 1952; Barton et al., 2001). The s.c. PTZ seizure test is considered suitable test to assess the anticonvulsive effect of non-convulsive seizures such as absence or myoclonic seizures. For this test, a convulsive dose of PTZ (that causes clonic seizure in 97% animals for 3 seconds duration) is injected to an animal and observed for 30 minutes. Another popular model for drug screening is corneal kindling model of focal seizures (Matagne and Klitgaard, 1998; Patra et al., 2018) where kindling is done by stimulating the optic nerve through corneal electrodes (3 mA, 60 Hz, 3 seconds) until the animals reach a criterion of 5 consecutive secondarily generalised seizures (Stage 4 or 5, as described by Racine (Racine, 1972)). This is not an acute model and usually takes 10-15 days for animals to be kindled and ready for drug testing (Matagne and Klitgaard, 1998; Patra et al., 2018). The above-mentioned models are easy to generate, aid rapid screening of a large number of compounds and are able to predict the clinical efficacy of AEDs to a certain extent (White et al., 1995; Barker-Haliski et al., 2017). However, the major
drawback is that they do not reproduce the spontaneous recurrent seizures (SRS), thus cannot be considered as true models of epilepsy (Modebadze et al., 2016).

Currently the pilocarpine model of SRS is used in preclinical research to investigate effects of candidate drugs on SE, epileptogenesis, SRS or TLE (Andre et al., 2007; Francois et al., 2011; Furtado et al., 2011). In this model, a single large dose of pilocarpine (300-400 mg/kg) is used to induce SE in rats (Biagini et al., 2006; Goffin et al., 2007). Pilocarpine is a muscarinic M<sub>1</sub> acetylcholine receptor agonist, therefore activation of cholinergic neuron leads to an increased level of glutamate in hippocampus region. This increased level of glutamate then damages the fine excitatory and inhibitory neuronal networks resulting into manifestation of seizure (Smolders et al., 1997). NMDA receptors activation then helps in maintenance of seizure (Smolders et al., 1997). However, the most significant problem with this model lies in its high mortality rate during or immediately after SE induction (20-85%) (Andre et al., 2007; Mueller et al., 2009). Therefore, this model has been refined to reduce the mortality rate and to produce progressive neurodegeneration and epileptogenesis. During this refinement process, the lithium pilocarpine model was initially introduced where pre-administration of lithium chloride potentiates pilocarpine’s convulsant properties thereby manifestation of SE can be observed at a comparatively lower dose of pilocarpine (Jope et al., 1986; Mueller et al., 2009). A further refinement to this model is termed as the reduced intensity status epilepticus induced spontaneous recurrent seizures (RISE-SRS) where repeated low doses of pilocarpine are used instead of a single high dose along with administration of muscle relaxant like xylazine in between the SE process and finally a stop solution which helps the animals to recover from SE state (details given in chapter 2) (Glielen et al., 2001; Curia et al., 2008; Modebadze et al., 2016). The RISE-SRS model exhibits a similar disease progression and pathology as the traditional post-SE models, but with reduced
mortality during the immediate post-SE period (Modebadze et al., 2016). This model is thus suitable for testing candidate drugs to investigate their prolonged disease modification effects.

The conventional AED screening models do not represent the genetic epilepsy like Dravet syndrome as the former models are largely based on chemical or electrical insults. Currently, various mouse models are available which represent the loss of SCN1A gene function as commonly observed in human Dravet syndrome. These models include knock-out models which were developed following a targeted deletion of exon 1 (Scn1a<sup>tm1Kea</sup>) (Miller et al., 2014) and exon 26 (Scn1a<sup>tm1Wac</sup>) (Yu et al., 2006) of the mouse Scn1a gene. Further, specific point mutation knock-in mouse model such as Scn1a<sup>R1407X</sup> (Ogiwara et al., 2007), Scn1a<sup>R1648H</sup> (Martin et al., 2010) and Scn1a<sup>E1099X</sup> (Tsai et al., 2015) are also exist.

1.2 Comorbidities associated with epilepsy

When other distinct clinical diseases or syndromes are present alongside the index disease (e.g. epilepsy) then these associated diseases/syndromes are referred as comorbidities of that index disease (Feinstein, 1970). It is reported that ~50% of epileptic adult individuals have at least one comorbidity (Forsgren, 1992). According to Boro and Haunt “nearly every patient with epilepsy will experience a comorbid medical condition at some point during the course of treatment” (Boro and Haut, 2003). The comorbidities commonly associated with epilepsy includes psychiatric, cognitive and medical conditions such as depression, anxiety, suicidality, attention deficit hyperactivity disorder (ADHD) and autism, memory impairment, and motor deficits (Hesdorffer et al., 1996; Hesdorffer et al., 2006; Hesdorffer et al., 2012; England et al., 2012; Chang et al., 2011; Cleary et al., 2004). These comorbidities have significant consequences including high premature mortality, poor quality of life, increased medical expenses and poor prognosis of disease (Kwan et al., 2009; Fazel et al., 2013; Kanner et al., 2012). Moreover, the treatment of these comorbidities (e.g. antidepressant
therapy) sometimes results into poor seizure outcome (Coupland et al., 2011; Hill et al., 2015). Studies have indicated that AEDs could also worsen comorbidities which leads to discontinuation of antiepileptic therapy and thereby poor disease management (Perucca et al., 2009; Chen et al., 2017a; Chen et al., 2017b). Hence, it is imperative to understand the complex association between the comorbidities and epilepsy for a better therapeutic outcome. In this section, I will focus on the most common comorbidities associated with epilepsy.

1.2.1 Depression

It has been documented in epidemiological studies that depression is more prevalent in people with epilepsy compared to other diseases (Mendez et al., 1986). The lifetime prevalence rate of depression among people with epilepsy extends from 11 to 62% (Wiglusz et al., 2012). Interestingly, a meta-analysis conducted on 5,434 epileptic patients across the globe reported that depression is more prevalent in female than in male patients (26.4% vs 16.7%) (Kim et al., 2018). In paediatric epileptic patients, the rate of depression ranges from 30-70% (Plioplys, 2003; Kanner, 2003) which is especially higher in adolescent (Thome-Souza et al., 2004). The amygdala and the hippocampus, two key structures of the temporal lobe are associated with the pathology of depression (and also anxiety) (Jones et al., 2008; Lopes et al., 2016). It has been observed from imaging studies that individuals with TLE and depression have hippocampal atrophy (Rosso et al., 2005; Richardson et al., 2007). Moreover, the condition is aggravated if epilepsy and depression both persist together (Richardson et al., 2007; Gilliam et al., 2007). However, the relationship between epilepsy and depression is unlikely to be unidirectional as several reports suggested that people with depression are also with higher risk of developing epilepsy. For instance, data obtained from UK General Practice Research Database showed a significantly higher incidence-rate ratio of depression in people three years before they were diagnosed with epilepsy (Hesdorffer et al., 2012). Other studies have supported this notion and reported that people with depression have
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up to 7 times higher risk of developing epilepsy (Hesdorffer et al., 2006; Hesdorffer et al., 2000; Salpekar and Mula, 2018).

1.2.2 Anxiety

Prevalence of anxiety disorders among people with epilepsy is also significantly higher. As an example, data obtained from two United States national surveys showed that people with epilepsy have two times higher incidence of anxiety disorders than the general population (Ottman et al., 2011; Kobau et al., 2006). A large Canadian national population health survey also reported a similar trend (Tellez-Zenteno et al., 2007). According to this study, anxiety disorders were observed in 22.8% people with epilepsy in contrast to only 11.2% in general population without epilepsy (Tellez-Zenteno et al., 2007). Further, a national population based study in the UK using clinical interviews reported a strong association of epilepsy with anxiety disorders (Rai et al., 2012). However, it is interesting to note that the prevalence of anxiety amongst people with epilepsy also depends on the methods of diagnosis. For example, a meta-analysis conducted on published data until July 2016 involving 3,221 epileptic patients across the world showed that the prevalence of anxiety was only 8.1% when diagnosis of anxiety was based on unstructured clinical assessment, whereas this rate was 27.3% when structured clinical interviews were conducted (Scott et al., 2017). Notably, the two key structures of brain, amygdala and hippocampus are associated with both the anxiety and epilepsy, thus a bidirectional relationship cannot be ignored i.e. anxiety not only follows epilepsy, but vice-versa might be true (Jones et al., 2008; Lopes et al., 2016). This is supported by a longitudinal cohort study including 3,773 reported epilepsy cases from 1993-2005 in the UK general practice research database observed anxiety disorders were prevalent in epileptic patients before they were diagnosed with epilepsy (Hesdorffer et al., 2012). Again, a Swedish population based case-control study reported that patients hospitalised with anxiety disorders are at >2 times higher risk of developing epilepsy.
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(Adelow et al., 2012). Like depression, anxiety disorders are also found to be associated with drug related adverse effects (Jacoby et al., 2015; Gómez-Arias et al., 2012).

1.2.3 Suicidality

Patients with epilepsy are at higher risk of suicides. A multicentre study by Jones et al. reported a suicide rate of about 12% among individuals with epilepsy compared with 1.1-1.2% in the general population (Jones et al., 2003). Further, a large Danish population-based case-control study investigated 21,169 suicide cases where they reported a three-fold higher risk of suicide among the people with epilepsy than those without epilepsy (Christensen et al., 2007). In addition to the occurrence of suicide cases, the attempted suicide or suicidal intention are also higher among the people with epilepsy (Jones et al., 2003). Based on the meta-analysis of the data obtained from the clinical trials the United States Food and Drug Administration (US-FDA) reported that the treatment with AEDs enhance the suicidal thoughts among individuals with epilepsy (FDA, 2008). A population-based retrospective cohort study in the UK assessed the magnitude of the association between suicide attempts and epilepsy where they suggested a common underlying biology in between them (Hesdorffer et al., 2016).

1.2.4 ADHD

ADHD is a persistent pattern of inattention, hyperactivity and impulsivity which affects the normal functioning and development of children (APA, 2013). It is one of the most common comorbidities of epilepsy in children (Dunn and Austin, 2004; Thome-Souza et al., 2004). ADHD plays a significant impact on the quality of life of the children with epilepsy as it not only associated with academic and developmental disabilities but also exacerbates other comorbidities such as anxiety and depression (Kwong et al., 2016). The prevalence rate of ADHD in children with epilepsy has been reported from 30% to 40%
Moreover, approximately 2.5-5.5 times higher prevalence of ADHD has been observed in epileptic children than those without epilepsy (Aaberg et al., 2016). The relationship between epilepsy and ADHD is again bidirectional with 3.94 times higher rate of epilepsy in ADHD children compared to the children without ADHD (Chou et al., 2013). Further, AEDs such as valproate is associated with exacerbation of ADHD (Ahmed and Mohamed, 2015). ADHD if not treated, symptoms may persist through adulthood (Ettinger et al., 2015; Salpekar and Mishra, 2014). However, ADHD in epilepsy is often untreated because of the common concept that stimulants may reduce seizure threshold which further worsen the patients’ quality of life (Davis et al., 2010; Salpekar and Mishra, 2014).

### 1.2.5 Autism spectrum disorder

Autism spectrum disorder is a neurodevelopmental disorder characterised by social interaction and communication deficits and presence of restricted and repetitive behaviours (APA, 2013). Autism is one of the most common comorbidities seen in patients with epilepsy especially in children and adolescent (Rai et al., 2012; Selassie et al., 2014). The prevalence rate of autism among patients with epilepsy varied from 4 to 21% (Tuchman et al., 2013; Reilly et al., 2014; Rai et al., 2012). On the other hand, around 30% of autistic individuals develops epilepsy (Viscidi et al., 2013). Interestingly, epileptiform activities have often noticed in EEG recordings of autistic children even in the absence of manifestation of seizures (Rossi et al., 1995; Mulligan and Trauner, 2014). The reason behind this bidirectional relationship between epilepsy and autism may be attributed to involvement of common pathophysiology such as a deficit in interneuron function which results in reduced inhibitory GABA activity in both the cases (Jacob, 2016). Intellectual disability characterised by a low intelligence quotient score (IQ; <70) was reported in patients having both autism and epilepsy (Viscidi et al., 2013). A high prevalence of autism have been documented in
patients with epileptic encephalopathies including Dravet syndrome and Landau-Kleffner syndrome (Besag et al., 2016; Chepure et al., 2018; Rosander and Hallbook, 2015).

1.2.6 Cognitive impairment

Epileptic patients often experience cognitive deficits which severely affect their quality of life (Nau et al., 2018; de la Loge et al., 2016; Arinzechi et al., 2016; Verche et al., 2018). The extent of cognitive decline in epilepsy often determined by the type of epilepsy, the area of damage, presently active epilepsy, AED treatment, and age and reserve capacities of individual (Elger et al., 2004). Cognitive deficits across multiple domains have been reported in a range of epilepsy types. For instance, a significant decline in verbal memory (Griffith et al., 2003), executive function (Keller et al., 2009), working memory (Winston et al., 2013) and alertness (Liu et al., 2016) have been demonstrated in patients with TLE. Further, a meta-analysis conducted on the data obtained from studies on frontal lobe epilepsy patients reported a higher rate of cognitive problems, particularly in the areas involved in the functioning of frontal lobe such as executive functions, attention, and motor skills than general population without epilepsy (Verche et al., 2018). Severe cognitive and intellectual disability are often encountered by the children with epileptic encephalopathies like Dravet syndrome (Acha et al., 2015), Landau Kleffner syndrome (Riccio and Vidrine, 2017) and West Syndrome (Riikonen, 1996). It has been reported that patients with Dravet syndrome often have severe intellectual deficit, in majority of cases the intelligence quotient (IQ) scores remain below 50% (Akiyama et al., 2010). The degree of cognitive decline is also positively correlated with the duration of epilepsy i.e. longer the duration worse the cognitive outcome (Jokeit and Ebner, 2002). Further, several AEDs e.g. carbamazepine (Forsythe et al., 1991; Meador et al., 2001), phenobarbital (Farwell et al., 1990; Calandre et al., 1990), sodium valproate (Stores et al., 1992; Masur et al., 2013), topiramate (Brandl et al., 2010; Dooley et al., 1999) have been reported to be associated with memory impairment in epileptic patients.
However, the mechanism behind cognitive impairment in epilepsy is far more complex and perhaps bidirectional, similar to other comorbidities as described above (Keezer et al., 2016). Moreover, the parts of the brain and neuronal network associated with learning and memory are often associated with epilepsy, therefore a common pathophysiological pathway might be involved in both the conditions (Titiz et al., 2014; Verche et al., 2018).

1.2.7 Motor disorder and gait abnormalities

Apart from neurological comorbidities, motor impairment is also frequently reported in patients with epilepsy (Beckung and Urebrant, 1997; Gloersen et al., 2000; Reijs et al., 2010; Kowalski and Di Fabio, 1995; Ottman et al., 2011). A study conducted on 100 patients with focal motor epilepsy found that the most common type of motor abnormalities in those patients were myoclonus, dystonia, chorea, stereotypies, myoclonus-dystonia and tremor (Fasano et al., 2018). Another study conducted on 87 children with cryptogenic partial and idiopathic generalised epilepsies found a slowing of psychomotor speed in the epileptic children where the authors suggested that cortical inhibition due to excessive neuronal discharge in epilepsy might be the reason of this slowing of psychomotor speed (Boelen et al., 2005). Further, abnormal gait has also been reported in epileptic children especially those with Dravet syndrome (Gitiaux et al., 2016; Rodda et al., 2012). Additionally, treatment with AEDs such as phenobarbital, valproic acid, phenytoin and lacosamide have adverse effects on motor function and gait in the patients with epilepsy (Zaccara et al., 2004; Ristić et al., 2006; Zaccara et al., 2013; Bainbridge et al., 2017).

1.2.8 Other medical conditions

Several other medical conditions such as migraine (Ottman and Lipton, 1994), multiple sclerosis (Allen et al., 2013), diabetes (Weatherburn et al., 2017) and cardiovascular diseases (Rowan, 2005) may also be seen in patients with epilepsy. Moreover, allergy, asthma
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and osteoporosis although not directly linked to the epilepsy itself, are often linked to AED adverse effects (Tellez-Zenteno et al., 2005; Gaitatzis et al., 2012; Pack et al., 2003).

1.3 Cannabis and cannabinoids

Cannabis (also known as marijuana) has been used since prehistoric times for medicinal, recreational and religious purposes as documented in ancient texts from Mesopotamia, India and China (Abel, 1980). The use of marijuana in the treatment of convulsions also lie in reports from the Middle East that were ascribed to the scholar Al-Mayusi (Lozano, 2001) in 1100 and the historian, Ibn al-Badri in 1464 (Aldrich, 1997). However, it was not until 1649 that Nicholas Culpeper translated the ‘Pharmacopoeia Londonensis’ from Latin into English, and suggested marijuana as a treatment of ‘inflammation of the head’ (Crawford, 2002). Thereafter, there appears to be no further mention of this therapeutic use of marijuana until its introduction to Western medicine from India in the 19th century by William O’Shaughnessy (O’Shaughnessy, 1840). Here, alongside other reports from the same period describing the control seizures with marijuana extracts (McMeens, 1856; McMeens, 1860; Reynolds, 1868).

Cannabis is mainly obtained from an herbaceous annual plant Cannabis sativa (also known as Indian hemp) (Russo et al., 2008). This plant comprises more than 525 chemically active natural compounds including cannabinoids, flavonoids, terpenoids and alkaloids (Radwan et al., 2009). Of all these the cannabinoids are the most active compounds that belong to a C21 terpenophenolic class of compounds and are mainly found in the trichomes of female flowers (Elsohly and Gul, 2014). More than 100 cannabinoids have been identified so far from the cannabis plant which are popularly known as phytocannabinoids that include Δ9-tetrahydrocannabinol (Δ9-THC), cannabidiol (CBD), cannabidivarin (CBDV), cannabigerol (CBG), cannabinol (CBN), Δ9-tetrahydrocannabivar (Δ9-THCV) and
cannabichromene (CBC) (Elsohly and Gul, 2014). These phytocannabinoids primarily exert their effects by modulating the endogenous cannabinoid system (discussed below) that plays an important physiological role in the regulation of brain, immune and endocrine functions. Subsequently, several chemical compounds have been synthesised that can modulate the endocannabinoid system which are collectively known as synthetic cannabinoids.

In the scope of this project, here in this section I will discuss the endocannabinoid system and cannabinoids with a prime focus on their role in epilepsy. As this project mainly deals with CBD, its therapeutic potential in other diseases will also be briefly discussed.

1.3.1 The endocannabinoid system

The endocannabinoid system (Figure 1.3) plays an important physiological role in modifying excitatory and inhibitory synaptic transmission in the brain. The canonical endocannabinoid system consists of two G protein coupled receptors, CB1 and CB2, with endogenous ligands 2-arachidonoylglycerol (2-AG) and N-arachidonylethanolamide (anandamide or AEA), each with unique degradation machinery (Pertwee, 2006; Di Marzo et al., 1998). Of the two cannabinoid receptor subtypes, CB1 is most widely expressed in the central nervous system, particularly in the mossy cell-granule cell synapses of the hippocampus, microglia, astrocytes and oligodendrocytes (Di Marzo et al., 1998). Once thought to be exclusively expressed outside the central nervous system, current research suggests that CB2 receptors are also expressed in the brain (Li and Kim, 2015), mediating neuronal excitability (Kim and Li, 2015) and inflammation in microglia (Atwood and Mackie, 2010). Importantly, in addition to acting via the canonical cannabinoid receptors, the endocannabinoids can also act via interactions with other receptor types such as the G-protein coupled receptor, GPR55 and the transient receptor potential vanilloid receptor (type 1), TRPV1 (Armstrong et al., 2009).
Of importance in activity-dependent pathophysiological processes such as epileptogenesis and epilepsy, the synthesis of endocannabinoids typically occurs “on demand” from postsynaptic membrane phospholipids although pre-synthesised endocannabinoid reserves are also contained within intracellular storage organelles (Maccarrone et al., 2010; Min et al., 2010). However, most commonly, postsynaptic neuronal depolarisation triggers membrane phospholipid breakdown by the enzymes diacylglycerol lipase (DAGL) and N-acylphosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) to form 2-AG and AEA respectively (Bisogno et al., 2003; Okamoto et al., 2004). Following their synthesis, the endocannabinoids passively diffuse across the presynapse in a retrograde fashion to orthosterically bind to and activate presynaptically located CB₁ receptor to inhibit the release of glutamate or GABA from principal or GABAergic neurons respectively (Kano et al., 2009; Armstrong et al., 2009). The endocannabinoid-mediated inhibition of excitatory glutamate or inhibitory GABA release are respectively known as depolarised-induced suppression of excitation (DSE) or inhibition (DSI) (Armstrong et al., 2009).

Endocannabinoid signalling is thought to play an important role in epileptogenesis and the subsequently developed epilepsy. Supplementing depolarisation-induced postsynaptic synthesis, endocannabinoids are also synthesised following activation of metabotropic glutamate and muscarinic acetylcholine receptors (Maejima et al., 2001; Ohno-Shosaku et al., 2003). Therefore, in addition to the activity-dependent phasic control of neurotransmitter release described previously, this tonic control of endocannabinoid release mediated by G-protein couple receptors may play an important role in epileptogenesis (Armstrong et al., 2009). Furthermore, the complexities of endocannabinoid signalling also extend to the pharmacological properties of the two principal endocannabinoids. Here, 2-AG (Kᵢ=472 nM), while acting as a full agonist at CB₁ receptor, exhibits lower affinity for CB₁ receptor than
AEA (K_\text{i}=32 \text{nM}) which acts as a partial agonist although 2-AG levels are, on average ~170-fold higher in brain than AEA (Vemuri and Makriyannis, 2009). Therefore, overall effects on the development of seizures may be driven by relative levels of the endocannabinoids in particular brain areas (von Rüden et al., 2015; Sugiura et al., 2000). Finally, following dissociation from the CB_1 receptors, endocannabinoids are rapidly degraded (Alger, 2014); 2-AG and AEA are catabolised by the enzymes monoacylglycerol lipase (MAGL) and alpha/beta-hydrolase domain containing 6 (ABHD6) (Marrs et al., 2010) or fatty acid amide hydrolase (FAAH), respectively (McKinney and Cravatt, 2005; Dinh et al., 2002).

Furthermore, activation of TRPV1 by AEA can trigger enhanced glutamate release following an increase in intracellular Ca^{2+} concentrations (Bhaskaran and Smith, 2010), although the concentration of AEA required to activate TRPV1 is greater than that required for CB_1 receptor activation (Ross, 2003; Manna and Umathe, 2012). Moreover, TRPV1 activation rapidly leads to desensitisation which, in the presence of persistently raised AEA levels, could lead to reduced neuronal activity (Carletti et al., 2016).
Figure 1.3. Schematic diagram representing the endocannabinoid retrograde signalling mechanism (taken from (Zou and Kumar, 2018)). Endocannabinoids are mainly synthesised and released from postsynaptic terminals. 2-AG is synthesised from DAG by DAGLα, whereas AEA is synthesised from NAPE by NAPE-PLD. The endocannabinoids act retrogradely to activate CB₁ receptors (CB₁Rs) at presynaptic terminals. Activated CB₁Rs inhibit neurotransmitter (NT) release by suppression of calcium influx. 2-AG can also activate CB₁Rs at astrocytes to release glutamate. Following its action, 2-AG is degraded to arachidonic acid (AA) and glycerol by MAGL. In addition to CB₁Rs, AEA can activate the TRPV1. Finally, AEA is degraded to AA and ethanolamine (EtNH₂) by FAAH. NAPE-PLD may also expressed in presynaptic terminals. Thick arrows represent translocation; thin arrows represent enzymatic process; blunted arrow represent inhibition.
1.3.2 Synthetic cannabinoids

A number of synthetic cannabinoids have been tested preclinically to assess their role in seizures and epilepsy, and it has been discussed in detail in our review article (Rosenberg et al., 2017b). The synthetic cannabinoid URB597, an inhibitor of FAAH (degradative enzyme of AEA) has been shown to have anticonvulsive effect in a number of preclinical seizure models such as MES (Naderi et al., 2011), PTZ-induced (Vilela et al., 2013) and KA-induced (Shubina et al., 2015) models of seizure. Further, URB602 and WWL123, the inhibitors of MAGL and ABHD6 (degradative enzymes of 2-AG), respectively also demonstrate anticonvulsive action in PTZ-induced seizure model (Naderi et al., 2011; Naydenov et al., 2014). Moreover, AM404, an AEA reuptake inhibitor demonstrated to be anticonvulsant in KA-induced (Shubina et al., 2015) and PTZ-induced seizure models (Manna and Umathe, 2012). However, at a higher dose it exerts proconvulsant effect in PTZ-induced seizure model (Manna and Umathe, 2012).

Direct agonism of CB$_1$ receptor by WIN55,212 produced an anti-convulsant effect in MES (Wallace et al., 2001; Naderi et al., 2008; Luszczki et al., 2011) and PTZ-induced (Payandemehr et al., 2015; Naderi et al., 2011) rodent models of seizures. Another direct CB$_1$ receptor agonist, arachidonyl-2'-chloroethylamide (ACEA) also showed anticonvulsive effect in MES (Luszczki et al., 2006; Luszczki et al., 2010; Andres-Mach et al., 2012) and PTZ-induced (Bahremand et al., 2008; Naderi et al., 2015) models of seizure. However, a pro-convulsant effect of both WIN55,212 and ACEA have also been reported on PTZ seizures (Vilela et al., 2013). In addition to the role of CB$_1$ receptors in reducing seizures, recent studies suggest that activation of mixed CB$_1$/CB$_2$ receptors or CB$_2$ receptor alone, may regulate excitability in the hippocampus, increase excitatory transmission (Kim and Li, 2015), and trigger cell-type specific hyperpolarisation following sustained stimulation (Stempel et al., 2016). AM1241, a CB$_2$ receptor agonist has been reported to be pro-convulsant in PTZ-
induced seizure model (de Carvalho et al., 2016). On contrary, studies with mixed CB₁/CB₂ receptor agonists (e.g. anandamide, or closely related palmitoylethanolamide) demonstrate primarily anti-convulsive effects in MES (Lambert et al., 2001; Wallace et al., 2002) and PTZ-induced seizures (Aghaei et al., 2015).

1.3.3 Phytocannabinoids

1.3.3.1 Δ⁹-tetrahydrocannabinol (Δ⁹-THC)

Δ⁹-THC is the most abundantly present chemical constituents of cannabis and is primarily responsible for the psychoactive effects accompanying with cannabis smoking (Shen and Thayer, 1999). It exerts its action mainly by acting as a partial agonist on the two cannabinoid receptors i.e. CB₁ and CB₂ (Pertwee, 2008). However, non-cannabinoid receptor mediated action of Δ⁹-THC by acting as a positive allosteric modulator of opioid receptors (μ and δ) has also been demonstrated (Kathmann et al., 2006). The psychotropic and anticonvulsant effects of Δ⁹-THC are believed to be mediated by CB₁ receptor (Shen and Thayer, 1999). However, the anticonvulsant effect of Δ⁹-THC is widely disputed, and variable results have been demonstrated in both preclinical and clinical studies; a recent review compiled 34 studies involving 6 different animal species reported that Δ⁹-THC showed anticonvulsant in 21, proconvulsant in 1, mixed in 1 and no efficacy in 11 studies (Rosenberg et al., 2015). Δ⁹-THC showed a mixed response in humans as well; for example, a study conducted in five children treated with Δ⁹-THC reported that epilepsy was controlled in two of them whereas three patients showed no significant response (Davis and Ramsey, 1949). Apart from CB₁ mediated anticonvulsant effect, its potent anti-inflammatory and analgesic effects have been demonstrated in various preclinical models (Sofia et al., 1975; Chesher et al., 1973; Rock et al., 2018). Further, the beneficial effects of Δ⁹-THC to improve neuropathic pain associated with diabetes (Wallace et al., 2015; Selvarajah et al., 2010), HIV infection (Ellis et al., 2009; Abrams et al., 2007), brachial plexus avulsion (Berman et al.,
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2004) and multiple sclerosis (Wissel et al., 2006) have been reported in clinical trials. Due to its demonstrable efficacy as an analgesic, nabiximols containing a ratio of 1:1 Δ⁹-THC:CBD (Sativex®, oromucosal spray; GW Pharmaceuticals) has been approved in Europe and Canada for the treatment of pain associated with multiple sclerosis (Rog et al., 2007; Langford et al., 2013; Russo et al., 2016), cancer (Johnson et al., 2013) and rheumatoid arthritis (Blake et al., 2006). Further, dronabinol and nabilone, the two synthetic Δ⁹-THC are used for the treatment of nausea and vomiting associated with cancer chemotherapy (Badowski, 2017). Moreover, dronabinol is beneficial in the treatment of weight loss and loss of appetite in acquired immune deficiency syndrome (AIDS) and cancer patients (Badowski and Yanful, 2018).

Although Δ⁹-THC has so many beneficial effects, it is associated with several adverse effects which limit its use as a therapeutic agent. For example, Δ⁹-THC has been demonstrated to cause sedation, hypomotility (Katsidoni et al., 2013; Rock et al., 2015) and even catalepsy (Prescott et al., 1992) in rats. In humans, Δ⁹-THC has been reported to produce psychosis (Kraft et al., 2008; D’Souza et al., 2004), anxiety (D’Souza et al., 2004), euphoria (Wallace et al., 2007), slow response (Lee et al., 2013) and impaired decision making (Ramaekers et al., 2006). Δ⁹-THC also impairs episodic and working memories in human in a dose dependent manner (Curran et al., 2002; Hart et al., 2001). However, recent studies have shown low dose Δ⁹-THC is able to improve memory in old mice (Bilkei-Gorzo et al., 2017; Sarne et al., 2018) and provide protection against inflammation induced cognitive damage (Fishbein-Kaminetsky et al., 2014).

1.3.3.2 Cannabidivarin (CBDV)

CBDV is a non-psychoactive cannabinoid which has shown its anticonvulsive effect both in vitro and in vivo (Hill et al., 2012). CBDV has been demonstrated to reduce epileptiform local field potentials (LFPs) in rat hippocampal brain slices when induced by 4-aminopyridine (4-AP) and Mg²⁺ (Hill et al., 2012). Further, in a PTZ induced rat seizure
model, CBDV significantly attenuated the severity and mortality in the treated group (Hill et al., 2012). Moreover, CBDV reduced the tonic-clonic convulsion frequency in mouse models of audiogenic and maximal electroshock seizure comparable to sodium valproate (Hill et al., 2012). Encouragingly, CBDV does not produce any antagonistic effect to commonly available antiepileptic drugs and does not induce any significant motor side effect when tested by static beam and grip strength task (Hill et al., 2012). The mechanism through which CBDV exerts its anticonvulsant action is still not clear. CBDV has little affinity at CB1 receptor and it has been demonstrated that its anticonvulsant effect is CB1 receptor-independent (Hill et al., 2013). Further, CBDV has been shown to act through CB2 dependent pathways (Scutt and Williamson, 2007). Some in vitro studies also reported that CBDV activates TRPV2, transient receptor potential of ankyrin type 1 (TRPA1) and transient receptor potential of ankyrin type 2 (TRPA2) receptors, inhibits the L-α-lysophosphatidylinositol, transient receptor potential of melastatin type 8 (TRPM8) and diacylglycerol lipase-α, but whether these contribute to its anticonvulsant action is still not clear (Anavi-Goffer et al., 2012; De Petrocellis et al., 2012; De Petrocellis et al., 2011).

1.3.3.3 Cannabidiol (CBD)

CBD is a major non-psychoactive constituent of cannabis which exerts its therapeutic potential against several diseases including epilepsy (Thomas et al., 1998). Unlike Δ9-THC, CBD shows very low affinity towards CB1 and CB2 receptors which makes it non-psychoactive (Thomas et al., 1998; Bisogno et al., 2001). However, it has been reported that CBD might also act as a negative allosteric modulator of CB1 and CB2 receptors (Thomas et al., 2007). At sub- to low- micromolar concentrations, CBD acts as an antagonist of the GPR55 (Ryberg et al., 2007), TRPM8 channel (De Petrocellis et al., 2008), and the equilibrative nucleoside transporter (ENT), whereas it shows agonistic activity on the TRPA1 channel (De Petrocellis et al., 2011), the 5-HT1A receptor (Russo et al., 2005), the glycine...
receptors (Ahrens et al., 2009), and has a bidirectional effect on intracellular calcium (Ryan et al., 2009). On the other hand, at higher micromolar concentrations, CBD activates the TRPV1 and TRPV2 channels (De Petrocellis et al., 2011), and the peroxisome proliferator-activated receptor γ (PPARγ) (O'Sullivan et al., 2009). It also inhibits cellular uptake and FAAH-catalysed degradation of anandamide (Bisogno et al., 2001). Further, CBD prevents adenosine reuptake by blocking the equilibrative nucleoside transporter (Carrier et al., 2006). This broad range of action makes CBD a potential candidate for the treatment of several diseases which are discussed briefly below.

### 1.3.3.3.1 Epilepsy

The role of CBD in epilepsy has been demonstrated both in preclinical and clinical studies. CBD showed marked antiepileptiform properties in vitro in rat brain slices experiencing seizure-like activity induced by 4-AP or Mg$^{2+}$ removal (Jones et al., 2012). CBD also reduced both the seizure frequency and mortality in PTZ, penicillin and pilocarpine induced acute seizure model of rats (Chiu et al., 1979; Consroe et al., 1982; Hill et al., 2013; Hansen et al., 2009; Jones et al., 2010; Jones et al., 2012). Further, the anticonvulsive effect of CBD has recently been demonstrated independently by our group and Klein et al. in MES, 6 Hz psychomotor seizure, and PTZ acute seizure tests and corneal kindling model of chronic seizures in mice (Klein et al., 2017; Patra et al., 2018). Our group has also established that a single intravenous dose of CBD (10 mg/kg) attenuates the maximum seizure severity in a rat model of pilocarpine-induced SE (Patra et al., 2018). Further, CBD (100 mg/kg twice daily; i.p. for 5 consecutive days) has been shown to reduce seizures in animal models of Dravet syndrome (Kaplan et al., 2017). The same group also demonstrated that a single dose of CBD (10 mg/kg; i.p.) improved social deficit in these mice (Kaplan et al., 2017).

Several clinical studies reported positive effect of CBD on infantile spasms, Lennox-Gastaut syndrome, tuberous sclerosis complex and other intractable epilepsies in children.
(Hess et al., 2016; Tzadok et al., 2016; Hussain et al., 2015). An open-label Phase-2 study conducted on 137 patients with various epilepsies, reported that CBD exerted a 36.5% median reduction in monthly motor seizures and was safe and tolerable (Devinsky et al., 2016). Further, a randomised double-blind placebo-controlled Phase 3 clinical trial on 120 drug resistant Dravet syndrome patients reported CBD was well tolerable and reduced monthly median seizure frequency from 12.4 to 5.9 in comparison to placebo group where it was reduced from 14.9 to 14 (Devinsky et al., 2017). Additionally, two double-blind, placebo-controlled clinical trials on 171 (Thiele et al., 2018) and 225 (Devinsky et al., 2018a) drug resistant Lennox-Gastaut syndrome patients documented a significant reduction in frequency of monthly drop seizures (atonic, tonic or tonic-clonic seizures that could have led to a fall) in the CBD treated groups than the placebo controlled groups. Considering the tolerability of CBD with promising seizure reduction potential in patients with Dravet syndrome and Lenox-Gastaut syndrome, the US-FDA approved CBD (Epidiolex; GW Pharmaceuticals) for the treatment of these two diseases (FDA, 2018).

1.3.3.3.2 Anxiety, depression and psychosis

The anxiolytic and antidepressant potential of CBD has been extensively studied and widely reported in the literature. CBD treatment produces anxiolytic effects in rats exhibited by increased open arm visits in elevated plus maze (EPM) test (Campos and Guimaraes, 2008; Guimaraes et al., 1990; Campos and Guimaraes, 2009), increased number of punished licks in the Vogel conflict test (Moreira et al., 2006; Campos and Guimaraes, 2008) and reduced freezing behaviours in the contextual fear conditioning test (Bitencourt et al., 2008). CBD also attenuates anxiety-induced rapid eye movement (REM) sleep disruption in rats caused due to repeated combination tests involving open field and elevated plus maze tests (Hsiao et al., 2012). Further, CBD treatment decreases panic responses in a number of animal models of panic disorders such as the elevated T-maze (Soares Vde et al., 2010), electrical
stimulation of the dorsal portions of the periaqueductal grey matter (Campos et al., 2013) and prey vs predator paradigm (Uribe-Marino et al., 2012). CBD has been shown to have antidepressant like activity in healthy rodents in forced swim test (Reus et al., 2011; El-Alfy et al., 2010; Zanelati et al., 2010; Sales et al., 2018). Additionally, the antidepressant activity of CBD has been demonstrated in genetic (Shoval et al., 2016; Shbiro et al., 2018), olfactory bulbectomy (Linge et al., 2016), chronic unpredictable stress (Campos et al., 2013) models of depression. The anxiolytic properties of CBD is not limited to preclinical studies, it has been demonstrated in both healthy human volunteers (Crippa et al., 2004; Fusar-Poli et al., 2009) and social phobia patients (Crippa et al., 2011; Bergamaschi et al., 2011). CBD treatment also produces significant improvement of psychosis observed in schizophrenia (Leweke et al., 2012; McGuire et al., 2018; Leweke et al., 2018) and Parkinson’s disease (Zuardi et al., 2009) patients. The molecular mechanisms through which CBD exert its anxiolytic and antidepressant effects are still not fully understood. One of the possible mechanism is by facilitating the 5-HT1A signalling, as several studies has shown that 5-HT1A receptor antagonist WAY-100635 diminishes its anxiolytic and antidepressant activities (Campos and Guimaraes, 2008; Soares Vde et al., 2010). The anxiolytic and antipsychotic effect of CBD might also be attributed to the enhancement of the endocannabinoid signalling by inhibiting the metabolism and uptake of anandamide (Fogaca et al., 2018) or by the activation of the TRPV1 receptor to facilitate presynaptic release of glutamate (Campos and Guimaraes, 2009). In addition to enhanced endocannabinoids signalling, CBD may produce its antipsychotic action by acting as a partial agonist on dopamine D2 receptors similar to antipsychotic drug aripiprazole (Seeman, 2016).

1.3.3.3.3 Alzheimer’s disease

CBD has also shown to have protective role against Alzheimer’s disease in several animal models. For instance, CBD reduces amyloid β-induced expression of inducible nitric
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oxide synthase (iNOS), interleukin-1β (IL-1β), glial fibrillary acidic protein (GFAP) and S100 calcium binding protein B in rodent astrocytes, thus attenuates reactive gliosis and inflammation associated with Alzheimer’s disease (Esposito et al., 2007; Esposito et al., 2011). It has been suggested that CBD induced anti-inflammatory and neuroprotective properties are mediated through activation of PPARγ (Esposito et al., 2011). CBD treatment also prevents amyloid β-induced microglial activation in mice and reverses spatial memory deficit in Morris water maze test (Martin-Moreno et al., 2011). Chronic CBD treatment also attenuates object recognition memory and social recognition memory deficits in genetically modified mouse model (AβPP/PS1 mice) of Alzheimer’s disease (Cheng et al., 2014a; Cheng et al., 2014b). Studies have shown the efficacy of CBD and Δ⁹-THC combinations in reversing dementia in mouse models of complex frontotemporal dementia (PK+/TauVLW mice, taupathy) (Casarejos et al., 2013) and Alzheimer’s disease (AβPP/PS1 mice) (Aso et al., 2015; Aso et al., 2016).

1.3.3.3.4 Parkinson’s disease

Parkinson’s disease is a slow neurodegenerative disease associated with progressive death of nigrostriatal dopaminergic neurons that results into several motor abnormalities such as bradykinesia, rigidity and tremor (Sethi, 2002). CBD exerts neuroprotective effect in 6-hydroxydopamine-induced animal model of Parkinson’s disease and reduces progressive degeneration of nigrostriatal dopaminergic neurons (Lastres-Becker et al., 2005; Garcia-Arencibia et al., 2007). Although no large scale studies have been conducted on human Parkinson’s patients, few reports suggested an improvement of psychotic symptoms and REM sleep disorder in Parkinson’s disease patients (Chagas et al., 2014a; Zuardi et al., 2009).
1.3.3.5 Other diseases

In addition to the above, CBD has also demonstrated its efficacy in pain (King et al., 2017; Ward et al., 2011; Turri et al., 2018; Gamble et al., 2018), cancer (Shrivastava et al., 2011; Elbaz et al., 2015; Ramer et al., 2012; Massi et al., 2004), diabetes (Weiss et al., 2006; Weiss et al., 2008) and cardiovascular diseases (Durst et al., 2007; Walsh et al., 2010; Rajesh et al., 2010).

1.4 Objectives

As mentioned previously, epilepsy is a progressive chronic neurological disease characterised by seizures and associated comorbidities and almost one third of patients with epilepsy are refractory to one or more currently available AEDs. Not only that, the drug induced adverse effects are also alarming. Thus, an AED which can take control of both seizures and associated comorbidities is the need of the hour. As mentioned before, CBD has a multimodal action and it has shown its efficacy against a range of diseases and conditions. Further, several studies have demonstrated the beneficial effects of CBD against acute seizures, however the effect of chronic CBD administration on seizures and comorbidities associated with chronic epilepsy has never been studied. Moreover, the effect of chronic CBD treatment on survivability and comorbidities associated with any genetic form of epilepsy syndrome has never been evaluated. Therefore, this project was designed to investigate the effect of CBD on survivability, seizures and associated comorbidities using animal models of TLE and Dravet syndrome to cover both the acquired and genetic form of epilepsies, respectively. To fulfil the overall aim of this project, I conducted the following three studies.

1. First, I evaluated the effect of chronic CBD treatment in a RISE-SRS model of TLE to assess its role on seizures and associated comorbidities such as motor control, gait and cognition (Chapter 2).
2. Secondly, I assessed the effect of long-term CBD treatment in a $\text{Scn1a}^{-/-}$ mouse model of Dravet syndrome to investigate its role on survivability and welfare of $\text{Scn1a}^{-/-}$ mice. The welfare parameters assessed were weight change, and total neonatal welfare, natural activity, reflex/response to touch, orbital tightening and body condition scores (Chapter 3).

3. Finally, I investigated the effect of chronic CBD treatment in a $\text{Scn1a}^{+/-}$ mouse model of Dravet syndrome to assess its effect on survivability and comorbidities associated with Dravet syndrome such as motor dysfunction, gait abnormalities, social deficit, anxiety, depression and cognitive impairment (Chapter-4).
Chapter 2: Effect of CBD in reducing seizures and associated comorbidities in rat model of temporal lobe epilepsy
2.1 Introduction

Seizures, whilst the primary symptom, are not the only aspect of temporal lobe epilepsy (TLE) that affect a patient’s quality of life. A number of comorbidities (e.g. depression, anxiety, motor disorder, cognitive deficits, social dysfunction, etc.) also contribute to a reduced quality of life, in addition to the poor prognosis associated with TLE (England et al., 2012). Furthermore, commonly used antiepileptic drugs (AEDs) such as valproate, phenytoin, and lacosamide are also known to produce a variety of cognitive, psychiatric, and motor adverse effects (George et al., 2015; Zaccara et al., 2004); thus therapies that do not carry the potential for adverse effects liability are in significant clinical demand. In this chapter, I investigated the effect of cannabidiol (CBD) in modifying seizures, motor function and cognition in a rat model of TLE.

CBD has been shown to have antiseizure activity in several animal models (Do Val-da Silva et al., 2017; Jones et al., 2012; Rosenberg et al., 2017b; Klein et al., 2017). Given its anticonvulsant efficacy in Phase 3 clinical trials, in 2018 the U.S. Food and Drug Administration (US-FDA) approved CBD (Epidiolex, GW Pharmaceuticals) as a drug for the treatment of Dravet Syndrome and Lennox Gastaut Syndrome in patients older than 2 years old (Devinsky et al., 2017; FDA, 2018; Thiele et al., 2018).

Despite the evidence in support of an antiepileptic action of CBD, few studies have investigated the potential for CBD to modify epilepsy-related behavioural comorbidities (Do Val-da Silva et al., 2017; Jones et al., 2012; Devinsky et al., 2017; Thiele et al., 2018). Further, no preclinical study has yet shown that sustained exposure to CBD not only reduces seizure burden in a TLE model but can also attenuate the severity of the associated behavioural comorbidities. Therefore, I assessed the effects of chronic oral administration of CBD on spontaneous seizures and associated behavioural comorbidities in the reduced
intensity status epilepticus induced spontaneous recurrent seizures (RISE-SRS) model (Modebadze et al., 2016). The RISE-SRS model shares several common features with human TLE including the post-insult epileptogenesis process without causing an overall damage of the brain, a similar electrophysiological profile (as observed during in vitro recording from human brain tissues) and spontaneous recurrent seizures (Modebadze et al., 2016). This model is, therefore, ideal for long-duration disease modification studies with candidate investigational therapies. Of note, the present study utilised a clinically-relevant treatment design, as animals were only enrolled to receive CBD well-after the onset of SRS. The results of the present study provide the first demonstration in a preclinical model of TLE to suggest that CBD may exert potential disease-modifying effects on SRS and attendant behavioural comorbidities. The work in this chapter has contributed towards a peer-reviewed publication (Patra et al., 2018).

2.2 Methods

2.2.1 Animals and test substances

All experiments mentioned in this chapter were conducted at the University of Reading under license (70/7672) UK Home Office regulations (Animals Scientific Procedures Act, 1986) and approved by the Animal Welfare and Ethical review board at the University of Reading. The animals were maintained in room with a 12h:12h dark:light cycle at 21°C and humidity of 50 ± 10%, with ad libitum access to food and water throughout the study period. Male Wistar rats (>70 g; 3-4 weeks old; Harlan Envigo, UK) were used for the induction of reduced intensity status epilepticus (RISE). For this study, CBD (Batch No. 6046727R) was supplied by GW Pharmaceuticals Ltd (Cambridge, UK). All other drugs and chemicals were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise.
2.2.2 Induction of reduced intensity status epilepticus (RISE)

RISE-SRS was induced as per the method described by Modebadze et al. (Modebadze et al., 2016) followed by an initial recovery and epileptogenesis period. In brief, the animals were initially injected with a subcutaneous injection of lithium chloride (127 mg/kg; s.c.) and returned to their home cage for 24 hours. The following day (24 hours after lithium chloride injection), animals were injected with methyl scopolamine, a muscarinic cholinergic receptor antagonist (1 mg/kg; s.c.) to provide protection against the pilocarpine-induced peripheral side effects. After 30 minutes, the animals were injected subcutaneously with a low dose of pilocarpine (25 mg/kg; s.c.) and were observed for 30 minutes for the symptoms of seizure activity and scored in Racine’s scale (Table 2.1) (Racine, 1972). If the animals did not reach the Racine stage 4 i.e. ‘bilateral forelimb clonus and rearing’ they were injected with further doses of pilocarpine (50 and 75 mg/kg each; s.c.) at 30 minutes intervals until they exhibited this behaviour. Animals that did not reach Racine stage 4 after 3 pilocarpine injections were euthanised by a Schedule 1 method (CO₂ asphyxiation followed by cervical dislocation).

**Table 2.1. Racine’s seizure severity scale** (Racine, 1972)

<table>
<thead>
<tr>
<th>Score</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mouth and facial movement</td>
</tr>
<tr>
<td>2</td>
<td>Head nodding</td>
</tr>
<tr>
<td>3</td>
<td>Unilateral forelimb clonus</td>
</tr>
<tr>
<td>4</td>
<td>Bilateral forelimb clonus with rearing</td>
</tr>
<tr>
<td>5</td>
<td>Generalised motor convulsion with forelimb clonus, rearing, falling and loss of postural tone</td>
</tr>
</tbody>
</table>
Once the animal started exhibiting ‘bilateral forelimb clonus and rearing’, they were immediately injected with xylazine (2.5 mg/kg; i.m.), an α2 adrenoreceptor agonist that has sedative and muscle relaxant properties. The animals were kept in the xylazine-modified SE condition for a maximum of 1 hour to reduce seizure severity and prevent seizure-related mortality. During this period the animals were held by the experimenters, with cotton buds placed gently inside the mouth of the animals to avoid tongue biting. Hypermellose eye drops (Medicom Healthcare, UK) were applied periodically to the eyes to prevent dryness. One-hour post xylazine injection the SE was terminated by a subcutaneous injection of 1 mL/kg of ‘stop’ solution containing MK 801 (0.1 mg/kg; Tocris Bioscience, UK), diazepam (ethanolic solution; 2.5 mg/kg; Hameln Pharmaceuticals, UK) and MPEP (20 mg/kg; Axon Medchem, The Netherlands). The animals were held (in hands) for another 30 minutes until they stopped having symptoms of SE. If the SE continued beyond 30 minutes, a further injection of ‘stop’ solution was given and held for another 30 minutes.

Once the behavioural manifestation of SE attenuated, the animals were kept on a heat pad to maintain body temperature and allowed to recover. The animals were closely monitored until they become active, at which point they were injected subcutaneously with 0.5 mL of 5% sucrose on one side of the body and with 0.5 mL of 0.9% saline on the other side of the body for rehydration. The animals were then hand-fed with moist powdered food (standard lab chow) and returned to their home cage. Twice daily welfare checks were conducted for another 3 days and weight was monitored. Any weight loss of more than 10% across any 2 consecutive measurements then animals were euthanised by the above-mentioned Schedule 1 method.

The steps of RISE induction have been depicted in Figure 2.1.
Figure 2.1. Graphical representation of the induction process of reduced intensity status epilepticus (RISE) in rats. 1. The rats were first injected with lithium chloride (127 mg/kg; s.c.). 2. After 24 hours, they were injected with methyl scopolamine (1 mg/kg; s.c.). 3. 30 minutes later, they were injected with increasing doses of pilocarpine (≥25 mg/kg; s.c. maximum 3 doses) at 30 minutes interval until the severity of seizure reaches Racine scale stage 4. 4. Xylazine was injected (2.5 mg/kg; i.m.) to reduce the seizure severity and the animals were physically restrained for 60 minutes. 5. A ‘stop solution’ consist of diazepam (2.5 mg/kg), MK801 (0.1 mg/kg) and MPEP (20 mg/kg) was injected subcutaneously for the termination of status epilepticus (SE). 6. After 2 weeks of post-induction, the post seizure behavioural battery tests (PSBB) were conducted twice weekly for the confirmation of spontaneous recurrent seizures (SRS)/epilepsy.
2.2.3 Confirmation of epilepsy

The animals were confirmed as epileptic by either visual observation of bilateral seizure (Racine scale ≥3) or a total score ≥10 (using touch X pick up scores) from four consecutive post-seizure behavioural battery (PSBB) tests, as previously described (Modebadze et al., 2016). The PSBB tests were conducted twice a week (Tuesday and Friday). The tests consisted of two tasks e.g. touch task and pick up task; with the scores obtained from these two tasks multiplied together to obtain a total PSBB score. In the touch task, the animals were gently poked on the rump with a blunt object (back side of a marker pen used consistently throughout the study) and the responses were scored by a previously trained researcher (Table 2.2). During the pickup task, the rats were picked up by grasping around the body and the responses were scored by trained researcher (Table 2.3). However, only animals that were classified as epileptic within 4-8 weeks from the day of SE induction were used for the analysis of seizures and comorbidities in the RISE-SRS model to minimize any age-related variability (Figure 2.2).

<table>
<thead>
<tr>
<th>Score</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No reaction</td>
</tr>
<tr>
<td>2</td>
<td>Rat turns toward instrument</td>
</tr>
<tr>
<td>3</td>
<td>Rat moves away from instrument</td>
</tr>
<tr>
<td>4</td>
<td>Rat freezes</td>
</tr>
<tr>
<td>5</td>
<td>Rat turns toward the touch</td>
</tr>
<tr>
<td>6</td>
<td>Rat turns away from the touch</td>
</tr>
<tr>
<td>7</td>
<td>Rat jumps (with or without vocalisation)</td>
</tr>
</tbody>
</table>
Table 2.3. Pick-up task in post seizure behavioural battery (PSBB) tests (Modebadze et al., 2016).

<table>
<thead>
<tr>
<th>Score</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Very easy pickup</td>
</tr>
<tr>
<td>2</td>
<td>Easy pickup with vocalisation</td>
</tr>
<tr>
<td>3</td>
<td>Some difficulty in pickup (rat rears and faces the hand)</td>
</tr>
<tr>
<td>4</td>
<td>Rat freezes</td>
</tr>
<tr>
<td>5</td>
<td>Difficult pickup (rat moves away)</td>
</tr>
<tr>
<td>6</td>
<td>Very difficult pickup (rat behaves defensively or attacks the hand)</td>
</tr>
</tbody>
</table>

2.2.4 Experimental design

To study the effect of CBD on seizures and associated comorbidities, 20 epileptic rats were randomly allocated into two groups with 10 animals daily orally treated with 200 mg/kg CBD (Batch No. 6046727R) and 10 animals daily orally treated with vehicle (3.5% Kolliphor® HS, Sigma-Aldrich, Poole, UK). 10 age-matched, vehicle-treated non-epileptic control rats were also studied. The CBD-treated group was initially treated for 1 week with vehicle followed by 1 week with a titrating dose of CBD (starting at 50 mg/kg and increasing by 50 mg/kg in every 2 days) to reach the final daily dose of 200 mg/kg. Thereafter 200 mg/kg dose was continued for 8 weeks until the end of all experiments (Figure 2.2). In line with the CBD-treated group both the vehicle-treated groups (non-epileptic/epileptic) were treated for 10 weeks with vehicle (Figure 2.2).
Figure 2.2. Study timeline. The study consisted of three groups: epileptic vehicle-treated, epileptic CBD-treated and non-epileptic vehicle-treated (n=10/groups). Following the confirmation of spontaneous recurrent seizures (SRS) the epileptic animals were randomised to either vehicle or CBD treatments. The CBD-treated animals were initially treated orally with vehicle for a week followed by one week of oral tapering dose of CBD (50-150 mg/kg/day). CBD at 200 mg/kg/day was then administered orally for 8 weeks until the completion of the study. The other two groups (epileptic and non-epileptic) were treated with vehicle (3.5% Kolliphor®) for 10 weeks. Epileptic animals were video monitored for the assessment of seizure burden and seizure burden ratio. All the animals were assessed on their motor (rotarod and gait tests) and cognitive function (hole-board test) during the last two weeks.
2.2.5 Assessment of seizures

All epileptic animals were video monitored 24 hours a day for 7 weeks (Figure 2A). Twenty CCTV cameras (TP-101BK, Topica, Taiwan) were established and connected to a PC, with video footage recorded using Zoneminder (v1.2.3; Triornis Ltd., Bristol, UK) software. Video footage obtained during the light phase (07:00 to 19:00) from initial 4 days (at the beginning of treatment; first bin) and final 4 days (before beginning of the behavioural experiments; final bin) were coded offline to analyse behaviour. The light phase was selected for coding as in this model ~65% spontaneous seizures take place during this phase (Farrimond et al., 2009).

A blinded independent researcher was trained to identify and code severity of convulsive behaviour using a modified Racine scale (Table 2.4) as previously described (Sheffler et al., 2009). Any score of ≥ 3 were recorded as these reflected clearly identifiable motor convulsions. Both the severity and frequency of seizures are equally important in estimating the seriousness of the disease, therefore seizure burden in each animal was calculated from i) first, multiplying each seizure severity with its frequency (number of occurrence) ii) then adding up all the scores obtained in step (i) (Barker-Haliski et al., 2015). The seizure burden ratio was calculated from the seizure burden by using the formula:

\[
\text{Mean seizure burden ratio} = \frac{\text{mean seizure burden in final bin}}{\text{mean seizure burden in first bin}}
\]

A higher seizure burden ratio therefore indicates a worsening of the disease over time.
Table 2.4 Modified Racine scale used for coding seizure behaviour for TLE animal model; adapted from (Sheffler et al., 2009).

<table>
<thead>
<tr>
<th>Score</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal behaviour</td>
</tr>
<tr>
<td>1</td>
<td>Becoming motionless</td>
</tr>
<tr>
<td>2</td>
<td>Forelimb and/or tail extension, rigid posture</td>
</tr>
<tr>
<td>3</td>
<td>Myoclonic jerks or repetitive movements with head bobbing or “wet dog shakes”</td>
</tr>
<tr>
<td>3.5</td>
<td>Unilateral or bilateral forelimb clonus</td>
</tr>
<tr>
<td>4</td>
<td>Bilateral forelimb clonus and rearing</td>
</tr>
<tr>
<td>5</td>
<td>Bilateral forelimb clonus, rearing and occasional falling</td>
</tr>
<tr>
<td>5.5</td>
<td>Bilateral forelimb clonus, continuous rearing and falling</td>
</tr>
<tr>
<td>6</td>
<td>Tonic-clonic seizure with loss of postural tone</td>
</tr>
</tbody>
</table>

2.2.6 Assessment of motor coordination

Motor coordination was assessed using an accelerating rotarod (Ugo Basile rotarod model 7750) where each animal was placed on a rotating rotarod apparatus that linearly accelerated from 5 to 33 rpm over 5 minutes. Latency to fall from the rod (in seconds) was recorded, with a maximum score of 300 seconds. Each animal performed 3 trials and average latency to fall from the rod was entered into the statistical analysis. The animals were returned to their home cage after each trial and an interval of 2 minutes was given between two consecutive trials. Animals were habituated to the stationary rotarod for 2 minutes/day for two consecutive days before the test day.

2.2.7 Assessment of gait

Effect of the disease/treatment on cerebellar function was assessed by monitoring of gait patterns. A non-toxic ink was used to mark the hind paws of the rats as they walked on white paper (100 cm × 10 cm) lining the floor of a transparent custom-made Plexiglas tunnel (100 ×10 ×10 cm) attached to a darkened box at its end. Animals were habituated to the
runway for two consecutive days before completing two trials on the 3rd day. Left and right stride length were measured by the distance between two successive ipsilateral foot prints, whereas the stride width was measured from the distance between one paw print with its contralateral stride length at right angle (Wecker et al., 2013). The first and last foot prints were not considered for evaluation (Wecker et al., 2013). The mean value of each parameter obtained from the two trials represents a biological replicate in statistical test.

2.2.8 Assessment of cognitive function

A hole-board apparatus made of Plexiglas (70×70×45 cm), mounted on a table of 72 cm above the floor level was used to assess the cognitive function of the animals. The apparatus consisted of equally spaced 16 holes on its floor each of 2.5 cm in diameter. In this experiment, each rat was placed in the center of the hole-board and allowed to freely explore the apparatus for 10 min. Four accessible baits (Cheerios®, Nestle) were kept in four holes, randomly selected for each animal, but kept constant across all test days. Olfactory cues were eliminated by placing inaccessible Cheerios® in all other holes. Prior to onset of testing, animals were habituated to the hole-board for four days (two days without bait to let the animals acclimatise to the test arena followed by two days with accessible bait in all the 16 holes to habituate the animals to the rules of the task). Animals were food deprived for 4-5 hours before tests to enhance motivation to complete the task. Testing was conducted on three consecutive days with animals performing five trials each day. Head dips were recorded by a video camera (Sony DCR-SX21E) for later analysis. A head dip was scored when the head was introduced into the holes at least to eye level of the rodent. Errors consisted of head-dipping a hole that was baited with an inaccessible Cheerios® (reference memory error; RME); or re-dipping a hole that had previously been baited (working memory error; WME).
2.2.9 Statistical analysis

All statistical analyses of behavioural data from the RISE-SRS rats were performed using GraphPad Prism 6 software (GraphPad Software, Inc., USA). The normal distribution of the values was tested using the D’agostino-Pearson normality test. Seizure burden data obtained from epileptic animals (Section 2.2.5) were analysed by Wilcoxon matched pair test (non-parametric), whereas seizure burden ratio was analysed by Mann-Whitney test (non-parametric). Fisher’s exact test was further conducted to compare the number of animals from both the epileptic groups whose seizure was improved (seizure burden ratio <1). Data obtained from accelerating rotarod (Section 2.2.6), gait (Section 2.2.7) and hole-board (Section 2.2.8) tests were analysed by one-way ANOVA. If a significant main effect of group was found, then Holm-Sidak’s post hoc multiple comparison test was conducted to compare the non-epileptic vehicle-treated group with the epileptic vehicle-treated group (to observe the effect of disease), and the epileptic vehicle-treated with the epileptic CBD-treated group (to observe the disease modifying effect of CBD). Multiple comparisons were corrected in all cases. In the hole-board test, two animals from each of the epileptic vehicle-treated and epileptic-CBD treated groups did not respond (did not consume any Cheerios® during the habituation period), hence were excluded from this test. All data were presented as mean ± SEM. In all cases, p<0.05 was considered as the level of significance.

2.3 Results

2.3.1 CBD reduced long-term seizure burden in epileptic rats

To evaluate the effect of chronic CBD administration on disease progression, the effect of treatment upon seizure burden and seizure burden ratio was examined. Here, the epileptic vehicle-treated rats showed a robust increase in seizure burden from a median value of 25.75 (IQR=18.94-39.34) to 30.13 (IQR=21.00-49.91) over the course of the study.
Chapter 2

(Wilcoxon matched pair test, \(W=53, p<0.01;\) Figure 2.3A). On the other hand, CBD-treated epileptic rats did not demonstrate a time-dependent increase in seizure burden (Wilcoxon matched pair test, \(W=-31, p=0.13;\) Figure 2.3B) observed by a change of median value from 28.50 (IQR=26.81-39.75) to 24.75 (IQR=19.69-35.94) in the same time period.

![Figure 2.3](image)

**Figure 2.3. Effect of chronic administration of vehicle/CBD on seizure burden in epileptic rats.** A. Seizure burden before and after vehicle treatment. Median seizure burden was increased significantly in this group at the end of the observation period. B. Seizure burden before and after CBD treatment. CBD-treated rats showed no change in median seizure burden. Data were analysed by Wilcoxon matched pairs signed rank test; \(n=10/\)group; **\(p<0.01.\)**
Interestingly, the seizure burden ratio was significantly greater in vehicle-treated epileptic animals compared to CBD-treated epileptic animals (n=10/group; Mann Whitney test, U=22, p<0.05; Figure 2.4A). Moreover, a significantly greater number of CBD-treated rats (7 of 10) showed an improvement in disease severity (seizure burden ratio below 1) compared to vehicle-treated rats (1 of 10) (Fisher’s exact test, p<0.05, Figure 2.4B).

**Figure 2.4.** Dot plot and bar diagram showing effect of vehicle/CBD treatment on seizure burden ratio in epileptic rats. A. Median seizure burden ratio of epileptic vehicle-treated and epileptic CBD-treated animals recorded during a 7-week continuous video monitoring period. CBD significantly reduced the seizure burden ratio compared to the vehicle-treated counterpart (n=10/group; data were analysed by Mann Whitney test; *p<0.05). Data are expressed as median, min to max, and interquartile range (IQR). B. Number of epileptic vehicle-treated and epileptic CBD-treated animals with seizure burden ratio below or above 1 (Data were analysed by Fisher’s exact test; *p<0.05).
2.3.2 CBD attenuated motor incoordination associated with epilepsy

Motor coordination of the animals was tested using the accelerating rotarod. A significant difference in time spent on the rotarod was observed among the groups (One-way ANOVA; \( F_{(2,27)} = 5.996; \ p < 0.01 \)). Holm Sidak’s \textit{post hoc} test revealed that both the non-epileptic vehicle-treated (130.40 ± 3.99 seconds; \( p < 0.05 \)) and epileptic CBD-treated (149.00 ± 13.10 seconds; \( p < 0.01 \)) animals spent significantly more time on the rod, compared to the epileptic vehicle-treated group (97.87 ± 12.13 seconds). This indicates that chronic oral CBD administration attenuated the TLE-induced motor dysfunction seen in vehicle-treated animals (Figure 2.5).
Figure 2.5. Dot plot showing effect of chronic vehicle/CBD administration on motor coordination of rats in accelerating rotarod test. Mean time spent by non-epileptic vehicle-treated, epileptic vehicle-treated and epileptic CBD-treated animals on accelerating rotarod (n=10/group). Non-epileptic vehicle-treated and epileptic CBD-treated group spent significantly more time on the accelerating rotarod compared to the epileptic vehicle-treated group. Data are expressed as mean ± SEM; data were analysed by one-way ANOVA with Holm-Sidak’s *post hoc* test; *p<0.05, **p<0.01.

2.3.3 CBD did not exert any adverse effect on gait in epileptic rats

The gait test was performed to assess left stride length (LSL), right stride length (RSL) and stride width (SW) of the animals. The mean values of LSL (Figure 2.6A) of non-epileptic vehicle-treated, epileptic vehicle-treated and epileptic CBD-treated animals were respectively 144.10 ± 5.33, 143.80 ± 5.38 and 145.20 ± 5.85 millimetres, whereas the mean
values of RSL (Figure 2.6B) in these groups were respectively 142.80 ± 4.64, 144.80 ± 4.97 and 147.30 ± 5.64 millimetres (n=10/group). Further, SW (Figure 2.6C) in these groups were respectively 41.58 ± 1.27, 44.30 ± 1.19 and 43.34 ± 1.94 millimetres (n=10/group). No significant differences between the groups were observed for any of these parameters (One-way ANOVA; for LSL $F_{(2,27)}=0.019$, for RSL $F_{(2,27)}=0.198$, for SW $F_{(2,27)}=0.842$; p>0.05; Figure 2.6A-C). These data show that chronic CBD administration does not have any adverse effect on gait of the TLE animals.

Figure 2.6. Dot plots showing effect of chronic vehicle/CBD administration on gait of rats. A. Left stride length (millimeters) of non-epileptic vehicle-treated, epileptic vehicle-treated, and epileptic CBD-treated rats (n=10/group). No significant difference in mean left stride length was observed among the groups. B. Right stride length (millimeters). No significant difference in mean right stride length was observed among the groups. C. Stride width (millimeters). No significant difference in mean stride width was observed among the groups. Data are expressed as mean ± SEM; data were analysed by one-way ANOVA with Holm-Sidak’s post hoc test.
2.3.4 CBD attenuated working memory deficits associated with epilepsy

To assess the effect of CBD on cognitive performance, animals were challenged on the hole-board test of reference and working memory.

A significant difference in number of reference memory errors (RME) was observed between groups (One-way ANOVA; \( F_{(2,23)}=8.83; \ p<0.01 \)). The Holm-Sidak’s post hoc test revealed that epileptic vehicle-treated rats (2.72 ± 0.38; \( n=8 \)) made significantly more RME compared to the non-epileptic vehicle-treated rats (1.11 ± 0.09; \( n=10; \ p<0.001 \); Figure 2.7A). However, no significant difference was observed in between epileptic CBD-treated (1.95 ± 0.34; \( n=8 \)) and epileptic vehicle-treated rats (Holm-Sidak’s post hoc test; \( p=0.07 \); Figure 2.7A). Interestingly, no significant difference was observed in between epileptic CBD-treated and non-epileptic vehicle-treated animals (Holm-Sidak’s post hoc test; \( p>0.05 \)).

Similar to RME, a significant difference in working memory errors (WME) was found among the groups (One-way ANOVA; \( F_{(2,23)}=11.28; \ p<0.001 \)). The Holm-Sidak’s post hoc test showed that epileptic vehicle-treated rats (1.87 ± 0.33) made significantly more WME compared to both non-epileptic vehicle-treated (0.99 ± 0.09; \( p<0.01 \)) and epileptic CBD-treated (0.58 ± 0.05; \( p<0.001 \); Figure 2.7B) rats. The data obtained from hole-board test thus showed that chronic CBD-treatment attenuated the working memory deficit associated with epilepsy in RISE-SRS rats without producing any adverse effect on their reference memory.

A further correlation analysis was carried out between seizure burden and RME/WME, however no correlation was found between seizure burden and RME/WME (Appendix-1).
Figure 2.7. Dot plots showing the effect of chronic administration of vehicle/CBD on spatial memory of rats in hole-board test. A. Reference memory errors (RME) in non-epileptic vehicle-treated (n=10), epileptic vehicle-treated (n=8), and epileptic CBD-treated (n=8) groups. CBD did not significantly affect the reference memory compared to the non-epileptic vehicle-treated group. B. Working memory errors (WME). CBD significantly improved WME in epileptic animals compared to the epileptic vehicle-treated animals. Data are expressed as mean ± SEM; data were analysed by one-way ANOVA with Holm-Sidak’s multiple comparison test; **p<0.05, ***p<0.001.

2.4 Discussion

In this study, I demonstrated for the first time that chronic oral administration of CBD improves seizure burden ratio, motor comorbidities, and cognitive function in the RISE-SRS model of TLE in rats after the onset of symptomatic seizures. My study further supports a growing body of evidence to demonstrate that CBD is a well-tolerated and effective therapy
for chronic seizures (Devinsky et al., 2017), as well as demonstrating the potential that chronic oral administration of CBD may confer disease-modifying effects in a preclinical model of TLE. As mentioned in the introduction, this work has been published in the journal *Epilepsia* (Patra et al., 2018).

TLE is one of the most common forms of acquired epilepsy in humans (Jefferys and Jiruska, 2009). A number of FDA-approved antiepileptic drugs, such as valproate, phenobarbital and eslicarbazepine, have demonstrated disease-modifying potential in preclinical models of chronic seizure (Potschka et al., 2014; Brandt et al., 2006; Brandt et al., 2010). Further, CBD has been demonstrated to reduce seizure frequency and mortality in PTZ, penicillin and pilocarpine induced acute rat models of seizures (Chiu et al., 1979; Consroe et al., 1982; Hill et al., 2013; Hansen et al., 2009; Jones et al., 2010; Jones et al., 2012). Not only that, in two independent studies conducted by Klein et al., and our group have shown that CBD is anticonvulsive in MES, 6 Hz psychomotor seizure, and PTZ acute seizure tests and corneal kindling model of chronic seizures in mice (Klein et al., 2017; Patra et al., 2018). Moreover, we proved that intravenous CBD (10 mg/kg) administration is able to reduce maximum seizure severity in a rat model of pilocarpine-induced SE (Patra et al., 2018). Nevertheless, this is the first study to demonstrate the effect of CBD in a preclinical TLE model. Here, I showed that the vehicle-treated epileptic rats exhibited a notable, time-dependent increase in disease severity over the course of the study. CBD did not significantly reduce seizure burden after seven weeks of treatment; however, it did markedly modify the natural disease course following SE, as demonstrated by no overall increase in the seizure burden from the first to final seizure monitoring bin (Figure 2.4). Interestingly, seizure burden ratio was significantly lower in epileptic CBD-treated animals compared to their vehicle-treated counterparts, which suggests that CBD is disease-modifying in this model. It is also observed that a significantly greater number of CBD-treated animals (70%) showed a
significant improvement in disease severity (seizure burden ratio below 1) compared to vehicle-treated animals (10%).

A number of antiepileptic drugs (e.g. phenobarbital, valproic acid, phenytoin etc.) are reported to have adverse effect on motor function characterised by dyskinesia in people with epilepsy (Zaccara et al., 2004; Ristić et al., 2006; Zaccara et al., 2013; Bainbridge et al., 2017). Moreover, patients on phenytoin and valproic acid treatment sometimes exhibit Parkinsonism (Zaccara et al., 2004). While prior reports suggested that CBD is well-tolerated in non-epileptic rats (Jones et al., 2012), no studies have yet been conducted to examine the long-term effects of chronic CBD administration on tolerability and motor function in epileptic animals. Furthermore, no study has yet administered CBD for disease modification purposes after the onset of SRS. In this regard, the present study employed a clinically-realistic treatment scenario in a preclinical model of TLE to demonstrate that chronic oral administration of CBD is associated with notable anticonvulsant efficacy, minimal adverse effects liability, and disease-modifying potential well after the onset of symptomatic seizures.

It is well-known that humans and animals with epilepsy are more sensitive to adverse effects of antiepileptic drugs (Klitgaard et al., 2002), thus the present findings that chronic oral administration of CBD in rats with epilepsy was not associated with any adverse effects further supports the potential of this agent for chronic clinical use in epilepsy patient populations. In addition to these drug-induced adverse effects on motor function, motor deficits are one of the most common comorbidities exhibited by patients with epilepsy (Boelen et al., 2005). Here, I used two well-validated models of motor function, the rotarod and gait tests, to assess fine motor control and the gait of animals, respectively (Wecker et al., 2013; Krishnakumar et al., 2009). Epileptic vehicle-treated animals fell from the accelerating rotarod sooner than the non-epileptic vehicle-treated ones, indicating that SRS produced significant motor dysfunction related to balance. Similar findings have been previously
reported using the rotarod test where motor dysfunction was exhibited by pilocarpine induced epileptic rats (Krishnakumar et al., 2009). Importantly though, CBD-treated epileptic animals remained on the accelerating rotarod for a significantly longer time than the vehicle-treated epileptic animals. Thus, I showed for the first time that CBD is not only well-tolerated by epileptic animals after prolonged oral administration, but that CBD reduces the severity of motor deficits induced by epilepsy.

Epileptic rats performed comparably to healthy animals in the gait test which is consistent with lack of reports citing gait disorders in adult epileptic patients with TLE. However, AEDs such as valproic acid and lacosamide have detrimental effect on gait in human patients (Ristić et al., 2006; Bainbridge et al., 2017). I therefore wanted to eliminate the possibility that CBD may cause gait deficits. Interestingly in this study, chronic oral administration of CBD did not produce any changes in stride length or width in the epileptic rats. This result thus demonstrates that the dose of CBD necessary to reduce seizure burden was not associated with gait impairing effects in rats with SRS. Clinical trials conducted on Dravet Syndrome and Lennox Gastaut Syndrome patients also did not report any gait disturbances following CBD administration (Devinsky et al., 2017; Thiele et al., 2018). Whether these potential side effects of CBD are also absent in the general TLE patient population remains to be defined.

Cognitive deficits are one of the most common comorbidities associated with TLE (Rzezak et al., 2017; Realmuto et al., 2015). For example, patients with TLE often exhibit poor executive control and working memory deficits (Lima et al., 2017), whilst amnesia or accelerated long-term loss of memory is also frequently reported in patients with TLE (Miller et al., 2017). Cognitive function in animals is typically assessed using spatial memory tasks measuring reference memory and working memory errors (Douma et al., 1998; Vawter et al., 1997). Reference memory can be defined as long-term storage of acquired information that
remains constant over successive training sessions, while working memory is a form of short-term memory that refers to storage and manipulation of information acquired within a trial session (Olton, 1979). Several spatial memory tasks e.g. radial arm maze, Morris water maze and hole-board, have been used to evaluate these two types of memory in rodents (Decker, 2006; van der Staay, 1999). Here, I employed the hole-board task where epileptic vehicle-treated rats exhibited impairment of both the working and reference memory aspects of the hole-board test. These findings are comparable with previous studies of spatial or hippocampus-dependent memory in rats with TLE, e.g. a delayed nonmatching to position task (Schipper et al., 2016), eight arm radial maze (Wolf et al., 2016), and Morris water maze (Kalemenev et al., 2015). However, reference memory of CBD-treated epileptic animals was not significantly different compared to both non-epileptic and epileptic vehicle-treated animals, which suggests that although CBD could not improve the reference memory, it provided protection against the epilepsy-induced reference memory impairment in these animals. Remarkably, the present study showed that chronic oral administration of CBD improved working memory function in epileptic animals compared to their vehicle-treated counterparts. The beneficial effects of CBD on memory have also been reported which will be discussed in greater detail in chapter 5 (Osborne and Solowij, 2017; Martin-Moreno et al., 2011; Cheng et al., 2014a; Hindocha et al., 2015). Here, I demonstrated that memory deficits were improved by CBD which is a novel finding for TLE in rats. Although SRS are the primary cause of cognitive decline in TLE (van Rijckevorsel, 2006); the improved cognitive function in the CBD-treated epileptic group might be due to a seizure-independent mechanism rather than solely by seizure reduction e.g. anti-inflammatory/neuroprotective action (Schiavon et al., 2014; Pazos et al., 2012; Cassol et al., 2010). A further detailed investigation is thus warranted to shed light on the mechanism by which CBD improved the presently tested behavioural comorbidities.
2.5 Conclusion

In this study, I established that oral administration of CBD attenuated seizures, reduced motor deficits and improved cognitive function without affecting gait in a RISE-SRS rat model of TLE. Moreover, this study demonstrated that CBD has disease modifying effect in TLE and highlighted that it is a potential candidate drug for the treatment of spontaneous symptomatic seizures of TLE. Notably, this work has recently been published in the peer reviewed journal *Epilepsia*.
Chapter 3: Effect of CBD in improving survivability and neonatal welfare in Scn1a-/- mouse model
3.1 Introduction

In the previous chapter, I demonstrated the efficacy of cannabidiol (CBD) against seizures and associated comorbidities in rat model of temporal lobe epilepsy (TLE). In this chapter, I will report the role of CBD on survivability and neonatal welfare in Scn1a−/− mouse model of Dravet syndrome which reproduces ataxia, seizures and premature mortality as observed in human patients.

As mentioned in Chapter 1, the SCN1A gene encodes the Nav1.1 subtype of voltage-gated sodium channels which regulates the inhibitory network of the hippocampal GABAergic interneurons (Malo et al., 1991; Yu et al., 2006). Therefore, a loss of function of this gene due to mutation leads to abnormal interneuron firing and subsequent disinhibition to promote the severe myoclonic epilepsy observed in Dravet Syndrome (Cheah et al., 2012; Yu et al., 2006). The present study employed Scn1a−/− mice that exhibit seizures and associated behavioural symptoms due to lack of Nav1.1 channel expression at GABAergic inhibitory interneurons (Yu et al., 2006). The Scn1a−/− mice differ from human Dravet syndrome by their complete loss of expression of Nav1.1 channels, but they reproduce the ataxia, seizures and premature mortality commonly observed in Dravet syndrome patients (Yu et al., 2006; Kalume et al., 2007). These mice start exhibiting symptoms such as seizures, ataxia, poor righting reflex from postnatal day 9 (P9) onwards which become progressively worse over time, with animals dying before they reach the age of P16 (Yu et al., 2006; Miller et al., 2014; Kalume et al., 2007). Due to extreme severity and early mortality, this model is not suitable for studying complex behavioural comorbidities but does allow rapid assessment of antiepileptic drug (AED) efficacy to justify a larger study (Chapter 4).

In addition to fast AED screening, the Scn1a−/− mouse model is also useful for the evaluation of a number of well-established parameters such as natural activity,
reflex/response to touch, total neonatal welfare, orbital tightening and body condition that reflect the health and wellbeing of animals (Ullman-Culleré and Foltz, 1999; Wolfensohn and Lloyd, 2007; Langford et al., 2010). For instance, the natural activity and reflex/response to touch (righting reflex) provide important information on the neonatal motor function (Wolfensohn and Lloyd, 2007), while orbital tightening is a potential indicator of pain in animals (Langford et al., 2010). Overall health of the animals can be assessed by neonatal welfare and body condition scores (Wolfensohn and Lloyd, 2007; Ullman-Culleré and Foltz, 1999). In the present study, these welfare parameters were therefore used to assess the neonatal comorbidities associated with Scn1a<sup>−/−</sup> mouse model.

The efficacy of CBD has been demonstrated in several animal models of seizures and epilepsy (Do Val-da Silva et al., 2017; Jones et al., 2012; Kaplan et al., 2017) including the rat model of TLE (Chapter 2). However, a disease modifying effect of CBD has never been assessed in Scn1a<sup>−/−</sup> mice. Furthermore, as stated earlier, the Scn1a<sup>−/−</sup> mice present with a severe epilepsy phenotype that results in premature death with no currently available antiepileptic drugs reported to be efficacious in extending their survival. Conventional antiepileptic drug diazepam improved the seizures in these mice but failed to extend their survival (Yu et al., 2006). This study was therefore designed to investigate the effect of CBD in Scn1a<sup>−/−</sup> mouse model to evaluate its potential to extend the survivability and modify disease associated comorbidities in the Scn1a<sup>−/−</sup> mice.

### 3.2 Methods

#### 3.2.1 Animals and test substances

The present experiment was performed following UK Home Office regulations (Animals (Scientific Procedures) Act, 1986) under licence 70/8397 “Mouse Model of Dravet Syndrome” and was approved by the Animal Welfare and Ethics Review Board at the
University of Reading. All mice were maintained in 12h:12h light:dark cycle (experiments were conducted during light cycle 8:00-20:00 h), a room temperature of 21°C and humidity of 50 ± 10 %, with ad libitum access to food and water. 129S-Scn1a<sup>tm1Kea/Mmjax</sup> heterozygote mice (Jackson Laboratory, USA) were maintained in the BioResource Unit at the University of Reading (UK) and bred together to obtain Scn1a<sup>-/-</sup> and wild type (WT) animals (Figure 3.1). Scn1a<sup>-/-</sup> and wild type (WT) animals were maintained in the Unit as described earlier before entering the study and commencing drug treatment as outlined below. The maternal behaviour of the dams was also assessed simultaneously to ensure that any of the parameters observed in the study animals (Scn1a<sup>-/-</sup>/WT mice) were not affected by the dam’s behaviour (See 3.2.4.2). At the end of the study, animals were humanely killed by a Schedule 1 method (cervical dislocation). CBD (batch no. 6046727R) was supplied by GW Pharmaceuticals (Cambridge, UK). All other chemicals were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.
Figure 3.1. Schematic diagram showing breeding and experimental design for this experiment. Male and female 129S-Scn1a<sup>tm1Kea/Mmjax</sup> heterozygote (Scn1a<sup>+/−</sup>) mice were crossed to obtain Scn1a<sup>+/+</sup> (WT) and Scn1a<sup>−/−</sup> mice used in the study. Tissue collection (tail clippings) and genotyping was conducted in between postnatal day 2 (P2) and P4. On P5 these animals were randomised into four groups i.e. WT vehicle-treated, WT CBD-treated, Scn1a<sup>−/−</sup> vehicle-treated and Scn1a<sup>−/−</sup> CBD-treated (n=10/group). The animals were treated twice daily subcutaneously with either vehicle (ethanol: Kolliphor<sup>®</sup>: 0.9% saline=2:1:17) or CBD (100 mg/kg) from postnatal day 8 (P8) onwards until P25/death (whichever was earlier). Twice daily welfare was conducted to assess body weight, total neonatal welfare, natural activity, reflex/response to touch, orbital tightening and body condition scores. Survivability was also assessed.
3.2.2 Genotyping of animals

Approximately 1-2 mm of tail tip was clipped from each animal at P2-4. DNA was extracted by incubating each sample at 75°C for 5 minutes followed by 95°C for 10 minutes (deactivation step) with 20 μL 5X PCRBIO Rapid Extract Buffer A (1u/μL), 10 μL 10X PCRBIO Rapid Extract Buffer B (PCR Biosystems, USA) and 70 µL PCR grade water. The volume was then adjusted by adding 900 µL PCR grade water followed by centrifugation at 16000g for 1 minute. The supernatant containing mouse DNA was collected and kept at -20°C until use.

1 μL DNA sample solution was amplified in 20 μL PCR reaction mixture containing 0.4 μL each of: 25 µM forward primer (AGT CTG TAC CAG GCA GAA CTT G) and reverse primers (Rwt: CCC TGA GAT GTG GGT GAA TAG; Rmut: AGA CTG CCT TGG GAA AAG CG), 10 μL taq mix red (PCR Biosystems, USA) and 7.8 μL Rnase-free PCR grade water. The reaction condition included an activation step at 94°C for 2 minutes followed by 15 cycles of denaturation (94°C for 2 seconds), annealing (65°C for 15 seconds) and extension (68°C for 21 seconds); and another 15 cycles of denaturation (94°C for 15 seconds), annealing (60°C for 15 seconds) and extension (72°C for 21 seconds) which was followed by a final extension at 72°C for 2 minutes.

After the reaction was complete, samples (10 μL) were loaded in 2% agarose gel (stained with 2 μL Syber Safe DNA gel stain, Invitrogen, USA) in 1X TAE (Promega, USA) buffer and run for 2 minutes at 70V in a gel electrophoresis apparatus (BIORAD, USA). The gel was then observed under a UV transilluminator (Syngene, UK; Figure 3.2).
Figure 3.2. Example result from PCR used for genotyping animals produced via breeding of the 129S-Scn1a\textsuperscript{tm1Kea/Mmjax} transgenic mouse line. Mouse tail DNA was amplified with primers for the wild type (WT) and mutated (Scn1a\textsuperscript{-/-}) Scn1a alleles. The presence of a 357bp band indicates the WT allele, and a 200bp band indicates the Scn1a\textsuperscript{-/-} allele. -ve: negative control; +ve: positive control.

3.2.3 Experimental design

Following genotyping, animals were randomly divided into four groups WT vehicle-treated, WT CBD-treated, Scn1a\textsuperscript{-/-} vehicle-treated and Scn1a\textsuperscript{-/-} CBD-treated (n=10/group). They were injected subcutaneously twice daily with either CBD (100 mg/kg) or its vehicle (ethanol: Kolliphor\textsuperscript{®}: 0.9% saline=2:1:17) from P8 until P25 or death (whichever was earlier). A twice daily welfare check was conducted throughout the entire duration of the study. Drug administration was conducted at 0800 h and followed by welfare checks. Conversely, afternoon welfare checks were conducted from 1600 h and followed by drug administration in order to provide the maximum possible time between doses. The experimental timeline is depicted in Figure 3.1.
3.2.4 Assessment of welfare scores

3.2.4.1 Neonatal welfare

Welfare scoring of neonates was conducted twice daily using a blinded spreadsheet that lacks the information on the genotype of the animals and the treatment (CBD/vehicle) given to them, to ensure the experimenter remained blind to both treatment and genotype. Neonatal welfare scoring (Table 3.1) was based upon a previously validated standardised approach used widely in murine models (Wolfensohn and Lloyd, 2007; Langford et al., 2010; Ullman-Culleré and Foltz, 1999). Here, the parameters used for the welfare assessment were: weight, natural activity (NA; 0-3), reflex/response to touch (RT; 0-3), orbital tightening (OT; 0-2; Figure 3.3), body condition score (BC; 1-3) and surface temperature (ST; 0-2; data not shown). Finally, a total neonatal welfare score (TNW; range 0-8) was calculated by adding together scores from NA, RT, and ST.
Table 3.1. Welfare assessment sheet for neonatal mice; adapted from (Wolfensohn and Lloyd, 2007; Ullman-Culleré and Foltz, 1999; Langford et al., 2010)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Observations</th>
<th>Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) Natural activity (NA)</td>
<td>Wriggling ++ (highly active)</td>
<td>0</td>
</tr>
<tr>
<td>i) Natural activity (NA)</td>
<td>Wriggling + (active)</td>
<td>1</td>
</tr>
<tr>
<td>i) Natural activity (NA)</td>
<td>Not active but can move</td>
<td>2</td>
</tr>
<tr>
<td>i) Natural activity (NA)</td>
<td>Still</td>
<td>3</td>
</tr>
<tr>
<td>ii) Reflexes/respond to touch (RT)</td>
<td>Righting reflex +++ (time taken to return back on its feet: &lt;1 second)</td>
<td>0</td>
</tr>
<tr>
<td>ii) Reflexes/respond to touch (RT)</td>
<td>Righting reflex ++ (time taken to return back on its feet: 1-3 seconds)</td>
<td>1</td>
</tr>
<tr>
<td>ii) Reflexes/respond to touch (RT)</td>
<td>Righting reflex + (time taken to return back on its feet: 4-10 seconds)</td>
<td>2</td>
</tr>
<tr>
<td>ii) Reflexes/respond to touch (RT)</td>
<td>No righting reflex - (unable to return back on its feet within 10 seconds)</td>
<td>3</td>
</tr>
<tr>
<td>iii) Surface temperature (ST)</td>
<td>Warm (~35°C)</td>
<td>0</td>
</tr>
<tr>
<td>iii) Surface temperature (ST)</td>
<td>Partial cold (dorsal/ventral part of the body)</td>
<td>1</td>
</tr>
<tr>
<td>iii) Surface temperature (ST)</td>
<td>Cold</td>
<td>2</td>
</tr>
<tr>
<td>iv) Total neonatal welfare score (TNW; Sum of scores i to iii)</td>
<td>mouse is emaciated, skeleton is extremely prominent, vertebrae distinctly segmented</td>
<td>1</td>
</tr>
<tr>
<td>v) Body condition (BC)</td>
<td>Mouse is underconditioned, segmented vertebral column evident, dorsal pelvic bones are palpable</td>
<td>2</td>
</tr>
<tr>
<td>v) Body condition (BC)</td>
<td>Mouse is well-conditioned, vertebrae and dorsal pelvis not prominent, palpable with slight pressure</td>
<td>3</td>
</tr>
<tr>
<td>vi) Orbital tightening (OT)</td>
<td>Not present</td>
<td>0</td>
</tr>
<tr>
<td>vi) Orbital tightening (OT)</td>
<td>Moderately present</td>
<td>1</td>
</tr>
<tr>
<td>vi) Orbital tightening (OT)</td>
<td>Obviously present</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 3.3. Scoring of orbital tightening in mice to assess pain. Orbital tightening is the closing of the eyelid or narrowing of orbital area, a wrinkle might also be visible around the eye. Adapted from National Centre for the Replacement Refinement and Reduction in Animals in Research (NC3R) and Langford et al (Langford et al., 2010).

3.2.4.2 Dam welfare

A dam welfare score (range: 0-6; Table 3.2) was also measured to assess maternal behaviour where parameters e.g. milk in neonate’s stomach (0-2), nest building (0-2) and retrieval of young (0-2) were checked. These parameters were scored in comparison to the other WT C57BL/6 mice (not included in the study) maintained in the animal house. A high dam welfare score (5-6), indicative of a dam exhibiting weak maternal behaviours or impaired recovery from gestation and birth, was undesirable and increased the likelihood of rejection of neonates and so presented a confounding factor for a given litter in a study. In the present study, dam scores remained 0 throughout the study and so the responses of the pups were not considered to have been affected by variations in maternal behaviours.
Table 3.2. Welfare assessment sheet for dam; adapted from (Wolfensohn and Lloyd, 2007).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Observations</th>
<th>Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk in stomach (only applicable when the body colour is pink)</td>
<td>Milk is visible</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Milk is slightly visible</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>No visible milk in stomach</td>
<td>2</td>
</tr>
<tr>
<td>Nest building</td>
<td>Good nest making</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Some nest making</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>No nest</td>
<td>2</td>
</tr>
<tr>
<td>Retrieval of young</td>
<td>Always</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sometimes</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Never</td>
<td>2</td>
</tr>
<tr>
<td>Total Score</td>
<td></td>
<td>0-6</td>
</tr>
</tbody>
</table>

3.2.5 Assessment of survivability

Due to UK Home Office limitations imposed upon the use of this model of epilepsy due to its severity, animal suffering was minimised by employing a validated, welfare scoring system (mentioned in section 3.2.4.1) alongside a mathematical model to predict death (Appendix I; developed by M. Bazelot, GW Pharmaceuticals, UK). In this way, any animal for which the model predicted death could be sacrificed 0.5 day before enduring the maximal severity of the disease. The model used an algorithm to predict death based on prior data obtained from untreated Scn1a<sup>−/−</sup> mice (n=19) that exhibited the maximum severity of the disease and died a natural death (data not shown). In this algorithm, the thresholds for each parameter (TNW, NA, RT, OT, BC scores) to predict death were obtained using the following procedure: (i) each parameter, measured every half day from birth for each animal, is averaged with a moving mean with a 1.5 day window; (ii) the least severe score for each parameter observed across the 19 animals over 0.5 day before their death was found; (iii) each of the 5 parameters exhibited by the animals in the study were compared to scores.
obtained in (ii) twice a day; (iv) if each of the 5 parameters reached their respective threshold defined in (ii) at least once since P8, the animal would undergo a Schedule 1 procedure (cervical dislocation) within 0.5 day. Additionally, surface temperature (ST) threshold was employed such that if the sum of the ST scores over the last 1.5 days, was equal to or greater than 3, the animal would be killed by Schedule 1 procedure (cervical dislocation) within 0.5 day.

3.2.6 Statistical analysis

Welfare parameters were analysed using SPSS 24 (IBM SPSS Statistics®, UK), whilst survivability data were analysed using GraphPad Prism 6 software (GraphPad Software, Inc., USA). Data obtained from welfare parameters were compared using a three-way ANOVA to observe the main effects of treatment, genotype and time, and their two-way and three-way interactions. If significant two-way interactions were found the Bonferroni’s post hoc test was conducted on any treatment×genotype interactions to assess the effect of CBD treatment on different genotypes (WT/Scn1a−/−). The Bonferroni’s post hoc test was also conducted for a significant three-way treatment×genotype×time interaction to compare the effect of CBD treatment with vehicle treatment at every time point of welfare assessment in both the WT and Scn1a−/− groups. In all cases, the post hoc test was corrected for multiple comparisons. Data from 2.2% welfare scores were outliers and were excluded from further analysis (±2.5*SD) (Miller, 1991). For the survivability data, survival curves from Scn1a−/− vehicle-treated and CBD-treated groups were compared using a Mantel-Cox test. No WT animals died during the study, so survivability curves were not compared. All the data are expressed as mean ± SEM. In all cases, p<0.05 is considered as the level of significance.
Chapter 3

3.3 Results

3.3.1 CBD administration improved neonatal welfare

3.3.1.1 Weight

The mean weights of WT vehicle-treated, WT CBD-treated, Scn1a−/− vehicle-treated and Scn1a−/− CBD-treated group were respectively 7.41 ± 0.09, 7.34 ± 0.09, 4.85 ± 0.09 and 5.43 ± 0.09 g. A three-way ANOVA showed main effects of treatment (F(1,612)=9.06; p<0.01), genotype (F(1,612)=683.41; p<0.001) and time (F(16,612)=13.01; p<0.001) on weight. Overall, the weight was higher in CBD-treated animals (6.39 ± 0.06 g) compared to the vehicle-treated animals (6.13 ± 0.06 g) and in WT animals (7.38 ± 0.06 g) compared to the Scn1a−/− animals (5.14 ± 0.06 g). Here, no three-way interaction among treatment×genotype×time was observed (F(16,612)=0.02; p>0.05; Figure 3.4A). Among the two-way interactions, the genotype×time (F(16,612)=18.36; p<0.001) and treatment×genotype (F(1,612)=13.96; p<0.001) interactions were found to be significant, whilst the treatment×time interaction was not significant (F(16,612)=18.36; p>0.05). The Bonferroni’s post hoc analysis was conducted for treatment×genotype which showed that CBD treatment increased weight only in Scn1a−/− mice (p<0.001) but it did not produce any effect in WT mice (p>0.05).

3.3.1.2 Total neonatal welfare (TNW)

The mean TNW scores in WT vehicle-treated, WT CBD-treated, Scn1a−/− vehicle-treated and Scn1a−/− CBD-treated group were respectively 0.39 ± 0.04, 0.24 ± 0.04, 3.66 ± 0.04 and 2.85 ± 0.04. Main effects of treatment (F(1,612)=128.78; p<0.001), genotype (F(1,612)=4850.12; p<0.001) and time (F(16,612)=57.89; p<0.001) on TNW scores was found. Overall, scores were better in CBD treated animals (1.55 ± 0.03) compared to vehicle treated animals (2.03 ± 0.03) and in WT animals (0.32 ± 0.03) compared to the Scn1a−/− animals (3.26 ± 0.03). A significant three-way interaction among treatment×genotype×time was observed
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(F_{16,612}=5.46, p<0.001). Two-way interactions were observed for treatment\times genotype (F_{1,612}=62.74; p<0.001), treatment\times time (F_{16,612}=2.19; p<0.01) and genotype\times time (F_{16,612}=112.22; p<0.001). Bonferroni’s post hoc test was conducted for treatment\times genotype and treatment\times genotype\times time interactions. The post hoc analysis for treatment\times genotype revealed that CBD treatment significantly improved the TNW score both in WT (p<0.05) and Scn1a\textsuperscript{-/-} mice (p<0.001). The post hoc comparison for treatment\times genotype\times time interactions revealed that CBD delayed the worsening of welfare scores in Scn1a\textsuperscript{-/-} mice from P12 to P16 compared to the vehicle-treated Scn1a\textsuperscript{-/-} mice on respective days (p<0.01; Figure 3.4B). This post hoc test further showed that CBD improved TNW score in WT animals from P8-P8.5 i.e. in first day of treatment compared to the WT vehicle-treated animals on respective occasions (p<0.05; Figure 3.4B).
Figure 3.4. Plots showing chronic administration of CBD to wild type (WT) and Scn1a<sup>−/−</sup> mice on weight and total neonatal welfare (TNW) scores. A. Weight of WT vehicle-treated, WT CBD-treated, Scn1a<sup>−/−</sup> vehicle-treated and Scn1a<sup>−/−</sup> CBD-treated mice from postnatal day 8 (P8) onwards (n=10/group). CBD had no effect on weight in WT mice compared to the vehicle-treated WT mice. No three-way interaction of treatment×genotype×time was observed on weight. B. TNW scores. CBD significantly improved the TNW score in WT mice from P8 to P8.5 compared to the vehicle-treated WT mice. CBD significantly improved the TNW score in Scn1a<sup>−/−</sup> mice from P12 onwards compared to the vehicle-treated Scn1a<sup>−/−</sup> mice. Data are expressed as mean ± SEM; data were analysed by a three-way ANOVA with Bonferroni’s post hoc test; #p<0.05; **p<0.01; ***p<0.001; # represents WT vehicle-treated vs WT CBD-treated; * represents Scn1a<sup>−/−</sup> vehicle-treated vs Scn1a<sup>−/−</sup> CBD-treated.

3.3.1.3 Natural activity (NA)

The mean NA score in WT vehicle-treated, WT CBD-treated, Scn1a<sup>−/−</sup> vehicle-treated and Scn1a<sup>−/−</sup> CBD-treated group were respectively 0.24 ± 0.02, 0.21 ± 0.02, 1.64 ± 0.02 and 1.35 ± 0.02. A three-way ANOVA revealed the main effect of treatment (F<sub>(1,612)=47.24</sub>; p<0.001), genotype (F<sub>(1,612)=3090.07</sub>; p<0.001) and time (F<sub>(16,612)=28.82</sub>; p<0.001) on NA
scores. Taken together, the NA score was better in CBD-treated animals (0.78 ± 0.02) compared to the vehicle-treated animals (0.94 ± 0.02) and in WT animals (0.22 ± 0.02) compared to the Scn1a−/− animals (1.49 ± 0.02). Further, a significant three-way interaction of treatment×genotype×time was observed here (F(16,612)=3.12, p<0.05). Two-way interactions were observed for treatment×genotype (F(1,612)=34.12; p<0.001), treatment×time (F(16,612)=2.30; p<0.01) and genotype×time (F(16,612)=81.61; p<0.001). Bonferroni post hoc analysis was conducted for treatment×genotype and treatment×genotype×time interactions. The post hoc test for treatment×genotype revealed that CBD improved natural activity only in Scn1a−/− mice (p<0.001) but not in WT mice (p>0.05). The post hoc analysis for treatment×genotype×time revealed that CBD significantly (p<0.05) delayed the worsening of NA scores in Scn1a−/− mice from P12 to P16 compared to the vehicle-treated Scn1a−/− mice on respective days (Figure 3.5A).

3.3.1.4 Reflex/response to touch (RT)

The mean RT score in WT vehicle-treated, WT CBD-treated, Scn1a−/− vehicle-treated and Scn1a−/− CBD-treated group were respectively 0.15 ± 0.02, 0.04 ± 0.02, 1.83 ± 0.02 and 1.42 ± 0.02. The main effect of treatment (F(1,612)=112.78; p<0.001), genotype (F(1,612)=3958.19; p<0.001) and time (F(16,612)=32.25; p<0.001) on RT scores was found in three-way ANOVA. Altogether the RT score was better in CBD-treated animals (0.73 ± 0.02) when compared to the vehicle-treated animals (1.00 ± 0.02) and in WT animals (0.10 ± 0.02) when compared to the Scn1a−/− animals (1.63 ± 0.02). The three-way interaction among treatment×genotype×time interaction was also noticed here (F(16,612)=5.19, p<0.001). The treatment×genotype (F(1,612)=38.05; p<0.001), treatment×time (F(16,612)=2.27; p<0.01) and genotype×time (F(16,612)=61.73; p<0.001) interactions were found to be significant. Bonferroni post hoc test was conducted for treatment×genotype and treatment×genotype×time interactions. The post hoc analysis for treatment×genotype
revealed that CBD significantly improved the RT score both in Scn1a−/− (p<0.001) and WT (p<0.01) mice. The post hoc analysis for treatment×genotype×time showed that CBD significantly improved (lower value) the RT score in Scn1a−/− group from P12 to P14.5 and on P16 compared to its vehicle-treated counterpart on respective days (p<0.01; Figure 3.5B). The Post hoc test for treatment×genotype×time further revealed that CBD significantly improved the RT score in WT animals from P8-P9.5 compared to the WT vehicle-treated mice on respective days (p<0.01; Figure 3.5B).
Figure 3.5. Plots showing chronic administration of CBD to wild type (WT) and Scn1a<sup>−/−</sup> mice on natural activity (NA) and reflex/response to touch (RT) scores. A. NA scores of WT vehicle-treated, WT CBD-treated, Scn1a<sup>−/−</sup> vehicle-treated and Scn1a<sup>−/−</sup> CBD-treated mice from postnatal day 8 (P8) onwards (n=10/group). CBD had no effect on NA score of WT mice. CBD significantly improved the NA score in Scn1a<sup>−/−</sup> mice from P12 onwards compared to the vehicle-treated Scn1a<sup>−/−</sup> mice. B. RT scores. CBD significantly improved the RT score in WT mice from P8 to P9.5 compared to the vehicle-treated WT mice. CBD significantly improved the RT score in Scn1a<sup>−/−</sup> mice from P12 to P14.5 and on P16 compared to the vehicle-treated Scn1a<sup>−/−</sup> mice. Data are expressed as mean ± SEM; data were analysed by a three-way ANOVA with Bonferroni’s post hoc test; #p<0.01; **p<0.01; ***p<0.001; # represents WT vehicle-treated vs WT CBD-treated; * represents Scn1a<sup>−/−</sup> vehicle-treated vs Scn1a<sup>−/−</sup> CBD-treated.

3.3.1.5 Orbital tightening (OT)

The mean OT score in WT vehicle-treated, WT CBD-treated, Scn1a<sup>−/−</sup> vehicle-treated and Scn1a<sup>−/−</sup> CBD-treated group were respectively 0.00 ± 0.00, 0.00 ± 0.00, 0.50 ± 0.02 and 0.25 ± 0.02. A three-way ANOVA showed the main effect of treatment (F<sub>(1,612)=56.67</sub>;
(p<0.001), genotype (F(1,612)=483.67; p<0.001) and time (F(16,612)=65.42; p<0.001) on OT score. Altogether, OT score was better in CBD-treated animals (0.12 ± 0.01) compared to vehicle-treated animals (0.25 ± 0.01) and in WT animals (0.00 ± 0.00) compared to Scn1a−/− animals (0.37 ± 0.01). A three-way treatment×genotype×time interaction was observed (F(16,612)=6.78; p<0.001). Further, significant two-way interactions for treatment×genotype (F(1,612)=56.66; p<0.001), treatment×time (F(16,612)=6.78; p<0.001) and genotype×time (F(16,612)=65.42; p<0.001) were observed. Bonferroni’s post hoc test was conducted for treatment×genotype and treatment×genotype×time interactions. The post hoc test for treatment×genotype revealed that CBD improved OT score in Scn1a−/− mice (p<0.001) no such change was observed in WT mice (p>0.05). The post hoc test for treatment×genotype×time revealed that the OT score was better (lower) in CBD-treated Scn1a−/− group from P13 until P16 compared to the vehicle-treated Scn1a−/− group on respective days (p<0.01; Figure 3.6A).

3.3.1.6 Body condition (BC)

The mean BC score in WT vehicle-treated, WT CBD-treated, Scn1a−/− vehicle-treated and Scn1a−/− CBD-treated group were respectively 3.00 ± 0.01, 3.00 ± 0.01, 2.71 ± 0.01 and 2.82 ± 0.01. The main effect of treatment (F(1,612)=36.37; p<0.001), genotype (F(1,612)=623.81; p<0.001) and time (F(16,612)=84.18; p<0.001) on BC score was observed in three-way ANOVA. Overall, the BC score was better in CBD-treated animals (2.91 ± 0.01) compared to vehicle-treated animals (2.86 ± 0.01) and in WT animals (3.00 ± 0.01) compared to Scn1a−/− animals (2.77 ± 0.01). A significant treatment×genotype×time three-way interaction was observed here (F(16,612)=4.82; p<0.001). The two-way interactions were observed for treatment×genotype (F(1,612)=36.37; p<0.001), treatment×time (F(16,612)=4.82; p<0.001) and genotype×time (F(16,612)=84.18; p<0.001). Bonferroni’s post hoc analysis was done for treatment×genotype and treatment×genotype×time interactions. The post hoc analysis for
treatment×genotype revealed that CBD treatment improved this score only in Scn1a−/− mice (p<0.001), but it did not exert any effect on WT animals (p>0.05). The post hoc analysis for treatment×genotype×time revealed that BC scores were higher (better) in CBD-treated Scn1a−/− mice from P13.5 until P15.5 compared to the vehicle treated Scn1a+/− mice on respective days (p<0.01; Figure 3.6B).

CBD thus well-tolerated by the WT mice and it delayed the worsening of the welfare scores in Scn1a+/− mice compared to their vehicle-treated counterpart.
Figure 3.6. Plots showing chronic administration of CBD to wild type (WT) and Scn1a<sup>−/−</sup> mice on orbital tightening (OT) and body condition (BC) scores. A. OT of WT vehicle-treated, WT CBD-treated, Scn1a<sup>−/−</sup> vehicle-treated and Scn1a<sup>−/−</sup> CBD-treated mice from postnatal day 8 (P8) onwards (n=10/group). CBD had no effect on OT score of WT mice. CBD significantly improved the OT score in Scn1a<sup>−/−</sup> mice from P13 onwards compared to the vehicle-treated Scn1a<sup>−/−</sup> mice. B. BC scores. CBD had no effect on BC score of WT mice. CBD significantly improved the BC score in Scn1a<sup>−/−</sup> mice from P13.5 to P15.15 compared to the vehicle-treated Scn1a<sup>−/−</sup> mice. Data are expressed as mean ± SEM; data were analysed by a three-way ANOVA with Bonferroni’s post hoc test; **p<0.01; ***p<0.001; * represents Scn1a<sup>−/−</sup> vehicle-treated vs Scn1a<sup>−/−</sup> CBD-treated.

### 3.3.2 CBD administration increased survivability

As expected, none of the WT animals died during the study. Survivability of the two Scn1a<sup>−/−</sup> groups were therefore analysed using the Mantel-Cox test. Here, the median survivability in the CBD-treated Scn1a<sup>−/−</sup> mice was significantly higher (16.25 days) compared to the vehicle-treated Scn1a<sup>−/−</sup> mice (15.5 days; X²=8.61; p<0.01; n=10/group; Figure 3.7).
Figure 3.7. Plot showing chronic administration of CBD on survival of Scn1a<sup>−/−</sup> mice. CBD treatment significantly (p<0.01) increased survival compared to the vehicle-treated Scn1a<sup>−/−</sup> group. Error bars are SEM; Data were analysed by Mantel-Cox test.

3.4 Discussion

In the present study, the effect of CBD on standard welfare parameters; such as natural activity, reflex/response to touch, total neonatal welfare, orbital tightening and body condition scores; was evaluated in both WT and Scn1a<sup>−/−</sup> mice. I showed that CBD was well-tolerated by the neonatal WT mice and that CBD delayed the worsening of these scores in such a severe animal model of epilepsy. Remarkably, it is established here that CBD treatment extended the survivability of Scn1a<sup>−/−</sup> mice; to date, this is the only drug that has increased survivability in this model.

In this study, WT vehicle-treated pups become fully active and responsive around P11-P12 from which stage the natural activity and reflex/response to touch scores were found to be zero. Interestingly, the reflex/response to touch score was found to be zero in the CBD-treated WT animals as early as P9 and was better (lower) from P8 to P9.5 compared to the vehicle-treated WT animals. Reflex is a good indicator of neurological development in mouse pups (Fox, 1965), however the effect observed in this study was so small that it was unlikely
that CBD expedited the developmental process in these mice. I further demonstrated that CBD was well-tolerated and had no adverse effects upon natural activity and reflex/response to touch and total neonatal welfare scores in neonatal WT mice throughout the observation period which establishes the fact that the beneficial effects of CBD in Scn1a<sup>−/−</sup> mice have not been influenced by any other confounding effects. The tolerability of CBD on motor function are well documented in adult rodent models of hepatic encephalopathy (Magen <i>et al.</i>, 2010), multiple sclerosis (Mecha <i>et al.</i>, 2013), schizophrenia (Peres <i>et al.</i>, 2016), catalepsy (Sonego <i>et al.</i>, 2016; Gomes <i>et al.</i>, 2013), seizures (Jones <i>et al.</i>, 2012; Klein <i>et al.</i>, 2017), epilepsy (Chapter 2) and Dravet syndrome (Kaplan <i>et al.</i>, 2017). However, the effect of CBD on motor function in neonates has not been previously demonstrated. The current findings thus extend our knowledge by highlighting that sustained exposure to CBD is safe and well-tolerated in neonates at the doses employed.

Although the neonates perceive pain and distress they are unable to behaviourally express them (Wolfensohn and Lloyd, 2007). It is therefore difficult to use conventional behavioural methodologies to assess the disease-related suffering in neonates. Here, I scored the natural activity and reflex/response to touch to determine the motor function and wellbeing of the pups as described previously in neonates (Wolfensohn and Lloyd, 2007). In the present study, both scores were significantly higher, indicating worsening symptomology, in the Scn1a<sup>−/−</sup> mice compared to the WT mice. Previously it has been reported that Scn1a<sup>−/−</sup> mice start exhibiting the symptoms of limb tremors from P9 which progressively deteriorated to loss of postural control nearer to death (Yu <i>et al.</i>, 2006), thus my results are in agreement with this study. As previously mentioned the total neonatal welfare score obtained from a combined scores of natural activity, reflex/response to touch and surface temperature and provides an overview of general health and wellbeing of these animals (Wolfensohn and Lloyd, 2007). A significantly higher total neonatal welfare score in the Scn1a<sup>−/−</sup> mice
compared to the WT mice therefore advocates that lack of Nav1.1 expression had a significant impact on overall health and welfare in Scn1a\(^{-/-}\) mice.

The current study further established that CBD treatment significantly delayed the worsening of natural activity and reflex/response to touch scores in Scn1a\(^{-/-}\) mice compared to their vehicle-treated counterparts. These findings thus illustrate that CBD has a protective effect against motor deficits induced by the Scn1a mutation. Notably, I already demonstrated in the previous chapter that CBD improves motor function in RISE-SRS rats, thus this is the second evidence of CBD’s beneficial role upon motor function in epilepsy. Children with Dravet syndrome often exhibit motor deficits (Aljaafari \textit{et al.}, 2017), therefore these results indicates that CBD may be beneficial to treat motor dysfunction in those children. However, more focussed behavioural assays using Scn1a\(^{+/-}\) mice, which survive longer to be tested on more conventional motor function tests (e.g. rotarod, static beam tests etc), are required to validate this and are described in a less severe model of Dravet syndrome in the next chapter.

In line with the changes in natural activity and reflex/response to touch score, similarly beneficial results were obtained for total neonatal welfare score i.e. CBD treatment significantly delayed the worsening of this score in Scn1a\(^{-/-}\) animals compared to their vehicle-treated counterparts. This suggests an improvement in the overall health status of the mutant animals after CBD treatment.

Children with Juvenile myoclonic epilepsy often experience headache as a comorbidity associated with seizures (Dedei Daryan and Guveli, 2018; Ertem \textit{et al.}, 2017; Rozen, 2011). Both human and animals exhibit orbital tightening (closing of the eyelid or narrowing of orbital area) as a pain related physiological sign (Prkachin, 1992; Langford \textit{et al.}, 2010). So, this serves as a valuable parameter for the assessment of pain. Hence, the orbital tightening was measured in this phenotype as an indicator of pain. The orbital tightening score was found to be significantly higher, indicating worst symptoms, in Scn1a\(^{-/-}\)
animals compared to the WT animals. Prolonged CBD treatment showed no effect on the orbital tightening scores of WT animals. CBD treatment has been reported to produce analgesia in several rodent models such as nerve injury (De Gregorio et al., 2019), osteoarthritis (Philpott et al., 2017) and surgical incision (Genaro et al., 2017) in rats, and nerve injury (Casey et al., 2017), corneal injury (Thapa et al., 2018) and chemotherapy induced pain (King et al., 2017) in mice. However, the analgesic effect of CBD in epilepsy model has never been studied. Here, CBD significantly delayed the worsening of the orbital tightening score in Scn1a−/− animals compared to their vehicle-treated cohorts. This highlights that CBD may have analgesic properties in neonatal epilepsy. Of note, this is the first study that indicates a possible analgesic effect of CBD in any epilepsy model.

Body condition score is considered as a sensitive and reliable parameter to assess the health status of animals. It is a widely used technique for routine health check-up of laboratory, farm and pet animals (Ullman-Culleré and Foltz, 1999; Roche et al., 2006; Sapowicz et al., 2016). This method was also used to assess the health in several animal disease condition such as polycystic kidney disease in rats (Hickman and Swan, 2010), cancer in dogs (Michel et al., 2004) and hyperthyroidism in cats (Peterson et al., 2016). In our laboratory, body condition scoring is used for monitoring the health of epileptic rats. The scoring is done by overserving and palpating the flesh covering the bony protuberances of hips and vertebral column (Ullman-Culleré and Foltz, 1999). The present study showed that the body condition score was significantly lower (worse) in Scn1a−/− mice compared to their WT counterparts. This corroborates a previous study which reported a lack of body fat in post-mortem examination of Scn1a−/− mice (Yu et al., 2006). In this study, treatment with CBD in WT animals did not produce any effect on the body condition score. Interestingly, here CBD significantly delayed the worsening of body condition score in Scn1a−/− mice compared to the vehicle-treated Scn1a−/− mice. Previous study on Scn1a−/− mice demonstrated
that hand feeding could improve the survivability in these mice up to 2.5 days (Yu et al., 2006). Therefore, it might be argued that CBD increased the feed intake here which overall improved the body condition score in Scn1a−/− mice. However, this is highly unlikely as CBD did not produce any impact on weight over the course of the study in neither WT nor Scn1a−/− mice compared to their respective vehicle-treated cohorts. Further, several studies proved that CBD has no effect on food intake or weight in adult healthy mice (Wiley et al., 2005; Riedel et al., 2009). Therefore, this is justified to interpret that the delay in worsening of body condition score by CBD-treatment in Scn1a−/− mice did not rely on increased food intake but may reflect an overall disease modification property of CBD.

The present study further demonstrated that the vehicle-treated Scn1a−/− mice died prematurely with an overall medial survivability of 15.5 days which was expected with this severe epilepsy phenotype (Yu et al., 2006; Kalume et al., 2007). Interestingly, here CBD treatment significantly extended the survivability of Scn1a−/− mice when compared to their vehicle-treated counterparts (median survivability 16.25 days vs 15.5 days). This result is highly promising as standard antiepileptic drugs, such as diazepam, fail to extend survivability (Yu et al., 2006). An extension of survivability in this model was only shown after hand feeding (Yu et al., 2006) but as discussed in the previous paragraph CBD induced extension of survivability was not due to increased feeding but possibly due to its anticonvulsant and disease modifying property. This hypothesis is supported by a previous study which established the anticonvulsant effect of CBD in a similar but less severe model with haploinsufficiency of NaV1.1 channels (Kaplan et al., 2017). Overall, this is the first study which demonstrates the beneficial effect of CBD upon the survival of Scn1a−/− mice.
3.5 Conclusion

In this study, I characterised the Scn1a<sup>−/−</sup> mouse model using well established neonatal welfare scores and assessed the effect of CBD on welfare and survivability of Scn1a<sup>−/−</sup> mice. The welfare parameters were found to be significantly worse in the Scn1a<sup>−/−</sup> mice compared to their WT counterparts. Notably, this is the first study to demonstrate that CBD-treatment improves the welfare parameters and extends the survivability of Scn1a<sup>−/−</sup> mice. CBD is also the first drug that exerted such positive effect in this model. The Scn1a<sup>−/−</sup> mice shares several common features with human Dravet syndrome patients, therefore CBD may be useful to prevent premature mortalities and neonatal comorbidities associated with Dravet syndrome.

Despite reproducing several key features of human Dravet syndrome, the Scn1a<sup>−/−</sup> mice differ with the human patients genotypically by their complete loss of voltage-gated Nav1.1 expression and phenotypically by the extreme disease severity. Further, due to early mortality, this model limits the use of standard behavioural tests to assess intricate comorbidities such as motor dysfunction, social deficit, anxiety, depression and cognitive impairment associated with Dravet syndrome. This thesis therefore extended in the next chapter to investigate the effect of CBD using the Scn1a<sup>+/−</sup> mouse model that phenotypically and genotypically resembles the human Dravet syndrome.
Chapter 4: Effect of CBD in reducing premature mortality and comorbidities in Scn1a\(^{+/-}\) mouse model of Dravet syndrome
4.1 Introduction

In the previous chapter, I showed that cannabidiol (CBD) improved the welfare parameters and extended the survivability of Scn1a⁻/⁻ mice. However, due to early mortality and extreme severity of the model it was not possible to assess intricate behavioural comorbidities. In this chapter, I therefore evaluated the effect of chronic CBD treatment on survivability and comorbidities associated with Dravet syndrome using a less severe Scn1a⁺/- mouse model that phenotypically and genotypically resembles the human Dravet syndrome.

As mentioned in Chapter 1, premature mortality is common in Dravet syndrome patients (Sakauchi et al., 2011; Cooper et al., 2016). In addition, they exhibit multiple comorbidities including psychomotor delay, gait abnormality, hyperactivity, attention deficits, autism, sleep disorders, anxiety, depression, language impairment and severe cognitive deficits that have profound adverse effects upon the quality of life (Black and Gaebler-Spira, 2016; Brunklaus et al., 2011; Genton et al., 2011; Li et al., 2011). The current ‘gold’ standard treatment for Dravet syndrome includes the combination of two or more antiepileptic drugs in the therapeutic regimen, such as benzodiazepines, valproic acid, topiramate, levetiracetam and stiripentol (Morimoto et al., 2018; Devinsky et al., 2017). However, these drugs often fail to adequately control seizures and are also associated with severe drug-induced motor and psychiatric adverse effects including anxiety, depression and memory impairments (Ristić et al., 2006; Chen et al., 2017a). As mentioned in previous chapters, CBD has been approved by the United States Food and Drug Administration (USFDA) for the treatment of Dravet syndrome patients, however CBD’s effect on the premature mortality and behavioural comorbidities associated with Dravet syndrome is yet to be established (FDA, 2018). Of note, in the previous chapter, I demonstrated the preliminary evidence of CBD’s efficacy on a severe Scn1a⁻/⁻ mouse model of Dravet syndrome.
The widely used preclinical model of Dravet syndrome involves Scn1a<sup>+/−</sup> mice which resembles the human disease form (Miller et al., 2014; Yu et al., 2006; Han et al., 2012; Hawkins et al., 2017). Interestingly, the phenotype of the Scn1a<sup>+/−</sup> mice largely depends on their background strain with Scn1a<sup>+/−</sup> mice on a 129S background strain failing to demonstrate seizures and premature mortality that can be observed on C57BL/6 background (Yu et al., 2006). However, the reproductive performance of the Scn1a<sup>+/−</sup> mice on C57BL/6 background strain is very poor which limits their use in any preclinical studies (Yu et al., 2006). Fortunately, hybrid Scn1a<sup>+/−</sup> offspring obtained from a cross of 129S×C57BL/6 strains recapitulate several features of Dravet syndrome including seizures, premature mortality and other comorbidities such as social deficit, anxiety and memory impairments (Han et al., 2012; Yu et al., 2006), thus they are considered as a standard model to study drug effect on Dravet syndrome (Kaplan et al., 2017; Anderson et al., 2017; Hawkins et al., 2017).

Efficacy of several conventional AEDs have previously been tested in this model. For example, a single intraperitoneal dose of carbamazepine (20 mg/kg), levetiracetam (10 mg/kg) or topiramate (40 mg/kg) was shown to prevent premature mortality (Hawkins et al., 2017). Further, clonazepam (0.0625 mg/kg) was reported to protect the social deficits and memory impairment in Scn1a<sup>+/−</sup> mice following single intraperitoneal dosing (Han et al., 2012). Not only that, CBD has recently been shown to improve seizures (100 mg/kg twice daily for 8 days; i.p.) and social deficit (10 mg/kg single dose; i.p.) in these mice (Kaplan et al., 2017). However, none of these studies used chronic doses of AEDs to observe their effect on comorbidities which are ether induced, made worse or both by several AEDs as mentioned before. The present study was therefore designed to observe the effect of chronic CBD treatment on premature mortality and several comorbidities such as motor impairment, social deficit, anxiety, depression and cognitive dysfunction in Scn1a<sup>+/−</sup> mouse model of Dravet syndrome.
4.2 Methods

4.2.1 Animals and test substances

These experiments were performed following UK Home Office regulations (Animals (Scientific Procedures) Act, 1986) and was approved by the Animal Welfare and Ethics Review Board at the University of Reading. All mice were maintained in 12h:12h dark:light cycle, a room temperature of 21°C and humidity of 50 ± 10 %, with ad libitum access to food and water. The animals were group housed throughout the experiment except for 3 days during sucrose preference test when each animal was individually housed. This experiment was conducted in dark cycle (dim red light, 8:00-20:00 h). Male 129S-Scn1a\textsuperscript{tm1Kea/Mmjax} heterozygote mice (Jackson Laboratory, USA) maintained in the BioResource Unit, University of Reading (UK) were bred with female wild type C57BL/6 mice (Charles River, UK) to obtain Scn1a\textsuperscript{+/-} and wild type (WT) littermate mice used in this experiment. At the end of the study, animals were humanely killed by a Schedule 1 method (cervical dislocation).

CBD with batch no. 070214 was supplied by GW Pharmaceuticals (Cambridge, UK). All other chemicals were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

4.2.2 Experimental design

Here, Scn1a\textsuperscript{+/-} were randomly divided into two groups and subcutaneously injected with either CBD (100 mg/kg twice daily; n=12) or its vehicle (ethanol: Kolliphor\textsuperscript{®}: 0.9% saline=2:1:17; n=29) from P8 onwards until P52 or death (whichever was earlier). Similarly, wild type (WT) littermate mice (n=11) were injected with vehicle for the entire period of the study. Given that a significant number of deaths (~60%) were predicted to occur between P20-P27 in vehicle-treated Scn1a\textsuperscript{+/-} a larger initial group size was utilised to obtain a minimum n=10 animals/group for behavioural assessment from P35 onwards. Of note, I accounted the possibility that CBD-treated animals might also die, therefore in the first run
equal group sizes were taken. However, the mortality was higher in the vehicle-treated
$Scn1a^{+/\text{-}}$ group than the CBD-treated $Scn1a^{+/\text{-}}$ group, so in subsequent runs, a greater number
of animals were included in the vehicle-treated $Scn1a^{+/\text{-}}$ group.
Male 129S-Scn1a<sup>tm1K<sub>e</sub>a/Mmjax</sup> heterozygote (Scn1a<sup>+/−</sup>) mice were crossed with female C57BL/6 WT (Scn1a<sup>+/+</sup>) mice to obtain Scn1a<sup>+/+</sup> (WT) and Scn1a<sup>+/−</sup> mice used in the study. Tissue collection (tail clippings) and genotyping was conducted in between postnatal day 2 (P2) and P4. These animals were randomised into three groups i.e. WT vehicle-treated (n=11), Scn1a<sup>+/−</sup> vehicle-treated (n=29) and Scn1a<sup>+/−</sup> CBD-treated (n=12). The animals were treated twice daily subcutaneously with either vehicle (ethanol: Kolliphor<sup>®</sup>: 0.9% saline=2:1:17) or CBD (100 mg/kg) from postnatal day 8 (P8) onwards until P52/death (whichever was earlier). Since a higher number of deaths were predicted from P20-27, an initial larger group size was considered for vehicle-treated Scn1a<sup>+/−</sup> mice to obtain a minimum n=10 animals/group for behavioural assessments such as social interaction (P35-37), sucrose preference (P39-P41), elevated plus maze (P42) and motor function and radial arm maze (P45-P52) tests.

Figure 4.1. Schematic representation of breeding and experimental design for this experiment. Male 129S-Scn1a<sup>tm1K<sub>e</sub>a/Mmjax</sup> heterozygote (Scn1a<sup>+/−</sup>) mice were crossed with female C57BL/6 WT (Scn1a<sup>+/+</sup>) mice to obtain Scn1a<sup>+/+</sup> (WT) and Scn1a<sup>+/−</sup> mice used in the study. Tissue collection (tail clippings) and genotyping was conducted in between postnatal day 2 (P2) and P4. These animals were randomised into three groups i.e. WT vehicle-treated (n=11), Scn1a<sup>+/−</sup> vehicle-treated (n=29) and Scn1a<sup>+/−</sup> CBD-treated (n=12). The animals were treated twice daily subcutaneously with either vehicle (ethanol: Kolliphor<sup>®</sup>: 0.9% saline=2:1:17) or CBD (100 mg/kg) from postnatal day 8 (P8) onwards until P52/death (whichever was earlier). Since a higher number of deaths were predicted from P20-27, an initial larger group size was considered for vehicle-treated Scn1a<sup>+/−</sup> mice to obtain a minimum n=10 animals/group for behavioural assessments such as social interaction (P35-37), sucrose preference (P39-P41), elevated plus maze (P42) and motor function and radial arm maze (P45-P52) tests.
4.2.3 Assessment of survivability

As seizure related deaths in this model were unpredictable, animals were video monitored continuously (24h×7days) throughout the study and any mortality observed was cross checked with the available video footage to confirm the reason of death.

4.2.4 Assessment of motor function

Fine motor control in animals were assessed by the accelerated rotarod and static beam tests. Animals were habituated to the stationary rotarod for 2 min a day for 2 days. In the accelerated rotarod test each mouse was placed individually on a linearly accelerating rod (4-40 rpm over 5 minutes; LE8500, Letica Scientific Instruments, UK) and average latency to fall from the rod (maximum 300 seconds) was calculated from 3 consecutive trials (2 min interval between trials).

The static beam task was further employed to analyse balance and coordination (Sedy et al., 2008), where the animals were required to walk along a cylindrical elevated beam (100 cm long, 0.9 cm diameter and 50 cm height from floor) and enter a dark enclosure at the beam end. The mice were habituated to the task for three consecutive days before the test day. Each day of the habituation period, the animals were placed 30, 60 and 100 cm away from the enclosure and allowed to traverse along the beam. On the test day, each mouse performed two consecutive trials (2 minutes interval between trials) with a maximum given time of 2 minutes to complete the task (the nose entering the box was taken as task completion). The test was video monitored (Sony DCR-SX21E) and blinded offline analysis was conducted (Observer XT 12, Noldus, The Netherlands) to evaluate the average number of foot slips made from two consecutive trials.
4.2.5 Assessment of gait

Gait test was conducted to assess the cerebellar function of the animals (Patel and Hillard, 2001). In this test, the hind paws of each mouse were marked with a non-toxic ink and the mouse was allowed to walk on a white paper (50×10 cm) placed on the floor of a custom-made plexiglass tunnel (50×10×10 cm). To obtain the left and right stride length, the distance between two ipsilateral paw prints was measured, whereas stride width was calculated from the distance between a foot print and its contralateral stride length at right angle (Wecker et al., 2013). The initial and last foot prints were not considered in measurements. All the animals were habituated to the test procedures and the apparatus for 2 days prior to the test. On the day of test, two trials were conducted for each animal to obtain mean stride length (left or right) and width for that animal.

4.2.6 Assessment of social interaction

The social interaction test was conducted in the home cage of test mouse to assess the social behaviour of the animals (Sato et al., 2013). On test day, cage mate(s) were removed from the home cage of the test mouse and the mouse remained in isolation for 15 minutes. A novel wild type mouse of same strain, same sex and similar weight to the test mouse was then introduced to the home cage of the test mouse. Activity was video recorded (Sony DCR-SX21E) for 10 minutes and the obtained video files were blinded at the end of all experiments. Time spent in active interactions (e.g. close following, sniffing, allogrooming/social grooming and mounting) and number of rearing (lifting the front paws on the air) occasions were coded offline using Observer XT 12 (Noldus, Netherlands). Aggressive behaviours were not considered as social interactions and were not coded. In this test, a reduced social interaction is considered as autistic like behaviour (Sato et al., 2013), while increased rearing occasions is sign of defensive escapes (Kaplan et al., 2017).
4.2.7 Assessment of anxiety-like behaviour

The elevated plus maze (EPM) test was performed to assess the level of anxiety in animals (Chen et al., 2017c). The wooden test apparatus consists of two closed arms (50×10×40 cm) and two open arms (50×10 cm) connected via a central platform (10×10 cm) and raised at a height of 50 cm above the floor. Each animal was placed on the central platform facing towards an open arm. Activity was video recorded (Swann SRDVR-16440H, UK) for 5 minutes. The video files were blinded and coded offline at the end of all experiments using Observer XT 12 (Noldus, Netherlands). Time spent on open arms was inversely related to the level of anxiety.

4.2.8 Assessment of depression-like behaviour

The sucrose preference test was carried out to assess the depression-like behaviour (Serova et al., 2017). The animals were separately housed during this test. Here, 24 hours before the test, animals were trained to drink from two bottles each containing 2% sucrose. On the first day of test, the animals were provided with a pre-weighed bottle of 2% sucrose and another containing a pre-weighed volume of tap water. The positions of the bottles were swapped after 24 hours to avoid any side preferences. After 48 hours, both bottles were weighed, and sucrose preference was calculated by using the following formula.

\[
\text{Sucrose preference (\%)} = \frac{\text{Sucrose consumption}}{\text{Sucrose consumption} + \text{Water consumption}} \times 100
\]

4.2.9 Assessment of cognition

An 8-arm radial maze (each arm 60×10 cm; raised at 50 cm above the floor) was used to assess the reference memory (RM) and working memory (WM) of the animals. On four consecutive days, animals were given two 10-minute sessions of habituation to the test apparatus and rules of the test, separated by a 90 min interval. During the first two days of habituation, food rewards (1/4 Cheerios®, Nestle) were randomly scattered on the floor of the
apparatus covering all arms and food-troughs at the end of each arm. On the 3rd and 4th habituation day, food rewards were placed only in food troughs of four randomly selected arms (fixed for each animal during the habituation and test day). Food was withdrawn 4-6 hours before the trial (both during habituation and test days) to motivate the animals to locate the rewards and thus perform the task. On the test day two trials of 10 min were conducted at 90 min interval and the activity of the animals were video recorded for offline blinded coding after the end of experiment. Entry to a non-baited arm was considered as a reference memory error (RME), whereas re-entry to a previously baited arm from which the food was already taken is considered as a working memory error (WME). The mean WME or RME were calculated from the two test trials.

4.2.10 Statistical analysis

All data were analysed in GraphPad Prism 6 software. The percentage of animals from the Scn1a+/− vehicle-treated and Scn1a+/− CBD-treated groups that survived until the end of the study (P52) were compared by Fisher’s exact test. Survival curves of Scn1a+/− vehicle-treated and Scn1a+/− CBD-treated group were compared using a Mantel-Cox test. Further, data obtained from the comorbidity assessment were checked for normality by D'Agostino & Pearson omnibus normality test. Data obtained from rotarod, gait, social interaction (active interaction), EPM, sucrose preference, RAM (RME) tests were normally distributed and the differences between the three groups were analysed by one-way ANOVA. If a significant difference was found then Holm-Sidak post hoc test was conducted between the WT vehicle-treated and Scn1a+/− vehicle-treated groups (to assess any disease related deterioration), and between Scn1a+/− vehicle-treated and Scn1a+/− CBD-treated groups (to evaluate the effect of CBD in improvement/deterioration of the disease condition). On the other hand, data obtained from static beam, social interaction (rearing occasions), RAM (WME) were found to be non-parametric, thus were analysed by Kruskal-Wallis test. Upon observing a significant
difference among the groups, the Dunn’s post hoc test was employed to assess the differences between WT vehicle-treated and Scn1a+/− vehicle-treated groups, and between Scn1a+/− vehicle-treated and Scn1a+/− CBD-treated groups. Multiple comparisons were corrected in all cases. Parametric data are presented in scattered dot plot in the figures and are expressed as mean ± SEM. Non-parametric data are presented in box plot in the figures and are expressed as median, min to max, and interquartile range (IQR). In all cases, p<0.05 is considered as the level of significance.

4.3 Results

4.3.1 CBD increased survivability of Scn1a+/− mice

The mortality rate was highest between P20-P27 in Scn1a+/− mice except for a single animal from the Scn1a+/− vehicle-treated group which died at P47. I reviewed the recorded video footages and confirmed that tonic-clonic seizures were the cause of death in all cases. Approximately 66% (19 of 29) Scn1a+/− vehicle-treated animals died before the completion of the study in contrast to only 17% (2 of 12) Scn1a+/− CBD-treated animals (Fisher’s exact test; p<0.0001) (Figure 4.2A). Further, survivability was significantly (Mantel-Cox test; X²=5.94; p<0.05; Figure 4.2B) less in Scn1a+/− vehicle-treated group (median survival 24 days) compared to the Scn1a+/− CBD-treated group (more than 50% animals survived until the end of the study so median survivability could not be assessed).
Figure 4.2. Plots showing chronic administration of CBD on survival of Scn1a<sup>+/−</sup> mice. **A.** Percentage of Scn1a<sup>+/−</sup> vehicle-treated and Scn1a<sup>+/−</sup> CBD-treated animals that survived until the completion of experiment (P52). A significantly higher number of CBD treated animals survived until the end of experiment compared to the Scn1a<sup>+/−</sup> vehicle-treated animals. Data were analysed by Fisher’s exact test; ****p<0.0001. **B.** Survival curve. CBD treatment significantly increased survival of Scn1a<sup>+/−</sup> mice compared to the vehicle-treated Scn1a<sup>+/−</sup> mice. Error bars are SEM; Data were analysed by Mantel-Cox test; p<0.05.

4.3.2 CBD did not exert any adverse effect on motor function

Motor function was assessed by both the accelerating rotarod and static beam test. In accelerating rotarod test, the mean total time spent on the moving rod by the WT vehicle-treated (n=11), Scn1a<sup>+/−</sup> vehicle-treated (n=11) and Scn1a<sup>+/−</sup> CBD-treated (n=10) were respectively 90.33 ± 2.00, 97.76 ± 5.41 and 91.27 ± 5.14 seconds. No significant difference in time spent on rod was observed among the groups (One-way ANOVA; F<sub>(2,29)</sub>=0.86; p=0.44; Figure 4.3A).
On the other hand, in static beam test, the median number of foot slips made by the WT vehicle-treated (n=11), Scn1a\textsuperscript{+/-} vehicle-treated (n=11) and Scn1a\textsuperscript{+/-} CBD-treated (n=10) were respectively 0.00, 1.00 and 0.50. Here, a significant difference in number of foot slips was found (Kruskal-Wallis test, H\textsubscript{(2)} = 10.67; p<0.01). Dunn’s post hoc test revealed that Scn1a\textsuperscript{+/-} vehicle-treated group made significantly (p<0.01) more foot slips compared to the WT vehicle-treated group, however no significant difference was observed in between Scn1a\textsuperscript{+/-} vehicle-treated and Scn1a\textsuperscript{+/-} CBD-treated groups (p>0.05; Figure 4.3B). A further comparison between the WT vehicle-treated and the Scn1a\textsuperscript{+/-} CBD-treated groups revealed no significant difference in number of foot slips between these two groups (p>0.05).
Figure 4.3. Dot plot, and box and whisker plot showing chronic administration of CBD to *Scn1a*+/− mice on motor function. A. Mean time (seconds) spent on accelerated rotarod by the wild type (WT) vehicle-treated (n=11), *Scn1a*+/− vehicle-treated (n=11) and *Scn1a*+/− CBD-treated (n=10) groups. No difference was observed among the groups. Data were analysed by one-way ANOVA and are expressed as mean ± SEM. B. Median number of foot slips made in static beam test. CBD treatment did not increase the number of foot slips by the *Scn1a*+/− mice compared to both the WT vehicle-treated and *Scn1a*+/− vehicle-treated mice. Data were analysed by Kruskal-Wallis test followed by Dunn’s *post hoc* test and are expressed as median, min to max, and interquartile range; **p<0.01.

### 4.3.3 CBD did not produce any gait abnormalities

The gait test was conducted to assess left stride length (LSL), right stride length (RSL) and stride width (SW) of the animals. The mean values of LSL (Figure 4.4A) of WT vehicle-treated (n=11), *Scn1a*+/− vehicle-treated (n=11) and *Scn1a*+/− CBD-treated (n=10)
animals were respectively 69.55 ± 1.39, 67.79 ± 1.33 and 70.51 ± 2.07 millimetres, whereas the mean values of RSL (Figure 4.4B) in these groups were respectively 70.42 ± 1.03, 67.58 ± 1.84 and 70.18 ± 2.16 millimetres. Further, SW (Figure 4.4C) in these groups were respectively 20.52 ± 0.55, 19.64 ± 0.62 and 21.29 ± 0.64 millimetres. In the gait test, no significant change was observed for left stride length (One-way ANOVA, F(2,29)=0.73; p=0.49), right stride length (One-way ANOVA, F(2,29)=0.86; p=0.44) and stride width (One-way ANOVA, F(2,29)=1.87; p=0.17) among the WT vehicle-treated, Scn1a⁺/⁻ vehicle-treated and Scn1a⁺/⁻ CBD-treated groups (Figure 4.4).

Figure 4.4. Dot plots showing chronic administration of CBD to Scn1a⁺/⁻ mice on gait. A. Left stride length (millimeters) B. Right stride length (millimeters) and C. Stride width of the wild type (WT) vehicle-treated (n=11), Scn1a⁺/⁻ vehicle-treated (n=11) and Scn1a⁺/⁻ CBD-treated (n=10) groups. CBD did not produce any adverse effect on any of these parameters. All the data were analysed by one-way ANOVA and are expressed as mean ± SEM.

4.3.4 CBD improved social behaviour of Scn1a⁺/⁻ mice

The social interaction test was conducted to assess the active social interaction and rearing behaviour exhibited in the home cage of the test animals.
The time spent on active interaction was significantly differed among the groups (One-way ANOVA; F(2,29)=13.58; p<0.0001). The Scn1a<sup>+/−</sup> vehicle-treated animals (39.52 ± 7.82 seconds; n=11) spent significantly less time in performing active interaction with the stranger mouse compared to both WT vehicle-treated (142.10 ± 20.39 seconds; n=11) and Scn1a<sup>+/−</sup> CBD-treated (138.00 ± 16.89 seconds; n=10) animals (Holm-Sidak’s post hoc test; p<0.001; Figure 4.5A).

Further, a significant difference in number of rearing events was observed among the groups (Kruskal-Wallis test, H(2)= 16.18; p<0.001) with Dunn’s post hoc test revealing a significantly higher number of rearing occasions for Scn1a<sup>+/−</sup> vehicle-treated animals (median 38.00; IQR=19.00-47.00) compared to both WT vehicle-treated (median 12.00; IQR=3.00-21.00; p<0.05) or Scn1a<sup>+/−</sup> CBD-treated (median 3.50; IQR=2.00-6.75; p<0.001) animals (Figure 4.5B).
Figure 4.5. Dot plot, and box and whisker plot showing the effect of chronic administration of CBD to Scn1a+/− mice on active social interaction and rearing behaviour. A. Time (second) spent on active interaction in social interaction (SI) test by the wild type (WT) vehicle-treated (n=11), Scn1a+/− vehicle-treated (n=11) and Scn1a+/− CBD-treated (n=10) groups. CBD significantly increased the active interaction time compared to the Scn1a+/− vehicle-treated group. The data were analysed by one-way ANOVA with Holm-Sidak’s post hoc test and are expressed as mean ± SEM; ***p<0.001. B. Rearing events made in social interaction test. CBD treatment significantly reduced the number of rearing compared to the Scn1a+/− vehicle-treated group. The data were analysed by Kruskal-Wallis test followed by Dunn’s multiple comparison test and are expressed as median, min to max, and interquartile range (IQR). *p<0.05; ***p<0.001.
Chapter 4

4.3.5 CBD reduced anxiety-like behaviour in *Scn1a*+/− mice

The anxiety of the animals was assessed by the amount of the time spent on the open arms of an elevated plus maze (EPM). The time spent on the open arms differs significantly among the groups (One-way ANOVA, \( F_{(2,28)}=5.11; p<0.05 \)). The *Scn1a*+/− vehicle-treated animals (11.17 ± 3.31 seconds; \( n=11 \)) spent significantly less time on the open arms compared to both *WT* vehicle-treated (83.62 ± 22.69 seconds; \( n=11 \)) and *Scn1a*+/− CBD-treated (93.90 ± 28.09 seconds) animals (Holm-Sidak’s *post hoc* test; \( p<0.05 \); Figure 4.6A).

4.3.6 CBD reduced depression-like behaviour in *Scn1a*+/− mice

Depression-like behaviour is inversely correlated with sucrose preference (Murray *et al.*, 2013). In the present study, sucrose preference differed significantly among the groups (One-way ANOVA, \( F_{(2,29)}=8.37; p<0.01 \)). A Holm-Sidak’s *post hoc* test showed that the *Scn1a*+/− vehicle-treated animals (67.93 ± 4.42 %; \( n=11 \)) had less preference to sucrose over water in comparison to both *WT* vehicle-treated (84.91 ± 2.02 %; \( n=11; p<0.01 \)) or *Scn1a*+/− CBD-treated (80.82 ± 2.05 %; \( n=10; p<0.05 \)) animals (Figure 4.6B).
Figure 4.6. Dot plots showing the effect of chronic administration of CBD to Scn1a<sup>+/−</sup> mice on anxiety-like and depression-like behaviours. A. Mean time (seconds) spent on open arms in Elevated Plus Maze (EPM) test by the wild type (WT) vehicle-treated (n=11), Scn1a<sup>+/−</sup> vehicle-treated (n=11) and Scn1a<sup>+/−</sup> CBD-treated (n=10) groups. CBD treatment significantly increased the time spent on the open arms of EPM compared to the Scn1a<sup>+/−</sup> vehicle-treated group. B. Mean sucrose preference (%) in sucrose preference (SP) test. CBD significantly increased the sucrose preference compared to the Scn1a<sup>+/−</sup> vehicle-treated group. The data were analysed by one-way ANOVA with Holm-Sidak’s post hoc test and are expressed as mean ± SEM. *p<0.05; **p<0.01.

4.3.7 CBD improved cognition in Scn1a<sup>+/−</sup> mice

The reference memory (RM) and working memory (WM) function in the animals were assessed using an eight-arm radial arm maze (RAM) test.
A significant difference (One-way ANOVA, $F_{(2,28)}=29.54; p<0.0001$) in the number of reference memory errors (RME) was observed among the groups. The $Scn1a^{+/}$ vehicle-treated group ($11.15 \pm 1.17; n=10$) made significantly more RME compared to both WT vehicle-treated ($4.09 \pm 0.53; n=11$) and $Scn1a^{+/}$ CBD-treated ($3.6 \pm 0.45; n=10$) groups (Holm-Sidak’s post hoc test; $p<0.0001$; Figure 4.7A).

Further, working memory errors (WME) were significantly different among the groups (Kruskal-Wallis test; $H_{(2)}=15.22; p<0.001$). The Dunn’s post hoc analysis revealed that $Scn1a^{+/}$ vehicle-treated group (median 2.5; IQR 1.50-14.00) made significantly more WME compared to both WT vehicle-treated (median 0.5; IQR 0.00-1.00; $p<0.01$) and $Scn1a^{+/}$ CBD-treated (median 0.25; IQR 0.00-1.00; $p<0.001$) groups (Figure 4.7B).
Figure 4.7. Dot plot, and box and whisker plot showing chronic administration of CBD to Scn1a+/− mice on spatial memory in 8-arm radial arm maze test (RAM). A. Median reference memory errors (RME) by the wild type (WT) vehicle-treated (n=11), Scn1a+/− vehicle-treated (n=10) and Scn1a+/− CBD-treated (n=10) groups. CBD treatment significantly reduced RME of Scn1a+/− mice compared to the Scn1a+/− vehicle-treated mice. The data were analysed by one-way ANOVA with Holm-Sidak’s post hoc test and are expressed as mean ± SEM; ****p<0.0001. B. Median working memory errors (WME). CBD significantly reduced WME of Scn1a+/− mice compared to the Scn1a+/− vehicle-treated group. Data were analysed by Kruskal-Wallis test followed by Dunn’s multiple comparison test and are expressed as median, min to max, and interquartile range (IQR); **p<0.01, ***p<0.001.
4.4 Discussion

In this experiment, the effect of chronic CBD treatment upon premature mortality and comorbidities such as motor dysfunction, social deficit, anxiety, depression and memory impairment associated with Dravet syndrome using the $Scn1a^{+/−}$ mouse model was investigated. This mouse model appropriately represents the human Dravet syndrome both genetically by its haploinsufficiency of Na\textsubscript{V}1.1 channel, and phenotypically by exhibiting the characteristics such as seizures, premature mortality and behavioural comorbidities such as social deficit, anxiety and memory dysfunction. associated with the disease. Here, I demonstrated for the first time that CBD treatment has beneficial effect on survivability and disease associated comorbidities in this model.

In the present study, chronic CBD treatment significantly increased the survivability of $Scn1a^{+/−}$ mice in comparison to their vehicle-treated counterparts: 66% of $Scn1a^{+/−}$ vehicle-treated (19 of 29) animals died before the completion of the study compared to just 17% those treated with CBD (2 of 12). Not only that, the median survivability of $Scn1a^{+/−}$ vehicle-treated animals was only 24 days. Because of this higher mortality in vehicle-treated $Scn1a^{+/−}$ group a larger initial group size had to be employed to attain a minimum $n=10$ animals/group for behavioural experiments at the later part of the study. I further confirmed from the video recorded data that tonic-clonic seizure was the reason for death in $Scn1a^{+/−}$ mice, thus a significantly lower mortality in CBD-treated group suggests that CBD provided significant protection against seizure related premature mortality. Although exploring the mechanism of anticonvulsant action of CBD is beyond the scope of this study, it is worth highlighting that a previous study on $Scn1a^{+/−}$ mice suggested that CBD exerts its anticonvulsant action through GPR55 receptor antagonism (Kaplan et al., 2017).
As previously mentioned, seizures and premature mortality are not the only concern in Dravet syndrome. Comorbidities such as motor deficits including abnormal gait is frequently reported in patients with Dravet syndrome (Aljaafari et al., 2017; Fasano et al., 2014; Rilstone et al., 2012; Gitiaux et al., 2016). Not only that, several AEDs such as phenobarbital, valproic acid, phenytoin and lacosamide also shown to have motor adverse effects in epileptic patients (Zaccara et al., 2004; Ristić et al., 2006; Zaccara et al., 2013; Bainbridge et al., 2017). In this study, vehicle-treated Scn1a+/− mice did not demonstrate any motor deficit on the accelerating rotarod. So, I assessed a different component of motor behaviour on static beam where the vehicle-treated Scn1a+/− mice exhibited motor deficits by making significantly more foot slips compared to their WT counterparts. Here, CBD treatment had no adverse effect on motor function in the Scn1a+/− mice. This result corroborates the previous finding that Scn1a+/− mice did not show any motor dysfunction in accelerated rotarod test following 3 days of CBD treatment (100 mg/kg; i.p.) (Kaplan et al., 2017). I further showed in the gait test that there was no difference in either stride length (left/right) or stride width among the groups. It is therefore clear that CBD was well-tolerated by Scn1a+/− mice, and no adverse effect was observed in gait or motor function.

In addition to motor disorders, Dravet syndrome is linked to several neuropsychiatric comorbidities. For example, social deficit, a common feature of autism is often observed in Dravet syndrome patients (Berkvens et al., 2015; Wolff et al., 2006; Li et al., 2011). Although a case study involving only three children with Dravet syndrome reported an improvement in autistic behaviour following treatment with valproate, clobazam, levetiracetam and oxcarbazepine (Chepure et al., 2018), larger studies involving more patients are lacking in the literature. Conversely, several clinical studies have reported autistic traits in children prenatally exposed to valproic acid (Rasalam et al., 2005; Moore et al., 2000). In this study, I investigated the effect of CBD on the social behaviour of the Scn1a+/−
mice. I observed that the vehicle-treated Scn1a\textsuperscript{+/-} mice had significant social deficits compared to their WT counterparts which is in line with the previous studies conducted in this animal model (Kaplan et al., 2017; Han et al., 2012). These authors hypothesised that an impairment of GABAergic neurotransmission might be the underlying mechanism behind the social deficits in these mice. Han et al demonstrated that a single dose (0.0625 mg/kg; i.p.) of clonazepam, a positive allosteric modulator of the GABA\textsubscript{A} receptor could significantly rescue the impaired social behaviour of the Scn1a\textsuperscript{+/-} mice when administered 30 minutes before the three-chamber social interaction test (Han et al., 2012). Further to this, Kaplan et al. showed that a single low dose of CBD (10-20 mg/kg, i.p.) also improved social behaviour of the Scn1a\textsuperscript{+/-} mice in the same test and they anticipated the role of CBD in GABAergic neuromodulation (Kaplan et al., 2017). However, the dose and frequency of administration of CBD applied in the above mentioned study is impracticable in Dravet syndrome therapy in clinics where patients receive multiple doses of CBD (Devinsky et al., 2017; Devinsky et al., 2018b). In the present study, the CBD-treated Scn1a\textsuperscript{+/-} mice spent significantly more time in active social interaction compared to their vehicle-treated counterparts. I therefore demonstrated that CBD improved the social behaviour of Scn1a\textsuperscript{+/-} mice after chronic administration using a dose (100 mg/kg, twice daily, s.c.) already shown to have an anticonvulsant effect in these mice (Kaplan et al., 2017). Apart from this model, the efficacy of CBD has also been demonstrated in attenuating \textDelta\textsuperscript{9}-tetrahydrocannabinol (Malone et al., 2009) and MK-801-induced (Gururajan et al., 2012) social deficits in rats. However, results obtained from behavioural assessment alone has its own limitation to explore the mechanism of action of CBD in the restoration of social deficits. A more detailed study using electrophysiological and molecular biological approach is therefore warranted to gain the molecular insight of CBD’s role in it.
In addition to impaired social behaviour, anxiety and depression are also major problems for patients with Dravet syndrome, severely impacting their quality of life (Wang et al., 2018; Jain et al., 2018; Chen et al., 2018). Although antiepileptic drugs such as valproate, lamotrigine, clobazam, gabapentin, oxcarbazepine etc have been reported to improve these behavioural comorbidities, several antiepileptic drugs like levetiracetam, topiramate, zonisamide etc progressively worsen these comorbidities (Chen et al., 2017a). The present study was therefore conducted to observe whether CBD has any effect on anxiety/depression associated with Dravet syndrome employing Scn1a+/− mouse model. The Scn1a+/− mice exhibits anxiety-like behaviour in elevated plus maze (EPM) and open field tests (Han et al., 2012). Here, I observed a similar effect in EPM test where the vehicle-treated Scn1a+/− mice showed anxiety-like behaviour by spending significantly less time on the open arm compared to the WT animals. Further, a significantly higher number of rearing behaviours exhibited by the Scn1a+/− mice compared to the WT animals in the social interaction test could be due to their higher level of anxiety that compelled them to escape from the test situation (Mines et al., 2010). Interestingly, CBD-treated Scn1a+/− mice spent significantly more time on the open arm of EPM compared to the vehicle-treated Scn1a+/− mice. This illustrates that chronic CBD treatment has anxiolytic potential in this mouse model of Dravet syndrome. In addition, the reduced rearing numbers in the CBD-treated Scn1a+/− mice as opposed to the vehicle-treated Scn1a+/− mice in the social interaction test might be attributed to an anxiolytic effect of CBD. This is the first study to suggest an anxiolytic effect of CBD in an epilepsy model. However, anxiolytic effects of CBD have previously been reported in a chronic unpredicted stress model in mice using both EPM and novelty supressed feeding test (Fogaca et al., 2018; Campos et al., 2013).

In the present study, the conventional sucrose preference test was employed to assess the depression-like behaviour in the Scn1a+/− mice (Serova et al., 2017). This test is based
upon the concept that depression-like behaviour in rodents is inversely correlated with their sucrose preference (Murray et al., 2013). Here, the vehicle-treated $Scn1a^{+/−}$ mice showed the typical depression-like behaviour i.e. a reduced preference to sucrose over water when compared to the WT mice. Remarkably, chronic CBD-treatment exerted antidepressant effect in the $Scn1a^{+/−}$ mice which exhibited an increased (normalised) sucrose preference in comparison with their vehicle-treated cohorts. The antidepressant action of CBD has previously been documented in genetic (Shoval et al., 2016), olfactory bulbectomy (Linge et al., 2016) and chronic unpredictable stress (Campos et al., 2013) models of depression. However, similar to the anxiolytic potential of CBD, its antidepressant effect has also never been studied in any epilepsy model, thus I am the first to demonstrate such effect of CBD in a mouse model of Dravet syndrome. Previous studies in naïve rodents have proposed that CBD exerts its antidepressant action via modulating the 5-HT neurotransmission or endocannabinoid signalling (Linge et al., 2016; Campos et al., 2013). Nevertheless, the underlying pathology of depression in epilepsy is complex and still unclear which necessitates a detailed mechanistic study to gain a better understanding on the antidepressant effect of CBD.

Cognitive deficit is also a frequently reported comorbidity in Dravet syndrome patients which immensely depreciate their quality of life (Olivieri et al., 2016; Acha et al., 2015; Villeneuve et al., 2014). Several domains of cognitive functions including visual attention, executive functions, and verbal, visual and working memories have found to be impaired in these patients (Pascalicchio et al., 2007; Acha et al., 2015; Roebling et al., 2009). Dravet syndrome patients also have significant intellectual deficits including very low intelligence quotient (IQ) which adversely affect their social life (Akiyama et al., 2010; Villeneuve et al., 2014). Furthermore, the existing antiepileptic drugs such as phenobarbital, phenytoin, topiramate etc. also possess negative impact on cognition in people with epilepsy (Chen et
Previously it has been established that the $Scn1a^{+/−}$ mice exhibit spatial memory deficit in both context dependent fear conditioning test and Barnes circular maze test (Han et al., 2012). Here it was observed that the vehicle-treated $Scn1a^{+/−}$ mice made significantly more reference and working memory errors compared to the WT mice in the radial arm maze (RAM) test. Therefore, in agreement with the previous study by Han et al, I showed that $Scn1a^{+/−}$ mice have spatial memory deficits. Several hypotheses have been proposed to explain the underlying pathology of memory impairment in epilepsy. Seizure is a major contributory factor for cognitive decline during developmental process (Khan et al., 2010; Ben-Ari and Holmes, 2006) and this appears to be true in the Dravet syndrome animal model where animals exhibit seizures from an early age. Further, seizure related disruption of neural plasticity, an important factor for memory formation has also been demonstrated in other animal model of epilepsies (Zhou et al., 2011; Schubert et al., 2005; Lenck-Santini and Scott, 2015). In contrast, a previous study demonstrated that the memory deficits in $Scn1a^{+/−}$ mice might be due to the attenuation of GABAergic neurotransmission and this could be rescued by clonazepam, a selective GABA$_A$ modulator (Han et al., 2012). Similarly, another study established that a selective knock-down of Nav1.1 channel at basal forebrain of rats resulted in memory deficits without manifestation of seizures, thus proving the concept that seizure is not the only contributory factor in memory loss exhibited by the $Scn1a^{+/−}$ mice (Bender et al., 2013). Moreover, removing cause of seizures does not always restore cognitive deficits in people with epilepsy (Helmaetdter et al., 2003). Interestingly in this study, CBD improved both the reference and working memory function of the $Scn1a^{+/−}$ mice compared to their vehicle-treated counterparts. A similar result was obtained in Chapter 2 where CBD improved working memory deficits in RISE-SRS rats. This is further consistent with previous reports of CBD restoring cognitive deficits associated with bile-duct ligation (Magen et al., 2010) and $Δ$9-
tetrahydrocannabinol (THC) administration (Murphy et al., 2017) in mice, and iron overloading in rats (Fagherazzi et al., 2012), indicating this effect is consistent in different models of epilepsy and across species. Furthermore, a caregiver-reported quality of life in childhood epilepsy (QOLCE) survey stated an improved memory function in the patients with refractory childhood epilepsy following CBD treatment (Rosenberg et al., 2017a). Nonetheless, the beneficial effect of CBD on memory function of Scn1a+/− mice was studied here for the first time. Although the present study illustrates the memory impairment associated with the disease and restoration of this deficit upon chronic CBD treatment, an additional, more comprehensive study is required to understand the molecular mechanisms behind CBD’s role in memory improvement in this model.

4.5 Conclusion

This study is the first to demonstrate that chronic administration of CBD prevents premature mortality and improves behavioural comorbidities associated with Dravet syndrome in Scn1a+/− mice without the detrimental effect on motor function that is otherwise seen with current pharmacotherapy. In light with the recent FDA approval of CBD, such highly promising results obtained from the present study undoubtedly increases the chance of a wider acceptance of Epidiolex® (GW Pharmaceuticals, UK) among the patients with Dravet syndrome.
Chapter 5: General discussion and conclusion
5.1 General Discussion

In this chapter, I will summarise the overall key findings of this project and discuss their potential implications in a wider therapeutic context. I will also briefly outline the future scope of research in this area.

The primary objective of this project was to investigate the effect of CBD treatment on seizures, premature mortality and comorbidities associated with epilepsy. This was achieved by evaluating CBD in three different models of epilepsy. The RISE-SRS rat model of TLE was employed to assess the effect of chronic CBD administration on seizures, motor function, gait and cognition (chapter 2). The Scn1a⁻/⁻ mouse model which recapitulates several symptoms of Dravet syndrome was utilised to find out the effect of long-term CBD treatment on survivability and several welfare parameters such as natural activity, reflex/response to touch, total neonatal welfare, orbital tightening and body condition (chapter 3). Finally, the effects of chronic CBD administration on premature mortality and comorbidities such as motor dysfunction, social deficits, anxiety, depression and cognitive impairment were assessed in the Scn1a⁺/⁻ mouse model of Dravet syndrome (chapter 4).

In this project, chronic CBD treatment was found to reduce seizures in TLE animals and prevented premature mortality in the Scn1a⁺/⁻ mice. A long-term CBD administration also increased survivability and improved the welfare parameters of the neonatal Scn1a⁺/⁻ mice. Further, CBD was shown to improve social deficit and reduced the anxiety-like and depression-like behaviours in the Scn1a⁺/⁻ mice. Most importantly, chronic CBD treatment improved the cognition and provided protection against the epilepsy induced motor deficit without producing any adverse effect on gait in both the preclinical models of TLE and Dravet syndrome. Overall, in this project, I have demonstrated the novel findings that CBD-treatment can prevent premature mortality, reduce seizures and improve comorbidities
associated with epilepsy. In the coming sections, the beneficial effects of CBD in each of these domains and its wider implications will be discussed.

Although the anticonvulsant property of CBD is well-known (Do Val-da Silva et al., 2017; Jones et al., 2012; Klein et al., 2017), its effectiveness against spontaneous recurrent seizures (SRS), the primary feature of epilepsy has never been evaluated in any preclinical models of epilepsy. In this project, I demonstrated for the first time that CBD can reduce the SRS and has the disease modifying effect in epilepsy. Additionally, this result provides a strong basis for clinical trials of CBD on other epilepsies (especially TLE) beyond Dravet Syndrome and Lennox Gastaut Syndrome, for which CBD (Epidiolex®, GW Pharmaceuticals) has already gained the US-FDA approval (FDA, 2018).

Another major concern in epilepsy is premature mortality (Dravet et al., 2005; Genton et al., 2011) and not a single study has assessed the effect of CBD in the prevention of premature mortality. Therefore, the present project is also first of its kind which demonstrates that CBD prevents premature mortality in epilepsy using the Scn1a+/− mouse model of Dravet syndrome. This effect of CBD could be attributed to its anticonvulsant action as all deaths were confirmed to be associated with seizures and the anticonvulsant action of CBD has already been established in the Scn1a+/− mice (Kaplan et al., 2017). Interestingly, previous studies have reported that the Scn1a+/− mice have decreased heart rate variability (HRV) (Kalume et al., 2013) and they may die due to a seizure-triggered ventricular fibrillation along with bradycardia at seizure offset (Auerbach et al., 2013; Kalume et al., 2013). Therefore, it is possible that CBD might have also played a cardioprotective role, in addition to its anticonvulsant property, to prevent the premature mortality in a severe form of epilepsy, such as Dravet syndrome. This hypothesis is supported by previous studies which demonstrated the cardioprotective effect of CBD against myocardial ischemic reperfusion injury in rats (Durst et al., 2007; Walsh et al., 2010) and type-I diabetic cardiomyopathy in
mice (Weiss et al., 2006; Rajesh et al., 2010). However, this needs to be further investigated to understand the precise mechanism by which CBD prevents premature mortality in epilepsy.

This project further established that CBD treatment improved the orbital tightening in the neonatal epileptic animals, which is a noteworthy finding as it suggests a possible analgesic property of CBD. The analgesic effect of CBD in various models of pain induced by nerve injury (De Gregorio et al., 2019), osteoarthritis (Philpott et al., 2017), surgical incision (Genaro et al., 2017), corneal injury (Thapa et al., 2018) and chemotherapy (King et al., 2017) have been demonstrated. Interestingly, I observed that CBD was well-tolerated in the healthy neonatal animals. Therefore, considering the safety profile of CBD and its potential analgesic effect in neonates, CBD could be a promising pain-killer for children. Nevertheless, in order to gain better understanding on CBD’s spectrum of analgesia in neonates, a well-designed study needs to be conducted on different validated pain models induced by repeated formalin injections, needle pricks or nerve injury (Bhutta et al., 2001; Anand et al., 1999; Gong et al., 2018).

The protective role of CBD to attenuate motor deficits in adult epileptic animals was also established. Further, no adverse effect on gait of epileptic animals was observed following chronic CBD treatment in both the models studied in this thesis. The excellent motor-tolerability of CBD indeed proves its superiority over other AEDs such as phenobarbital, valproic acid, phenytoin and lacosamide which produce adverse effects on motor function and gait in the patients with epilepsy (Zaccara et al., 2004; Ristić et al., 2006; Zaccara et al., 2013; Bainbridge et al., 2017). Again, this result advocates the potential application of CBD as a combinational antiepileptic therapy with the above-mentioned AEDs as it would allow lowering the dose of the latter and this ultimately may minimise the chance
of drug induced comorbidities. Moreover, motor disorder including tremor, rigidity and bradykinesia are the common features of Parkinson’s disease (Sethi, 2002). Thus, CBD might have the potential to protect against motor symptoms associated with Parkinson’s disease and other disorders of motor function such as Huntington’s disease. As mentioned in chapter 1, some studies have already shown that CBD reduced progressive degeneration of nigrostriatal dopaminergic neurons in rat model of Parkinson’s disease (Lastres-Becker et al., 2005; Garcia-Arencibia et al., 2007). Further, a survey conducted on Parkinson’s disease patients in the Czech Republic reported 39 of 85 cannabis users had an improvement in motor symptoms associated with Parkinson’s disease (Venderova et al., 2004). Additionally, a few small-scale clinical studies informed that CBD improved the psychotic symptoms and quality of life without causing any adverse effect on motor symptoms in Parkinson’s disease patients (Zuardi et al., 2009; Chagas et al., 2014a; Chagas et al., 2014b). However, a large-scale clinical trial is warranted to find out the effect of CBD on motor symptoms associated with Parkinson’s disease.

Besides the protective role of CBD on motor deficits, its effectiveness in several neuropsychiatric comorbidities associated with epilepsy, including social deficits, has been illustrated in this project using the Scn1a+/− mice. Although a previous study with a single low dose of CBD (20 mg/kg; i.p.) has been shown to restore the social impairment in these animals, it is therapeutically irrelevant in the context of epilepsy where patients receive multiple anticonvulsant doses of AED for a long time (Kaplan et al., 2017). In this project, for the first time chronic anticonvulsant doses of CBD have been demonstrated to improve social deficits associated with epilepsy. It is important to note that, in these experiments, CBD treatment was started at an early stage of the disease, which suggests that an early intervention could prevent the social impairment associated with epilepsy even with the higher anticonvulsant dose. Interestingly, social interaction deficit is a common trait in autism
Chapter 5

(APA, 2013), therefore the results also cast a new light on the possible role of CBD in the treatment of autism.

Anxiety and depression are the most commonly observed comorbidities across all forms of epilepsy (Wiglusz et al., 2012; Ottman et al., 2011; Tellez-Zenteno et al., 2007). However, the role of CBD on these two comorbidities has never been evaluated in any preclinical epilepsy models. This project is therefore also the first to demonstrate that CBD attenuates the anxiety-like and depression-like behaviours associated with epilepsy. Further, AED-induced depression and anxiety disorders are often seen in epilepsy patients (Jacoby et al., 2015; Gómez-Arias et al., 2012). Treatment with conventional AEDs such as levetiracetam, zonisamide and phenobarbital causes multiple psychiatric comorbidities including anxiety and depression in patients with epilepsy (White et al., 2010; Herranz et al., 1988; Weintraub et al., 2007). Given CBD’s apparent anxiolytic/antidepressant effects it is tempting to speculate that CBD may be used as an alternative or in combination with these AEDs not only to help reducing seizures but also to prevent the drug induced comorbidities. The anxiolytic and antidepressant effect of CBD has previously been demonstrated in rodents using genetic (Shoval et al., 2016), olfactory bulbectomy (Linge et al., 2016), chronic unpredictable stress (Campos et al., 2013) models of depression and repeated combination tests model of anxiety (Hsiao et al., 2012). Anxiolytic effect of CBD has also been reported in social phobia patients (Crippa et al., 2011; Bergamaschi et al., 2011). In agreement with these studies my results suggest a possible application of CBD in the treatment of generalised anxiety and depression associated with other diseases.

The present project further established cognitive deficits in two conventional preclinical models of epilepsy, which are in accordance with the reported cases of cognitive impairment in various types of epilepsy patients (Acha et al., 2015; Riikonen, 1996; Riccio and Vidrine,
Most importantly, I established another novel finding that CBD restored the epilepsy induced reference and working memory deficits in both TLE and Dravet syndrome. Therefore, it seems reasonable to speculate that the role of CBD in improvement of cognition is not restricted to a specific type of epilepsy, but it has the potential to restore memory deficit associated with other types of epilepsies. Moreover, several AEDs such as phenobarbital, phenytoin and topiramate aggravate the adverse effect upon cognition (Chen et al., 2001; Mei et al., 2006; Wandschneider et al., 2017), thus CBD may essentially reduce their adverse effects if used in combination or perhaps replace these in certain circumstances.

The beneficial effect of CBD on different types of memory have previously been described in several preclinical and clinical studies (Osborne and Solowij, 2017; Martin-Moreno et al., 2011; Cheng et al., 2014a; Hindocha et al., 2015). For example, CBD has been demonstrated to attenuate working memory deficit in prenatal infection model in rats (Osborne and Solowij, 2017) and hepatic encephalopathy model in mice (Avraham et al., 2011; Magen et al., 2010; Magen et al., 2009). Furthermore, CBD associated improvements in spatial memory were evidenced on Morris water maze test in Alzheimer’s disease (Martin-Moreno et al., 2011), cerebral malaria (Campos et al., 2015) and hypoxic brain injury (Schiavon et al., 2014) models in mice. As well as spatial memory, CBD has been reported to improve social recognition memory in rodents both in transgenic (Cheng et al., 2014a; Cheng et al., 2014b) and iron overload induced Alzheimer’s disease model (Fagherazzi et al., 2012). Interestingly, CBD has been demonstrated to improve Δ9-THC induced deficits in verbal and recognition memory (Morgan et al., 2012; Morgan et al., 2010; Hindocha et al., 2015), and working memory (Englund et al., 2013) in human volunteers. Therefore, in line with the previous studies my results suggest that CBD could be a potential candidate drug for the treatment of memory disorders.
Chapter 5

5.2 General Conclusion

Overall, I demonstrated the novel findings that CBD improves seizures, survivability and comorbidities associated with validated animal models of adult and childhood epilepsies. This is also the first study to establish that CBD has disease modifying role in both preclinical models of TLE and Dravet syndrome. Notably, the results of this project (Figure 5.1) contributed significantly towards the US-FDA approval of Epidiolex® (GW Pharmaceuticals) in 2018 (FDA, 2018). This project also highlights the further potential for CBD to reduce comorbidities associated with epilepsy and its current treatment. Taken together this project enhance the existing knowledge on CBD and its role in epilepsy.
**Figure 5.1.** Pictorial illustration of the overall findings of this project. Effect of CBD-treatment was investigated in RISE-SRS rat model of TLE, and *Scn1a*−/− and *Scn1a*+/− mouse models of Dravet syndrome. In RISE-SRS rat model of TLE, CBD reduced seizures, improved motor function and cognition without affecting the gait. Further, in *Scn1a*−/− mouse model, CBD extended survivability and improved several welfare parameters. Finally, in *Scn1a*+/− mouse model of Dravet syndrome, CBD reduced premature mortality, improved social behaviour and cognition, and reduced anxiety-like and depression-like behaviours without producing any motor adverse effects. These results formed a core part of the development and approval of Epidiolex® (GW Pharmaceuticals) in 2018.
5.3 Future scope of research

The primary objective of this PhD project was to evaluate the effect of CBD on seizures, premature mortality and comorbidities associated with epilepsy. I establish that CBD reduces premature mortality and improves seizures and associated comorbidities. Nonetheless, several questions remain unanswered which need to be investigated in future studies.

It has been demonstrated that CBD improved spontaneous seizures, but it is still not clear how CBD exerted this role. Inclusion of *in vivo* electrophysiology techniques in future studies with similar experimental design may provide us with more information on CBD’s role in modifying brain network activities in the epileptic animals. For example, electrocorticography (ECoG) or EEG could be conducted in the *Scn1a*+/− mouse model of Dravet syndrome during P21-27 (peak mortality period) to demonstrate that CBD can reduce the epileptiform activity in *Scn1a*+/− mice. Of note, I have collected and preserved the brain tissues from each of my experiments for future use. In order to gain a better understanding of the molecular mechanism of actions of CBD, these tissues could be utilised for immunohistochemistry and Western blot analysis to map the distribution, expression and the intracellular signalling pathways of several important receptor proteins such as GPR55, PPARγ and adenosine A2A that have previously been reported to be involved in CBD’s antiepileptogenic and anticonvulsant action (O’Sullivan *et al.*, 2009; Ryberg *et al.*, 2007). It is to be mentioned that some of the blood and brain tissue samples derived from this project have been used to implicate the 1 carbon cycle in the mechanism of action of CBD and this work has currently been submitted to the journal *PNAS* (Perry *et al.*, unpublished work).

Here in this project, CBD prevented the premature mortality in epileptic animals possibly due to its well-known anticonvulsant effect. To further investigate, whether this
preventive effect of CBD could also be attributed to its cardioprotective action, electrocardiography (ECG) may be conducted in a similar experimental set up alongside already proposed EEG.

Although this project establish that CBD improves the comorbidities associated with epilepsy, it suffers from some limitations due to the lack of mechanistic insights which needs to be explored in future. Previous studies on healthy animals have shown that CBD exerts its anxiolytic and antidepressant effects by enhancing the 5-HT$_{1A}$ signalling (Campos and Guimaraes, 2008; Soares Vde et al., 2010), therefore in future studies (involving similar protocol as used in my project) 5-HT$_{1A}$ receptor antagonist like WAY-100635 could be administered before the relevant behavioural tasks. An absence of beneficial effect on these parameters will indicate that CBD acts on 5-HT$_{1A}$ receptors to demonstrate its anxiolytic and antidepressant effects in epilepsy. Further, it has been proposed that CBD’s anti-inflammatory and neuroprotective action play important role in memory protection in Alzheimer’s disease (Esposito et al., 2007; Esposito et al., 2011). Therefore, the expression of inflammatory mediators such as iNOS, IL-1β, GFAP and PPARγ can be evaluated from the preserved brain tissues by both immunohistochemistry and Western blot analysis to shed light on CBD’s role in attenuating epilepsy-induced memory deficits.

I demonstrated CBD’s beneficial role only in spatial memory, therefore future studies are recommended to explore whether CBD can also improve other types of memories such as fear and recognition memories. This could be studied by fear conditioning (Kemppainen et al., 2006) and novel object recognition tests (Pearson et al., 2014) using the same experimental paradigm used in the present project. Further, it might be interesting to see whether CBD acts on the acquisition and/or retrieval step of the memory process using specially designed memory tasks.
In this project, the animals were chronically treated with CBD until the end of experiment. This approach allowed us to explore the disease modifying effect of CBD, however inclusion of a washout period for CBD at the end of experiment (instead of sacrificing them) followed by repeating the behavioural tasks would allow us to gain insight into the possible curative role of CBD against the comorbidities and further demonstrate that if the effect is truly disease modifying or not.

Finally, having observed the positive effects of CBD on motor and psychiatric comorbidities, future studies to assess the role of CBD on several other neurological and psychiatric disorders such as Parkinson’s disease, Alzheimer’s disease, generalised anxiety disorders, depression and autism would also be interesting.


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Appendix-I

(Seizure vs memory correlation)
Figure I. Dot plot showing the correlation of seizure burden with memory function. No correlation was found between seizure burden and A. reference memory function in epileptic vehicle-treated animals. B. working memory function in epileptic vehicle-treated animals. C. reference memory function in epileptic CBD-treated animals. D. working memory function in epileptic CBD-treated animals. n=8/group; data were analysed by linear regression.
Appendix-II
(Survivability model script)
function varargout = EndpointGui2(varargin)

gui_Singleton = 1;

gui_State = struct('gui_Name', mfilename, ...
    'gui_Singleton', gui_Singleton, ...
    'gui_OpeningFcn', @EndpointGui2_OpeningFcn, ...
    'gui_OutputFcn', @EndpointGui2_OutputFcn, ...
    'gui_LayoutFcn', [], ...
    'gui_Callback', []);

if nargin && ischar(varargin{1})
    gui_State.gui_Callback = str2func(varargin{1});
end

if nargout
    varargout{1:nargout} = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end

% End initialization code - DO NOT EDIT

% --- Executes just before EndpointGui2 is made visible.
function EndpointGui2_OpeningFcn(hObject, eventdata, handles, varargin)
    handles.output = hObject;
    guidata(hObject, handles);

    function varargout = EndpointGui2_OpeningFcn(hObject, eventdata, handles)
    varargout{1} = handles.output;

% % % _________________________________________Objects_____________________________

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Survivability model script

function edit1_Callback(hObject, eventdata, handles)
function edit1_CreateFcn(hObject, eventdata, handles)
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
set(hObject,'BackgroundColor','white');
end

function edit2_Callback(hObject, eventdata, handles)
function edit2_CreateFcn(hObject, eventdata, handles)
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
set(hObject,'BackgroundColor','white');
end

function edit3_Callback(hObject, eventdata, handles)
function edit3_CreateFcn(hObject, eventdata, handles)
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
set(hObject,'BackgroundColor','white');
end

function NWSedit_Callback(hObject, eventdata, handles)
function NWSedit_CreateFcn(hObject, eventdata, handles)
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
set(hObject,'BackgroundColor','white');
end

function NAedit_Callback(hObject, eventdata, handles)
function NAedit_CreateFcn(hObject, eventdata, handles)
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
set(hObject,'BackgroundColor','white');
end
Survivability model script

set(hObject,'BackgroundColor','white');
end

function RTedit_Callback(hObject, eventdata, handles)
function RTedit_CreateFcn(hObject, eventdata, handles)
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
set(hObject,'BackgroundColor','white');
end

function OTedit_Callback(hObject, eventdata, handles)
function OTedit_CreateFcn(hObject, eventdata, handles)
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
set(hObject,'BackgroundColor','white');
end

function BCSedit_Callback(hObject, eventdata, handles)
function BCSedit_CreateFcn(hObject, eventdata, handles)
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
set(hObject,'BackgroundColor','white');
end

function Weightcrit_Callback(hObject, eventdata, handles)
function Weightcrit_CreateFcn(hObject, eventdata, handles)
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
set(hObject,'BackgroundColor','white');
end

function BCS_checkbox_Callback(hObject, eventdata, handles)
function OT_checkbox_Callback(hObject, eventdata, handles)
function RT_checkbox_Callback(hObject, eventdata, handles)
function NA_checkbox_Callback(hObject, eventdata, handles)
function NWS_checkbox_Callback(hObject, eventdata, handles)
function Weight_checkbox_Callback(hObject, eventdata, handles)
function SurfTemp_checkbox_Callback(hObject, eventdata, handles)

%%% _________________Loading parameters___________________________

%%% 1. Loading file

function Load_File_Callback(hObject, eventdata, handles)
function Load_xl_file_Callback(hObject, eventdata, handles)
    %uiope
    [FileName,FilePath] = uigetfile('*.xls','File Selector');
    global Param

    try
        cd(FilePath)
        Param = xlsread(FileName);
    catch
        disp(strcat('you have to select a file or it does not work'))
    end

%%% _________________Build model_________________________________

function Go1_Callback(hObject, eventdata, handles)

%%% 1. Averaging values

    global Param
global DeadAnimalNb
global DeadAnimalind

ind = find(Param(:,11) == 1);
DeadAnimalind = [];
for i = 1:length(ind)
    DeadAnimalind = cat(1,DeadAnimalind,Param(ind(i),1));
end

DeadAnimalNb = length(DeadAnimalind);

AvParam = Param;

for i = 1:max(Param(:,1))
    ind = find(Param(:,1) == i);
    for j = 3:8
        for k = 3:length(ind)-1
            AvParam(ind(k),j) = mean(Param(ind(k)-2:ind(k),j));
        end
    end
end

%% 2.Death Day
DeathDay = [];
for i = 1:DeadAnimalNb
    ind = find(Param(:,1) == DeadAnimalind(i));
    DeathDay = cat(2,DeathDay,max(Param(ind,2)));
end

%% 3.Death delay calculation
Survivability model script

DayBefDeath = [];
AnimalN = 0;
for i = 1:max(Param(:,1))

    if isempty(find(DeadAnimalind(:) == i))
        ind = find(Param(:,1) == i);
        for j = 1:length(ind)
            DayBefDeath = cat(1,DayBefDeath,NaN);
        end
    else
        AnimalN = AnimalN + 1;
        ind = find(Param(:,1) == i);
        for j = 1:length(ind)
            DayBefDeath = cat(1,DayBefDeath,DeathDay(1,AnimalN)-Param(ind(j),2));
        end
    end
end

global Param2
Param2 = cat(2,Param(:,1),DayBefDeath,AvParam(:,4:8),Param(:,3),Param(:,9));

%% 3.Criterions calculation

CritMatrix = [];

global DelayParam
DelayParam = str2double(get(handles.edit2,'string'));
ind = find(Param2(:,2) == DelayParam);

global NWScrit
NWScrit = min(Param2(ind,3));

%disp(strcat('NWS criterion >= ',num2str(NWScrit)))
%set(handles.NWSedit,'string',num2str(NWScrit))

CritMatrix = cat(2,CritMatrix,Param2(ind,3));

global NAcrit
NAcrit = min(Param2(ind,4));

%disp(strcat('NA criterion >= ',num2str(NAcrit)))
%set(handles.NAedit,'string',num2str(NAcrit))

CritMatrix = cat(2,CritMatrix,Param2(ind,4));

global RTcrit
RTcrit = min(Param2(ind,5));

%disp(strcat('RT criterion >= ',num2str(RTcrit)))
%set(handles.RTedit,'string',num2str(RTcrit))

CritMatrix = cat(2,CritMatrix,Param2(ind,5));

global OTcrit
OTcrit = min(Param2(ind,6));

%disp(strcat('OT criterion >= ',num2str(OTcrit)))
%set(handles.OTedit,'string',num2str(OTcrit))

CritMatrix = cat(2,CritMatrix,Param2(ind,6));

global BCScrit
BCScrit = max(Param2(ind,7));

%disp(strcat('BCS criterion <= ',num2str(BCScrit)))
%set(handles.BCSedit,'string',num2str(BCScrit))

CritMatrix = cat(2,CritMatrix,Param2(ind,7));

global Weightcrit
Survivability model script

Weightcrit = max(Param2(ind,8));

%disp(strcat('Weight criterion <= ',num2str(Weightcrit)))
%set(handles.Weightcrit,'string',num2str(Weightcrit))

CritMatrix = cat(2,CritMatrix,Param2(ind,8));

global critVec

critVec = cat(1,NWScrit,NAcrit,RTcrit,OTcrit,BCScrit,Weightcrit);

CritDev = [];
for i = 1:6
    if i <= 4
        CritDev = cat(2,CritDev,critVec(i)-nanstd(CritMatrix(:,i)));
    else
        CritDev = cat(2,CritDev,critVec(i)+nanstd(CritMatrix(:,i)));
    end
end

NWScrit = CritDev(1);
set(handles.NWSedit,'string',num2str(CritDev(1)))

NAcrit = CritDev(2);
set(handles.NAedit,'string',num2str(CritDev(2)))

RTcrit = CritDev(3);
set(handles.RTedit,'string',num2str(CritDev(3)))

OTcrit = CritDev(4);
set(handles.OTedit,'string',num2str(CritDev(4)))

BCScrit = CritDev(5);
set(handles.BCSedit,'string',num2str(CritDev(5)))

Weightcrit = CritDev(6);
set(handles.Weightcrit,'string',num2str(CritDev(6)))


% 4. Death delay calculation
Survivability model script

NWSCheck = get(handles.NWS_checkbox,'value');
NACheck = get(handles.NA_checkbox,'value');
RTCheck = get(handles.RT_checkbox,'value');
OTCheck = get(handles.OT_checkbox,'value');
BCSCheck = get(handles.BCS_checkbox,'value');
WeightCheck = get(handles.Weight_checkbox,'value');
SurfCheck = get(handles.SurfTemp_checkbox,'value');

Vec = cat(1,NWSCheck,NACheck,RTCheck,OTCheck,BCSCheck,WeightCheck);
CheckVec = [];
for i = 1:length(Vec)
    if Vec(i) == 1
        CheckVec = cat(1,CheckVec,i);
    end
end

Results = [];
AnimalVec = [];
for i = 1:DeadAnimalNb
    AnimalVec = cat(1,AnimalVec,DeadAnimalind(i));
    ind = find(Param2(:,1) == DeadAnimalind(i) & Param2(:,2) <= 8);
    SurfTempValue = 0;
    for j = 1:length(ind)
        TempValue = 0;
        if SurfCheck == 1
            SurfTempValue = SurfTempValue + Param2(ind(j),9);
            if SurfTempValue >= 2
                disp(strcat('Animal nb :',num2str(DeadAnimalind(i)),'-- death delay is :',num2str(Param2(ind(j),2))))
            end
        end
    end
end
disp(strcat('Animal nb :',num2str(DeadAnimalind(i)),'-- body surface temp >= 2'))

Results = cat(1,Results,Param2(ind(j),2));
break
end
end

if j < length(ind)

for k = 1:length(CheckVec)
    if CheckVec(k) == 1 | CheckVec(k) == 2 | CheckVec(k) == 3 | CheckVec(k) == 4
        if max(Param2(ind(1):ind(j),CheckVec(k)+2)) >= critVec(CheckVec(k))
            TempValue = TempValue + 1;
        end
    elseif CheckVec(k) == 5 | CheckVec(k) == 6
        if min(Param2(ind(1):ind(j),CheckVec(k)+2)) <= critVec(CheckVec(k))
            TempValue = TempValue + 1;
        end
    end
end

if TempValue == length(CheckVec)
    disp(strcat('Animal nb :',num2str(DeadAnimalind(i)),'-- death delay is
        Results = cat(1,Results,Param2(ind(j),2));
break
end
elseif j == length(ind)

for k = 1:length(CheckVec)
    if CheckVec(k) == 1 | CheckVec(k) == 2 | CheckVec(k) == 3 | CheckVec(k) == 4

if max(Param2(ind(1):ind(j),CheckVec(k)+2)) >= critVec(CheckVec(k))
    TempValue = TempValue + 1;
end
elseif CheckVec(k) == 5 | CheckVec(k) == 6
    if min(Param2(ind(1):ind(j),CheckVec(k)+2)) <= critVec(CheckVec(k))
        TempValue = TempValue + 1;
    end
end

if TempValue == length(CheckVec)
    disp(strcat('Animal nb :',num2str(DeadAnimalind(i)),'--> death delay is :
    Results = cat(1,Results,Param2(ind(j),2));
    break
else
    disp(strcat('Animal nb :',num2str(DeadAnimalind(i)),'--> welfare scores are still
    Results = cat(1,Results,0);
    break
end
end
end
end

set(handles.edit1,'string',num2str(cat(2,AnimalVec,Results)))

function Go2_Callback(hObject, eventdata, handles)
global Param2

global DeadAnimalInd

global critVec

NWSCheck = get(handles.NWS_checkbox,'value');
NACheck = get(handles.NA_checkbox,'value');
RTCheck = get(handles.RT_checkbox,'value');
OTCheck = get(handles.OT_checkbox,'value');
BCSCheck = get(handles.BCS_checkbox,'value');
WeightCheck = get(handles.Weight_checkbox,'value');
SurfCheck = get(handles.SurfTemp_checkbox,'value');

Vec = cat(1,NWSCheck,NACheck,RTCheck,OTCheck,BCSCheck,WeightCheck);
CheckVec = [];
for i = 1:length(Vec)
    if Vec(i) == 1
        CheckVec = cat(1,CheckVec,i);
    end
end

global NWScrit

global NAcrit

global RTcrit

global OTcrit

global BCScrit

global Weightcrit

critVec = cat(1,NWScrit,NAcrit,RTcrit,OTcrit,BCScrit,Weightcrit);
AliveAnimalind = [];
for i = 1:max(Param2(:,1))
    if isempty(find(DeadAnimalind(:) == i))
        AliveAnimalind = cat(1,AliveAnimalind,i);
    end
end

Results = [];
AnimalVec = [];
for i = 1:length(AliveAnimalind)
    AnimalVec = cat(1,AnimalVec,AliveAnimalind(i));
    ind = find(Param2(:,1) == AliveAnimalind(i));
    if length(ind) < 17
        disp(strcat('Animal nb :',num2str(AliveAnimalind(i)),'---> welfare scores are still satisfactory'));
        Results = cat(1,Results,0);
    else
        if SurfCheck == 1
            SurfTempValue = sum(Param2(ind(16):ind(end),9) > 0);
            if SurfTempValue >= 3
                disp(strcat('Animal nb :',num2str(AliveAnimalind(i)),'---> body surface temp >= 3'))
                disp(strcat('Animal nb :',num2str(AliveAnimalind(i)),'---> needs to be killed ASAP'))
                Results = cat(1,Results,1);
            else
                TempValue = 0;
                for k = 1:length(CheckVec)
if CheckVec(k) == 1 | CheckVec(k) == 2 | CheckVec(k) == 3 | CheckVec(k) == 4

    if max(Param2(ind(16):ind(end),CheckVec(k)+2)) >= critVec(CheckVec(k))
        TempValue = TempValue + 1;
    end
elseif CheckVec(k) == 5 | CheckVec(k) == 6
    if min(Param2(ind(16):ind(end),CheckVec(k)+2)) <= critVec(CheckVec(k))
        TempValue = TempValue + 1;
    end
end
end

if TempValue == length(CheckVec)
    disp(strcat('Animal nb : ',num2str(AliveAnimalind(i)),'--> needs to be killed'))
    Results = cat(1,Results,1);
else
    disp(strcat('Animal nb : ',num2str(AliveAnimalind(i)),'--> welfare scores are still satisfactory'))
    Results = cat(1,Results,0);
end
end

else
    TempValue = 0;
    for k = 1:length(CheckVec)
        if CheckVec(k) == 1 | CheckVec(k) == 2 | CheckVec(k) == 3 | CheckVec(k) == 4
            if max(Param2(ind(16):ind(end),CheckVec(k)+2)) >= critVec(CheckVec(k))
                TempValue = TempValue + 1;
            end
        elseif CheckVec(k) == 5 | CheckVec(k) == 6
            if min(Param2(ind(16):ind(end),CheckVec(k)+2)) <= critVec(CheckVec(k))
TempValue = TempValue + 1;
end
end
end

if TempValue == length(CheckVec)
    disp(strcat('Animal nb :',num2str(AliveAnimalInd(i)),' --> needs to be killed'))
    Results = cat(1,Results,1);
else
    disp(strcat('Animal nb :',num2str(AliveAnimalInd(i)),' --> welfare scores are still satisfactory'))
    Results = cat(1,Results,0);
end
end
end

set(handles.edit3,'string',num2str(cat(2,AnimalVec,Results))
Appendix-III

(Published journal articles)
Cannabidiol reduces seizures and associated behavioral comorbidities in a range of animal seizure and epilepsy models

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Summary

Objective: Epilepsy is a progressive neurological disease characterized by recurrent seizures and behavioral comorbidities. We investigated the antiseizure effect of cannabidiol (CBD) in a battery of acute seizure models. Additionally, we defined the disease-modifying potential of chronic oral administration of CBD on associated comorbidities in the reduced intensity status epilepticus–spontaneous recurrent seizures (RISE-SRS) model of temporal lobe epilepsy (TLE).

Methods: We evaluated the acute antiseizure effect of CBD in the maximal electroshock seizure, 6-Hz psychomotor seizure, and pentylentetrazol acute seizure tests, as well as the corneal kindling model of chronic seizures in mice following intraperitoneal administration. Median effective or behavioral toxic dose was determined in both mice and rats. Next, we tested an intravenous preparation of CBD (10 mg/kg single dose) in a rat model of pilocarpine-induced status epilepticus. We defined the effect of chronic CBD administration (200 mg/kg orally) on spontaneous seizures, motor control, gait, and memory function in the rat RISE-SRS model of TLE.

Results: CBD was effective in a battery of acute seizure models in both mice and rats following intraperitoneal administration. In the pilocarpine-induced status epilepticus rat model, CBD attenuated maximum seizure severity following intravenous administration, further demonstrating CBD’s acute antiseizure efficacy in this rat model. We established that oral CBD attenuated the time-dependent increase in seizure burden and improved TLE-associated motor comorbidities of epileptic rats in the RISE-SRS model without affecting gait. Chronic administration of CBD after the onset of SRS ameliorated reference memory and working memory errors of epileptic animals in a spatial learning and memory task.

Significance: The present study illustrates that CBD is a well-tolerated and effective antiseizure agent and illustrates a potential disease-modifying effect of CBD on reducing both seizure burden and associated comorbidities well after the onset of symptomatic seizures in a model of TLE.

Williams and McNeish contributed equally.

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1 | INTRODUCTION

Epilepsy is a progressive, chronic neurological disorder characterized by recurrent seizures. Approximately 65 million people worldwide live with epilepsy, of whom ~30% are considered pharmacoresistant to the currently available anti-seizure drugs (ASDs). Seizures, although the primary symptom, are not the only aspect of epilepsy that affects a patient’s quality of life. Several comorbidities (eg, depression, anxiety, motor disorder, cognitive deficits, social dysfunction) also contribute to a reduced quality of life in addition to the poor prognosis associated with the disease. Furthermore, currently available ASDs are also known to produce a variety of cognitive, psychiatric, and motor adverse effects; thus, therapies that do not carry the potential to increase the adverse effects liability are in significant clinical demand.

Cannabis has been used since prehistoric times to treat several diseases, including epilepsy. However, the therapeutic benefits of whole cannabis are overshadowed by its psychoactive effects, which have limited its clinical use. More than 100 phytocannabinoids have been isolated, of which Δ⁹-tetrahydrocannabinol, cannabidiol (CBD), and cannabidivarin are considered most relevant in the treatment of epilepsy; however, Δ⁹-tetrahydrocannabinol has poor clinical potential due to its psychoactive and potential proconvulsive properties, which may limit chronic use.

CBD has been shown to have antiseizure activity in several animal models. Given its anticonvulsant efficacy in phase 3 clinical trials, the US Food and Drug Administration (FDA) in 2018 approved CBD (Epidiolex; GW Research) as a drug for the treatment of seizures associated with Dravet syndrome or Lennox-Gastaut syndrome in patients 2 years of age and older.

Here, we initially tested CBD in a battery of well-established preclinical seizure and epilepsy models following intraperitoneal administration to define CBD’s pharmacological profile and differentiate it from other ASD standards of care. Second, we investigated the efficacy of intravenous (IV) pretreatment with CBD in the rat pilocarpine-induced status epilepticus (SE) model. SE is one of the most common medical emergencies in patients with epilepsy, and is clinically defined as a seizure lasting >5 minutes or repetitive seizures within this time frame without regaining consciousness. The pilocarpine-induced SE model is typically sensitive to most ASDs when they are administered prior to or commensurate with SE onset; nonetheless, this model is useful to interrogate pharmacological efficacy in a severe seizure model. Finally, there is little information on the potential of CBD to modify epilepsy-related behavioral comorbidities despite its preclinical and clinical ability to provide acute seizure control.

Furthermore, no preclinical study has yet shown that sustained exposure to CBD not only reduces seizure burden in a temporal lobe epilepsy (TLE) model but can also attenuate the severity of the associated behavioral comorbidities. Therefore, as a final step we assessed the effects of chronic oral administration of CBD on spontaneous seizures and associated behavioral comorbidities in the newly developed reduced intensity SE–induced spontaneous recurrent seizures (RISE-SRS) model. The RISE-SRS model exhibits a similar disease progression and pathology as the traditional post-SE rat models, but with reduced mortality during the immediate post-SE period. This model provides a platform for the conduct of long-duration disease modification studies with candidate investigational therapies. Of note, the present study utilized a clinically relevant treatment design, as animals were only enrolled to receive CBD well after the onset of SRS. The results of the present study indicate that CBD exerts acute antiseizure efficacy by multiple routes of administration in several well-validated preclinical seizure and epilepsy models with a preclinical profile that is different from other prototype ASDs. Moreover, this present study provides the first demonstration in a preclinical model of TLE to suggest that CBD may exert potential disease-modifying effects on SRS and attendant behavioral comorbidities.

Key Points

- CBD is effective in a battery of acute seizure models following intraperitoneal administration
- CBD is effective in attenuating maximum seizure severity following intravenous administration in rats
- Oral CBD can attenuate time-dependent increase in seizure burden and motor comorbidities in a rat model of TLE
- Oral CBD can reverse epilepsy-induced cognitive deficits in a rat model of TLE
- This is the first study to demonstrate the disease-modifying effect of CBD on spontaneous recurrent seizure and associated comorbidities

**KEYWORDS**

cannabinoids, gait, maximal electroshock, memory, motor function, pilocarpine
2 | MATERIALS AND METHODS

All materials and methods are described in online Appendix S1.

3 | RESULTS

3.1 | CBD demonstrates acute antiseizure efficacy following intraperitoneal administration in a battery of well-established acute rodent seizure models

CBD (intraperitoneal [IP]) was initially evaluated for acute antiseizure efficacy in a battery of well-defined rodent seizure and epilepsy models (Table 1). These models have formed the basis for ASD discovery for decades\(^{17,18}\) and were thus employed to initially define the acute antiseizure efficacy of CBD relative to standard ASDs.\(^{17}\) For the purposes of the present study, we have also included the efficacy data for the ASDs phenobarbital (PB), valproic acid (VPA), and felbamate (FBM) as reported in the National Institute of Neurological Disorders and Stroke public database PANACHe (Table 1). The rotarod test was used to determine the potential for CBD to induce minimal motor impairment in mice and to calculate a median behaviorally impairing dose (TD50). Male mice were found to be impaired in their ability to perform on the rotarod test at a TD50 of 272 mg/kg (95% confidence interval [CI] = 241-303) when it was administered IP 1 hour prior to testing. In the antiseizure tests in mice, IP administration of CBD prior to electrical stimulation was found to block tonic extension seizures induced by maximal electroshock seizure (MES) in male mice with a median effective dose (ED50) of 80.0 mg/kg (95% CI = 65.5-96.0), yielding a protective index (PI; TD50/ED50) of 3.4. CBD was also effective in male mice against clonic seizures induced by subcutaneous administration of pentylenetetrazol when administered 1 hour prior to testing (ED50 = 120 mg/kg IP, PI = 2.3). CBD was found to protect male mice against the 6-Hz partial psychomotor seizure at two different currents following IP administration. With a 32-mA stimulation delivered 1 hour after drug administration, CBD had an ED50 of 144 mg/kg, yielding a PI for this test of 1.9. Importantly, CBD was also found to be effective and retained its potency at the 44-mA stimulation current in the 6-Hz test when administered 1 hour prior to electrical stimulation; ED50 at this current and time point of 173 mg/kg (PI = 1.6). Additionally, in male corneal kindled mice, the ED50 of CBD was determined to be 144 mg/kg (PI = 1.9). Thus, CBD demonstrates broad antiseizure efficacy in several well-validated mouse models of acute (MES, subcutaneous pentylenetetrazol, 6 Hz) and chronic (corneal kindled) seizures at doses well below the motor-impairing dose.

In male rats, visual evaluation of minimal motor impairment was scored by a trained investigator to determine the adverse effects of CBD on behavioral performance. Naive male rats were not found to be impaired on this test following administration of CBD doses up to 500 mg/kg; thus, a TD50 was determined to exceed 500 mg/kg (IP). In the antiseizure tests in rats, IP administration of CBD 2 hours prior to electrical stimulation was found to block tonic extension seizures induced by MES with an ED50 of 53.2 mg/kg, yielding a PI of >9.4. Thus, as demonstrated in male mice, CBD was effective in the rat MES test at a dose well below the motor-impairing dose. Based on this demonstration of efficacy and tolerability in several well-established acute seizure models in rats and mice, CBD was further evaluated in the pilocarpine-SE model and an etiologically relevant rat model of post-SE TLE.

3.2 | IV administration of CBD reduces severity of pilocarpine-induced SE

The pilocarpine-induced SE model is a well-established model of severe generalized seizures that is often used in the pursuit of novel ASDs. Based on the acute efficacy of CBD in the rat MES test following IP administration (Table 1), CBD was administered by the IV route 1 hour prior to the onset of SE to determine any potential for effect on maximal SE severity. This dose of CBD was found to significantly attenuate the maximum seizure severity (MSS; \(P < 0.05\)) compared to the vehicle-treated group (Figure 1A). To demonstrate the validity of the protocol and tractability of the model, we also evaluated the efficacy of a supratherapeutic dose of PB (30 mg/kg IV). Administration of PB 30 minutes prior to SE onset significantly reduced the MSS (\(P < 0.0001\)) relative to the vehicle-treated group (Figure 1B). It should be emphasized that the doses of CBD versus PB tested in this model were not pharmacologically equivalent (Table 1), as the IV dose of CBD was threefold lower than the rat MES ED50 (IP), whereas the dose of PB was 15-fold higher than the rat MES ED50 (IP). Of note, no mortality was observed in any of the experimental animals during the behavioral seizure monitoring period. Thus, CBD pretreatment was found to acutely attenuate MSS in this rat model of SE to a degree consistent with PB.

3.3 | Chronic oral administration of CBD attenuates seizure burden in the RISE-SRS model of TLE

3.3.1 | Chronic oral administration of CBD reduces long-term seizure burden

To evaluate the effect of chronic CBD administration on disease progression, we examined the effect of treatment
The seizure burden ratio was calculated from the seizure burden in each recording session by using the following formula: seizure burden ratio = \( \frac{\text{mean seizure burden in final bin}}{\text{mean seizure burden in first bin}} \). A higher seizure burden ratio therefore indicates a worsening of the disease over time (Figure 2B and 2C), as is typical of post-SE rat models of TLE.\(^{15,19} \) The seizure burden ratio was significantly greater in vehicle-treated epileptic animals compared to CBD-treated epileptic animals (n = 10 per group; Mann-Whitney test, \( U = 22, P < 0.05; \) Figure 2B). Moreover, disease severity was improved (seizure burden ratio < 1) significantly in 70% of the CBD-treated animals, in contrast to only 10% of vehicle-treated rats (Fisher’s exact test, \( P < 0.05; \) Figure 2C). CBD-treated epileptic rats did not demonstrate a time-dependent increase in seizure burden (Wilcoxon matched-pairs test, \( W = -31, P = 0.13; \) Figure 2E) observed by a change of median value from 28.50 (interquartile range [IQR] = 26.81-39.75) to 24.75 (IQR = 19.69-35.94), whereas vehicle-treated rats showed a robust increase in seizure burden from a median value of 25.75

<table>
<thead>
<tr>
<th>Antiseizure Test</th>
<th>CBD ED50, mg/kg</th>
<th>CBD TPE, h</th>
<th>PB ED50, mg/kg</th>
<th>PB TPE, h</th>
<th>VPA ED50 mg/kg</th>
<th>VPA TPE, h</th>
<th>FBM ED50 mg/kg</th>
<th>FBM TPE, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal electroshock, mouse</td>
<td>80 (65.5-96.0)</td>
<td>3.4</td>
<td>11.3 (9.39-13.7)</td>
<td>4.0</td>
<td>2</td>
<td>213 (138-274)</td>
<td>1.8</td>
<td>0.25</td>
</tr>
<tr>
<td>Subcutaneous pentylenetetrazol, mouse</td>
<td>120 (98.5-146)</td>
<td>2.3</td>
<td>1</td>
<td>13.9 (12.1-16.0)</td>
<td>3.3</td>
<td>0.5</td>
<td>305 (212-403)</td>
<td>1.3</td>
</tr>
<tr>
<td>6 Hz, 32 mA, mouse</td>
<td>144 (102-194)</td>
<td>1.9</td>
<td>1</td>
<td>14.8 (8.92-23.9)</td>
<td>3.1</td>
<td>0.5</td>
<td>139 (92.4-197)</td>
<td>2.8</td>
</tr>
<tr>
<td>6 Hz, 44 mA, mouse</td>
<td>173 (136-213)</td>
<td>1.6</td>
<td>1</td>
<td>No public data available</td>
<td>2</td>
<td>289 (242-384)</td>
<td>1.3</td>
<td>0.25</td>
</tr>
<tr>
<td>Corneal kindled mouse</td>
<td>115 (77.5-169)</td>
<td>2.4</td>
<td>1</td>
<td>9.42 (7.91-17.0)</td>
<td>4.8</td>
<td>0.5</td>
<td>174 (135-208)</td>
<td>2.2</td>
</tr>
<tr>
<td>Minimal motor impairment, TD50, mouse rotarod</td>
<td>272 (241-303)</td>
<td>2</td>
<td>2</td>
<td>45.5 (41.8-49.3)</td>
<td>0.25</td>
<td>390 (382-396)</td>
<td>0.25</td>
<td>452 (363-563)</td>
</tr>
<tr>
<td>Maximal electroshock, rat</td>
<td>53.2 (39.1-67.0)</td>
<td>9.4</td>
<td>2</td>
<td>2.61 (1.70-4.04)</td>
<td>15.7</td>
<td>2</td>
<td>212 (167-256)</td>
<td>2.2</td>
</tr>
<tr>
<td>Minimal motor impairment, TD50, rat open field analysis</td>
<td>&gt; 500 (ND)</td>
<td>ND</td>
<td>41.2 (37.0-46.6)</td>
<td>0.5</td>
<td>470 (431-497)</td>
<td>0.5</td>
<td>573 (285-886)</td>
<td></td>
</tr>
<tr>
<td>Maximal electroshock, formulation vehicle, mice</td>
<td>0/4 protected</td>
<td>1</td>
<td>Methylcellulose; no public data available</td>
<td>Methylcellulose; no public data available</td>
<td>Methylcellulose; no public data available</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Hz, 32 mA, formulation vehicle, mice</td>
<td>0/4 protected</td>
<td>1</td>
<td>Methylcellulose; no public data available</td>
<td>Methylcellulose; no public data available</td>
<td>Methylcellulose; no public data available</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal electroshock, formulation vehicle, rats</td>
<td>0/4 protected</td>
<td>1</td>
<td>Methylcellulose; no public data available</td>
<td>Methylcellulose; no public data available</td>
<td>Methylcellulose; no public data available</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CBD, cannabidiol; CI, confidence interval; ED50, median effective dose; FBM, felbamate; IP, intraperitoneal; ND, not done; PB, phenobarbital; PI, protective index; TD50, median behaviorally impairing dose; TPE, time to peak effect; VPA, valproic acid.

aData are from the National Institute of Neurological Disorders and Stroke PANACChE database (https://panache.ninds.nih.gov/ChemDetail.aspx?CHEM_ID=2&TEST_NO=7B#seereport).
3.3.2 Chronic oral administration of CBD to RISE-SRS rats attenuates the severity of behavioral comorbidities of TLE

Motor coordination

Motor coordination was tested with the accelerating rotarod test. A significant difference in time spent on the accelerating rod was observed among the groups (one-way analysis of variance [ANOVA], $F_2, 27 = 5.996$, $P < 0.01$). The Holm-Sidak post hoc test revealed that naïve vehicle-treated ($P < 0.05$) and epileptic CBD-treated ($P < 0.01$) animals spent significantly more time on the rod, compared to the epileptic vehicle-treated group. This motor test indicates that chronic oral CBD administration attenuated the TLE-induced motor dysfunction (Figure 3A).

Gait

The gait test was performed to assess left stride length, right stride length, and stride width of the animals. No significant differences among the groups were observed for any of these parameters (one-way ANOVA; for left stride length, $F_2, 27 = 0.019$; for right stride length, $F_2, 27 = 0.198$; for stride width, $F_2, 27 = 0.842$; Figure 3B-D). Furthermore, as animals were actively on CBD or vehicle therapy during this motor performance test, this study demonstrates that the dose of CBD necessary to reduce seizure burden was not associated with motor-impairing effects in rats with SRS.

Cognitive function

To assess the effect of CBD on cognitive performance, epileptic animals were challenged in the hole board test of reference and working memory. In this study, an age-matched cohort of nonepileptic, naïve rats was also included to determine whether chronic CBD administration could normalize behavioral performance. Chronic oral administration of CBD to epileptic rats was found to significantly reduce the number of reference memory errors (RMEs) and working memory errors (Figure 4). A significant difference in RMEs was observed among the groups (one-way ANOVA, $F_2, 42 = 15.06$, $P < 0.0001$). Here, the naïve vehicle-treated ($P < 0.0001$) and epileptic CBD-treated ($P < 0.05$) rats made significantly fewer RMEs (Figure 4A) compared to the epileptic vehicle-treated rats (Holm-Sidak post hoc test). However, the CBD-treated epileptic rats made significantly more RMEs when compared to the naïve vehicle-treated group (Holm-Sidak post hoc test, $P < 0.05$). Therefore, CBD failed to completely restore the reference memory in epileptic animals to the extent of their naive, nonepileptic controls. Working memory was similarly affected (one-way ANOVA, $F_2, 42 = 35.72$, $P < 0.0001$; Figure 4B), with the epileptic vehicle-treated group

(IQR = 18.94-39.34) to 30.13 (IQR = 21.00-49.91) in this same time period (Wilcoxon matched-pairs test, $W = 53$, $P < 0.01$; Figure 2D).
FIGURE 2  Chronic administration of cannabidiol (CBD) modifies the overall spontaneous seizure burden ratio and seizure burden in the reduced intensity status epilepticus (SE)–induced spontaneous recurrent seizures (SRS) rat model of chronic temporal lobe epilepsy up to 7 weeks postinsult. A, Study time line for the epileptic animals. PSBB, postseizure behavioral battery. B, Median seizure burden ratio of epileptic vehicle-treated and epileptic CBD-treated animals recorded during a 7-week continuous video monitoring period. CBD significantly reduced the seizure burden ratio compared to the vehicle-treated counterpart (n = 10/group; data were analyzed by Mann-Whitney test, *P < 0.05). Data are expressed as median, minimum to maximum, and interquartile range. C, Number of epileptic vehicle-treated and epileptic CBD-treated animals with seizure burden ratio < or > 1 (data were analyzed by Fisher’s exact test, *P < 0.05). D, Median seizure burden before and after vehicle treatment. Seizure burden was increased significantly in this group at the end of the observation period (n = 10; data were analyzed by Wilcoxon matched-pairs signed-rank test, **P < 0.01). E, Median seizure burden before and after CBD treatment. CBD-treated rats showed no change in seizure burden (n = 10; data were analyzed by Wilcoxon matched-pairs signed-rank test).
FIGURE 3  Chronic administration of cannabidiol (CBD) to spontaneously epileptic rats improves some indices of motor coordination 6 weeks after status epilepticus. A, Mean time (in seconds) spent on accelerated rotarod. The naive vehicle-treated and epileptic CBD-treated groups spent significantly more time on the accelerating rotarod compared to the epileptic vehicle-treated group (n = 10/group). B, There were no significant differences in mean left stride length (in millimeters) of naive vehicle-treated, epileptic vehicle-treated, and epileptic CBD-treated rats (n = 10/group). C, There were no significant differences in mean right stride length (in millimeters) between naive vehicle-treated, epileptic vehicle-treated, and epileptic CBD-treated rats (n = 10/group). D, There were no significant differences in mean stride width (in millimeters) of naive vehicle-treated, epileptic vehicle-treated, and epileptic CBD-treated rats (n = 10/group). Data are expressed as mean ± SEM. Data were analyzed by one-way analysis of variance with Holm-Sidak post hoc test. *P < 0.05, **P < 0.01.
demonstrating significantly more errors compared to the naive vehicle-treated group (Holm-Sidak post hoc test, \(P < 0.0001\)). Importantly, chronic CBD-treated rats demonstrated significantly fewer working memory errors compared to epileptic vehicle-treated animals (Holm-Sidak post hoc test, \(P < 0.0001\)). Interestingly, working memory performance for CBD-treated epileptic rats was superior to the naive vehicle-treated group (Holm-Sidak post hoc test, \(P < 0.05\)).

### 4 | DISCUSSION

In the present study, we have demonstrated the acute antiseizure efficacy of CBD in a battery of well-defined and established acute seizure tests in both mice and rats following IP administration. Our findings are consistent with other reports of the acute antiseizure efficacy of CBD in numerous preclinical seizure models.\(^{10,20}\) We have also demonstrated that acute IV administration of a subtherapeutic dose of CBD is able to significantly attenuate the MSS in the rat pilocarpine-induced SE model to a similar degree as a supratherapeutic dose of the prototype ASD, PB.\(^{22}\) Furthermore, we have demonstrated for the first time that chronic oral administration of CBD improves seizure burden ratio, motor comorbidities, and cognitive function in the RISE-SRS model of TLE in rats well after the onset of symptomatic seizures. Altogether, the present study further supports a growing body of evidence to demonstrate that CBD is a well-tolerated and effective therapy for acute and chronic seizures,\(^{11}\) as well as now demonstrating the potential that chronic oral administration of CBD may confer disease-modifying effects in an etiologically relevant preclinical model of TLE.

All of the FDA-approved ASDs exhibit acute antiseizure efficacy in one or more of the acute seizure and epilepsy models presently used for the evaluation of CBD.\(^{16,21}\) However, many of the FDA-approved ASDs are associated with significant motor adverse effects that may limit clinical utility. In this regard, we presently provide pharmacological data that suggest differentiation between CBD and the FDA-approved ASD, PB (Table 1). Of note, the activity profile of CBD in these acute seizure models is generally comparable to PB (Table 1). CBD is thus differentiated from numerous ASDs with broad-spectrum efficacy, including another broad-spectrum ASD, VPA (Table 1), because of this wide margin separating antiseizure efficacy and minimal motor impairment in numerous preclinical seizure models. The broad-spectrum efficacy and margin of safety of CBD are more directly comparable to FBM (Table 1), which, like CBD, is also approved for patients with Lennox-Gastaut syndrome.\(^{22}\) CBD presently demonstrated efficacy in the acute mouse MES, subcutaneous pentylenetetrazol, and 6-Hz assays at both 32- and 44-mA stimulus intensities. CBD also demonstrated a PI > 1.5 in the 6-Hz model of pharmacoresistant epilepsy at a 44-mA stimulus intensity, which substantially differentiates this compound from many other FDA-approved ASDs, including VPA.\(^{23,24}\) The activity of FBM is also preserved in the 44-mA version of the 6-Hz assay (Table 1); the PI of FBM is also much greater than CBD.
in this assay (4.6 for FBM vs 1.6 for CBD; Table 1). As
the 6-Hz stimulation intensity increases from 32 to 44 mA, most ASDs lose efficacy or are only effective at motor-
impairing doses\(^{16,17,23,25}\); thus, the finding that CBD retained efficacy and potency at both stimulation intensities
in this assay at doses well below the mouse TD50
(272 mg/kg IP) suggests that CBD is highly differentiated
from other FDA-approved ASDs (eg, PB and VPA; Table 1) and suggests potential for efficacy in pharmacore-
sistant patient populations akin to FBM. Unlike FBM, there
have been no reports to date of hepatotoxicity or aplastic
anemia with CBD use,\(^{26,27}\) but whether other clinical
adverse events will emerge with greater clinical use of this
agent remains to be determined.

The presently reported ED50 values in these acute mouse
assays align with work reported by Klein and colleagues
using CBD provided by the US National Institute of Drug
Abuse\(^{10}\) and by an independent laboratory using the same
CF-1 mouse strain.\(^{28}\) However, we presently report signifi-
cantly greater potency of CBD in the rat MES test than that
reported by Klein and colleagues (ED50 = 88.9 mg/kg [95%
CI = 69-124] IP\(^{10}\)), likely due to differences in compound
formulation, source, and purity. We also report that CBD
everred dose-dependent reductions in seizure score in the
corneal kindled mouse model of chronic focal seizures, fur-
ther suggesting that CBD is effective as an antiseizure agent
in an epileptic substrate (ie, kindled rodents). Altogether, the
presently reported pharmacological profile of CBD supports
further evaluation of its broad clinical utility for epilepsy.

IV administration of CBD also reduced MSS in the
pilocarpine-induced SE in rats (Figure 1A), consistent with
earlier findings demonstrating efficacy of a preclinical
CBD formulation administered via the IP route.\(^{9}\) We
believe this is the first demonstration of the efficacy of IV
CBD against the onset of SE, with this route of administra-
tion most commonly employed in the treatment of clinical
SE.\(^{6,9}\) Although further studies are needed to define
whether IV CBD can effectively reduce MSS after the
onset of SE (eg, against benzodiazepine-resistant SE), the
present results in the pilocarpine-SE model further support
the acute antiseizure efficacy of CBD in diverse preclinical
models of seizure.

TLE is one of the most common forms of acquired epi-
lepsy in humans.\(^{29}\) Therefore, we investigated the effect of
a clinically relevant CBD administration protocol in the
RISE-SRS model of TLE.\(^{15}\) We presently demonstrate that
CBD is disease-modifying in this model, where all animals
exhibit SRS before being assigned to treatment groups.
Specifically, chronic oral administration of CBD signifi-
cantly decreased the seizure burden ratio of epileptic ani-
mals and improved reference memory function. Although
CBD did not significantly reduce seizure burden after
7 weeks of treatment, it did markedly modify the natural
disease course following SE, as demonstrated by no overall
increase in the seizure burden from the first to final seizure
monitoring bin (Figure 2). These findings are in stark con-
trast to the vehicle-treated post-SE rats, which demon-
strated a notable, time-dependent increase in disease
severity (ie, seizure burden ratio increased). Disease sever-
ity, as characterized by a seizure burden ratio < 1, was
improved in 70% of the CBD-treated animals, in contrast
to only 10% of vehicle-treated rats attaining such a ratio
(Figure 2). Although several FDA-approved ASDs have
demonstrated disease-modifying potential in preclinical
models of chronic seizure,\(^{30,31}\) and CBD has been found to
be disease-modifying in a mouse model of Dravet syn-
drome,\(^{32}\) to our knowledge, this is the first study to demon-
strate such an effect with CBD in any preclinical TLE
model. The third-generation ASD topiramate has demon-
strated some potential for cognitive sparing and modification
of behavioral deficits when administered shortly after
SE onset,\(^{33,34}\) but no study has yet demonstrated such a
disease-modifying effect when treatment is initiated well
after the SE insult (8 weeks in present study). Although
our study did not include a CBD washout arm, the present
results demonstrate that chronic oral administration of CBD
is associated with long-term improvements in disease tra-
jectory in this rat model of epilepsy.

A number of ASDs (eg, PB, VPA, phenytoin) are
reported to have an adverse effect on motor function char-
acterized by dyskinesia in people with epilepsy.\(^{5,35-37}\)
Moreover, patients on phenytoin and VPA treatment some-
times exhibit parkinsonism.\(^{5}\) Although we presently
demonstrate that acute administration of CBD is well toler-
ated at doses up to 500 mg/kg (IP) in naive rats (Table 1),
in line with prior reports,\(^{39}\) no studies have yet been con-
ducted to examine the long-term effects of chronic CBD
administration on tolerability and motor function in epilep-
tic animals. Furthermore, no study has yet administered
CBD for disease modification purposes after the onset of
SRS. In this regard, the present study employed a clinically
realistic treatment scenario in a preclinical model of TLE
to demonstrate that chronic oral administration of CBD is
associated with notable antiseizure efficacy, minimal
adverse effects liability, and disease-modifying potential
well after the onset of symptomatic seizures.

It is well known that humans and animals with epilepsy
are more sensitive to adverse effects of ASDs;\(^{38}\) thus, our
present findings that chronic oral administration of CBD in
rats with epilepsy was not associated with any adverse
effects further supports the potential of this agent for chronic
clinical use in epilepsy patient populations. In addition to
these drug-induced adverse effects on motor function, motor
deficits are one of the most common comorbidities exhibited
by patients with epilepsy.\(^{39}\) Here, we used two well-vali-
dated models of motor function to assess the fine motor
control, balance, and gait of animals. Epileptic vehicle-treated animals fell from the accelerating rotarod sooner than the naïve vehicle-treated ones, indicating that SRS produced significant motor dysfunction related to balance. Similar findings were reported previously in the rotarod test, where motor dysfunction was exhibited by epileptic rats. In contrast, CBD-treated animals remained on the accelerating rotarod for a significantly longer time than the vehicle-treated epileptic animals. Thus, we show for the first time that CBD is not only well-tolerated by epileptic animals after prolonged oral administration, but that CBD reduces the severity of motor deficits induced by epilepsy.

Epileptic rats performed comparably to healthy animals in the gait test, which is consistent with gait disorders being rarely reported in adult epileptic patients with TLE. However, ASDs such as VPA and lacosamide have a detrimental effect on gait in human patients. It should be emphasized that animals were in the active CBD administration period during this motor test, suggesting that CBD at the dose tested did not confer any adverse effects on motor coordination. Our results demonstrate that chronic oral administration of CBD did not have any adverse effect on gait in rats with epilepsy. Clinical trials conducted on Dravet syndrome and Lennox-Gastaut syndrome patients also did not report any gait disturbances following CBD administration. Whether these side effects may also be absent in the general TLE patient population remains to be further determined.

Cognitive decline is a common comorbidity associated with TLE. For example, patients with TLE often exhibit poor executive control and working memory deficits, and amnesia or accelerated long-term loss of memory is also frequently reported in patients with TLE. Cognitive function in animals is typically assessed using spatial memory tasks measuring reference memory and working memory errors. Reference memory can be defined as long-term storage of acquired information that remains constant over successive training sessions, whereas working memory is a form of short-term memory that refers to storage and manipulation of information acquired within a trial session. Several spatial memory tasks (eg, radial arm maze, Morris water maze, hole-board task) have been used to evaluate these two types of memory in rodents. Here, we employed the hole-board task, where epileptic vehicle-treated rats exhibited impairment of both the working and reference memory aspects of the hole-board test. These findings are comparable with previous studies of spatial or hippocampus-dependent memory in rats with TLE, for example, a delayed nonmatching to position task, eight arm radial maze, and Morris water maze. In contrast, CBD-treated epileptic animals exhibited improved reference memory function compared to epileptic vehicle-treated animals; however, reference memory function was not restored to levels seen in healthy animals. Interestingly, our study shows that chronic oral administration of CBD improved working memory function compared to both epileptic vehicle-treated and naïve vehicle-treated animals. However, as we did not include a nonepileptic CBD treated group in our design, we are unable to ascertain whether CBD has memory enhancement properties in healthy animals. A memory-enhancement effect would be unusual, as previous studies using a radial arm maze have shown that CBD does not improve working memory function in healthy mice.

Given that the integrity of the blood-brain barrier is commonly disrupted in epilepsy, a limitation of the present study is that we did not determine whether higher CBD concentrations were present in the brains of these epileptic rats. Thus, the dose of CBD required to reach this concentration might not be therapeutically significant in healthy animals. Although SRS are the primary cause of cognitive decline in TLE, the improved cognitive function in the CBD-treated epileptic group might be due to a seizure-independent mechanism rather than solely by seizure reduction, for example, anti-inflammatory/neuroprotective action. However, further detailed investigation is thus warranted to shed light on the mechanism by which CBD improved the presently tested behavioral comorbidities.

Epidiolex (CBD) was approved by the FDA in 2018 for the treatment of seizures in the catastrophic pediatric encephalopathies Dravet syndrome and Lennox-Gastaut syndrome. The present study has demonstrated that CBD is also a good potential candidate drug for the treatment of spontaneous symptomatic seizures of TLE. Moreover, chronic oral administration of CBD also reduced severity of motor disorders and cognitive deficits typically associated with TLE, in line with prior demonstrations of disease-modifying effects in a mouse model of Dravet syndrome. Taken altogether, this study further illustrates that CBD not only is a potent and broad-spectrum ASD, but may also find use to attenuate cognitive deficits associated with TLE well after the onset of SRS. More detailed studies are thus required to investigate the potential mechanism of action for CBD as a disease-modifying agent for the patient with epilepsy.

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DISCLOSURE OF CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

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