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Evaluating the Therapeutic Potential of the Cannabinoid 2 Receptor in Epilepsy

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Evaluating the Therapeutic Potential of the Cannabinoid 2 Receptor in Epilepsy

By

Lindsey Shapiro

B.S., University of New Hampshire, 2014

Advisor: Andrew Escayg, Ph.D.

An abstract of

a dissertation submitted to the Faculty of the

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Abstract

Evaluating the Therapeutic Potential of the Cannabinoid 2 Receptor in Epilepsy

By Lindsey Shapiro

Epilepsy, characterized by recurrent unprovoked seizures as a result of excessive synchronous neuronal firing, is one of the most common neurological disorders. Approximately 30% of epilepsy patients do not achieve adequate seizure control with currently available medications, highlighting the need to identify novel molecular targets for anti-epileptic drug development. Cannabinoid 2 receptors (CB2Rs) have emerged as a potential therapeutic target in several neurological disorders, including stroke, traumatic brain injury, and neurodegenerative disease. CB2R agonists consistently reduce neuroinflammation, attenuate neuron loss, and improve behavioral outcomes across models of neurological disease. Furthermore, in vitro studies have identified that neuronally-expressed CB2Rs regulate neuronal excitability in the hippocampus and cortex, two brain regions which are involved in the generation and propagation of seizures. Despite accumulating evidence that CB2Rs influence physiological processes that are relevant to epilepsy, few studies have directly investigated the anti-epileptic potential of CB2R-targeted therapies. The goal of this dissertation was to evaluate the potential of CB2R modulation for the treatment of epilepsy. First, we established a direct relationship between CB2R modulation and seizure phenotypes by demonstrating enhanced seizure susceptibility with genetic knockout and pharmacological blockade of CB2Rs in mice. We next evaluated whether pharmacological enhancement of CB2R signaling might confer seizure resistance. Our results suggest that while the CB2R orthosteric agonist, JWH-133 does not increase seizure resistance, the novel CB2R positive allosteric modulator, Ec21a, confers robust seizure resistance. We demonstrate that Ec21a fulfils several clinical requirements including adequate brain uptake, lack of neurotoxicity, and the ability to maintain protection with repeated dosing. We also found that Ec21a is similarly seizure protective in a mouse line harboring the human *SCN1A* R1648H epilepsy mutation. To further characterize the potential of CB2R-targeted therapies in another clinically-relevant model, we evaluated the effects of CB2R ligands on a range of pathological features in a mouse model of mesial temporal lobe epilepsy, a common form of treatment-resistant epilepsy in adults. Overall, this study provided a comprehensive analysis of the role of CB2Rs in epilepsy and highlights the therapeutic value of allosteric modulation of CB2Rs as a potential treatment for refractory forms of epilepsy.

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ABBREVIATIONS

2-AG	2-arachidonoylglycerol
AD	Alzheimer's disease
AEA	*N*- arachidonoylethanolamine
AED	anti-epileptic drug
BBB	blood brain barrier
cAMP	cyclic adenosine monophosphate
CBD	cannabidiol
CBR	cannabinoid receptor
CB1R	cannabinoid 1 receptor
CB2R	cannabinoid 2 receptor
DAGL	diacylglycerol lipase
DS	Dravet syndrome
eCB	endocannabinoid
ECS	endocannabinoid system
EEG	electroencephalogram
FAAH	fatty acid amide hydrolase
GIRK	g-protein coupled inwardly rectifying potassium channel
GPCR	G-protein coupled receptor
GTCS	generalized tonic-clonic seizure
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
MJ	myoclonic jerk
MTLE	mesial temporal lobe epilepsy
NAPE-PLD	*N*-acyl-phosphatidylethanolamine phospholipase D
ND	neurodegenerative disease
PAM	positive allosteric modulator
PD	Parkinson's disease
PFC	prefrontal cortex
PTZ	pentylenetetrazol
SE	status epilepticus
TBI	traumatic brain injury
THC	tetrahydrocannabinol
VGCC	voltage-gated calcium channel
WT	wild-type

CHAPTER 1
INTRODUCTION

1.1 Overview

The overall goal of my dissertation research was to evaluate the potential of cannabinoid 2 receptor (CB2R) modulation as a therapy for treatment-resistant forms of epilepsy. In this introduction, I provide a general overview of epilepsy, highlighting the need to develop novel therapeutics for pharmacoresistant patients. I will also introduce the endocannabinoid system (ECS) and address how the ECS can be leveraged as a therapeutic target for a range of neurological disorders. In particular, I will focus on the growing interest in brain-expressed CB2Rs and discuss the potential for CB2R-targeted therapies to overcome persistent barriers to the effective treatment of pharmacoresistant epilepsy. Finally, I will conclude with a description of the specific goals of the research contained in this thesis.

1.2 Epilepsy

Epilepsy encompasses a heterogenous group of disorders which are characterized by spontaneous recurrent seizures as a result of repetitive and synchronous neuronal firing (ILAE, 1993). Epilepsy is one of the most common neurological disorders, affecting over 50 million individuals worldwide, with children and the elderly at highest risk (WHO, 2019). Approximately 30% of affected individuals are unable to achieve adequate seizure control with currently available anti-epileptic drugs (AEDs), and are thus considered refractory to treatment (Picot et al., 2008). In addition to poorly-controlled seizures, refractory forms of epilepsy are often accompanied by other comorbidities including psychiatric illness, learning and memory difficulties, sleep disturbances, and an increased risk for sudden unexpected death in epilepsy (Laxer et al., 2014; McCagh et al., 2009; Mohanraj et al., 2006).

1.2.1 Epilepsy Etiologies

The causes of epilepsy are extremely diverse. Many epilepsies arise from genetic mutations. While some mutations directly cause epilepsy, others result in structural, metabolic, or immune abnormalities in which epilepsy is a secondary result. Some forms of epilepsy are acquired as a consequence of brain injuries such as prolonged early-life febrile events, traumatic brain injury (TBI), or infection. Moreover, epilepsy can result from a combination of genetic and acquired causes. Epilepsies with any of the above etiologies may be refractory (ILAE, 2020). The work presented in this thesis will utilize mouse models of both genetic and acquired epilepsy to investigate the therapeutic potential of targeting CB2Rs across a range of etiologies.

1.2.2 Treatments for Epilepsy

There are nearly 30 AEDs which are currently used for the treatment of epilepsy (Wang and Chen, 2019). One common feature of epilepsy disorders is an imbalance of excitatory-inhibitory (E/I) signaling, leading to neuronal hyperexcitability. Thus, the mechanism of most AEDs is to either reduce excitability or increase inhibition, in order to restore E/I balance (Rogawski and Löscher, 2004; Wang and Chen, 2019). Sodium channel blockers (e.g. valproate, phenytoin) are the most frequently used class of AEDs, and serve to regulate intrinsic neuronal firing capacity, thereby decreasing neuronal excitation (Mantegazza et al., 2010; Remy et al., 2003; Van den Berg et al., 1993). Potassium channel openers (e.g. retigabine) and calcium channel blockers (e.g. ethosuximide) similarly regulate intrinsic excitability (Coulter et al., 1989; Tatulian et al., 2001). Other AEDs are designed to modulate synaptic transmission, either through an inhibition of synaptic excitation (e.g. leviteracetam) or an increase in synaptic

inhibition (e.g. benzodiazepenes, felbamate) (Lyseng-Williamson, 2011; Rho et al., 1997; Rudolph et al., 1999).

While AEDs are the most common approach to epilepsy treatment, there are some non-pharmacological options for individuals who do not respond to pharmacotherapy. For many patients, surgical resection is the next line of treatment. However, epilepsy surgery requires the accurate identification of the seizure focus, and thus, not all individuals are candidates for the procedure (Engel, 2019, Rugg-Gunn et al., 2020). When surgery is impossible or unsuccessful, vagus nerve stimulation (VNS) can be used as an add-on therapy to pharmacological treatment. However, VNS is primarily used for individuals with focal forms of epilepsy and is not a viable option for all patients (Gonzalez et al., 2019, Wheless et al., 2018). Lastly, the ketogenic diet is often used as a non-pharmacological approach to reduce seizure burden and/or reliance on AEDs. The ketogenic diet has proven to be particularly effective in several forms of severe pediatric epilepsy (Cai et al., 2017, Martin et al., 2016).

1.2.3 Barriers to Effective Epilepsy Treatment

Despite a large increase in the number of available AEDs over the last forty years, the percentage of epilepsy patients who fail to achieve adequate seizure control has remained relatively steady (Wang and Chen, 2019). Additionally, AEDs are often accompanied by unwanted side effects, including sedation, cognitive impairment, motor problems, and neuropsychiatric effects as a result of their broad actions in the brain (Perucca and Gilliam, 2012; Stephen et al., 2017). Furthermore, epilepsy patients can experience a number of comorbidities, including anxiety, depression, migraine, and learning and memory difficulties which current AEDs fail to attenuate (Guekht, 2017; Keezer et al., 2016).

Lastly, given that epilepsy develops in many patients following a precipitating brain injury (i.e. acquired forms of epilepsy), there is a need to identify therapies which might also be anti-epileptogenic (Yu et al., 2020a). At present, there are no effective drugs available for this purpose. There is thus a critical need to develop AEDs with novel mechanisms to treat the broad spectrum of clinical challenges which epilepsy patients face.

1.3 Overview of the Endocannabinoid System

The ECS has emerged as a potential therapeutic target for a number of neurological disorders, including epilepsy. A functional ECS is thought to be present in all vertebrates, and is broadly comprised of three major components: cannabinoid receptors (CBRs), endocannabinoids (eCBs), and the enzymes which synthesize and degrade eCBs (Elphick and Egertová, 2005). Below is an overview of the discovery of the ECS.

In the 1960s, unique chemical constituents of cannabis, Δ^9-tetrahydrocannabinol (Δ^9 THC) and cannabidiol (CBD), were discovered and termed phytocannabinoids (Mechoulam and Gaoni, 1965; Mechoulam and Shvo, 1963). More than twenty years later, high affinity binding sites for Δ^9 THC were discovered in the brain and called cannabinoid 1 receptors (CB1Rs) (Devane et al., 1988). A second cannabinoid receptor, CB2R, was soon identified and found to be highly expressed in peripheral immune tissues (Munro et al., 1993). Both CB1R and CB2R mediate the effects of a diverse set of endogenous, plant-derived, and synthetic ligands.

Next identified were eCBs, the endogenous agonists of CBRs (Devane et al., 1992; Mechoulam et al., 1995). The two best studied eCBs are *N*-arachidonoylethanolamine (AEA; anandamide) and 2-arachidonoylglycerol (2-AG). AEA and 2-AG are synthesized 'on demand' from membrane lipids (Felder et al., 1993; Sugiura et al., 1995) via the enzymes *N*-acyl-

phosphatidylethanolamine phospholipase D (NAPE-PLD), and diacylglycerol lipase (DAGL), respectively (Bisogno et al., 2003; Okamoto et al., 2004). AEA and 2-AG are predominately degraded by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (Cravatt et al., 1996; Dinh et al., 2002). While both 2-AG and AEA are agonists at CBRs, basal levels of 2-AG in the brain are about 1,000 times higher than AEA, and it is thus thought to be the predominant eCB in central nervous system (CNS) signaling (Di Marzo and De Petrocellis, 2012).

A graphical overview of the major ECS components in the CNS is provided in **Fig 1.1**. Since its initial discovery, a much broader network of receptors, ligands, enzymes, and pathways have been found to influence ECS function and are referred to as the "endocannabinoidome" (Di Marzo, 2018). However, given that the role of many of these proteins in ECS signaling is still poorly understood, they will be largely excluded from discussion in this thesis.

1.3.1 Distribution of the ECS

eCBs and related enzymes are expressed in neurons and glia throughout the CNS. CB1Rs are among the most abundant receptors in the brain, with high expression on excitatory and inhibitory terminals in brain regions such as cortex, hippocampus, cerebellum, and basal ganglia (Mackie, 2005). CB1Rs are also expressed on astrocytes, although at lower levels than on neurons (Bosier et al., 2013; Castillo et al., 2012).

CB2Rs were historically considered to be peripheral cannabinoid receptors, with early studies failing to find CB2R mRNA expression in human or rodent brain tissue (Galiègue et al., 1995; Schatz et al., 1997). More recent studies have challenged this view with the demonstration of CB2R mRNA and protein in the CNS of rodents, non-human primates, and humans using a

variety of experimental techniques (Gong et al., 2006; Lanciego et al., 2011; Li and Kim, 2015; Liu et al., 2009; Schmole et al., 2015). CB2Rs are predominately observed on microglia (Núñez et al., 2004; Schmole et al., 2015). However, CB2R expression has also been described on neurons in brain regions including cortex, hippocampus, and brainstem, although their expression on neurons in the healthy brain remains widely debated (Li and Kim, 2015; Schmole et al., 2015; Stempel et al., 2016). While limited work has been done to describe the subcellular localization of CB2Rs in neurons, it has been postulated that CB2Rs are expressed at the postsynaptic membrane (Stempel et al., 2016), although intracellular localization has also been described (den Boon et al., 2012). CB2Rs are highly inducible, with expression greatly increasing in the disease state, a mechanism which is thought to be a protective response during neuronal injury (Concannon et al., 2016; Yu et al., 2015). The distribution of the ECS in cells of the CNS is illustrated in **Figure 1.1**.

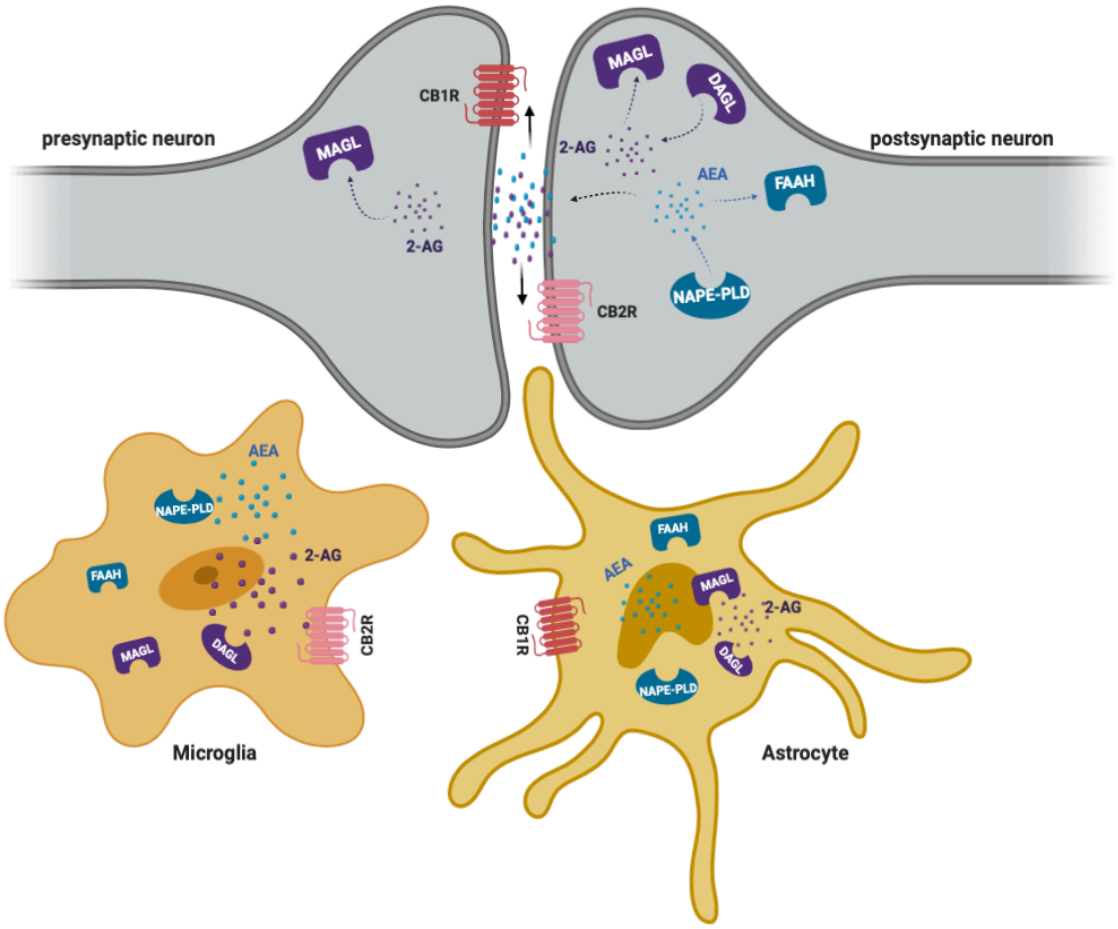

Fig 1.1 The endocannabinoid system (ECS) in the brain. The endocannabinoids 2-arachidonylglycerol (2-AG) and *N*-arachidonoylethanolamine (AEA) are produced 'on demand' in postsynaptic neurons, microglia, and astrocytes via the enzymes diacylglycerol lipase (DAGL) and *N*-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD). eCBs are degraded in neurons and glia via the enzymes monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH). eCBs exert effects on cannabinoid 1 receptors (CB1Rs) which are predominately located on presynaptic neurons and astrocytes, as well as cannabinoid 2 receptors (CB2Rs), predominately expressed on microglia and postsynaptic neurons.

1.3.2 Cannabinoid receptor signaling pathways

Both CB1Rs and CB2Rs are class A serpentine G-protein coupled receptors (GPCRs) which couple to $G_{i/o}$ proteins, leading to the modulation of a diverse array of signaling pathways (Howlett et al., 2002). Broadly, ligand binding leads to a G_i-protein dependent inhibition of adenylyl cyclase (AC) with subsequent reductions in cyclic adenosine monophosphate (cAMP) levels, resulting in a range of cellular effects including altered gene transcription, protein phosphorylation, and alterations to ion channel function (Felder et al., 1995; Mukherjee et al., 2004). CBRs are also coupled to mitogen-activated protein kinases (MAPK) such as extracellular signal-regulated kinase ½ (ERK ½), which plays an important role in cell proliferation, migration, and cell death (Bouaboula et al., 1995; Ibsen et al., 2017; Turu and Hunyady, 2010). Additionally, CBRs functionally couple to ion channels including inwardly-rectifying potassium channels (GIRKS) and voltage-gated calcium channels (VGCCs) to exert direct effects on cell excitability (Atwood et al., 2012; Ho et al., 1999; Mackie et al., 1995; McAllister et al., 1999; Szabó et al., 2014). In addition to G-protein dependent signaling mechanisms, CBRs also recruit β-arrestin, which mediates receptor internalization in addition to influencing ERK ½ signaling and ion channel coupling (Breivogel et al., 2013; Chen et al., 2014). In some circumstances, CB1Rs couple to other G-proteins in a cell-type and ligand-specific manner, an effect which has not been described for CB2Rs (Navarrete and Araque, 2008). An overview of major $G_{i/o}$ signaling pathways for CBRs is provided in **Fig 1.2**.

Importantly, CBRs display strong functional selectivity, wherein different agonists activate distinct signaling pathways upon binding. Thus, while each of the above pathways are involved in CBR signaling, the extent to which they are activated highly depends on the specific ligand and the context in which it is used (Atwood et al., 2012).

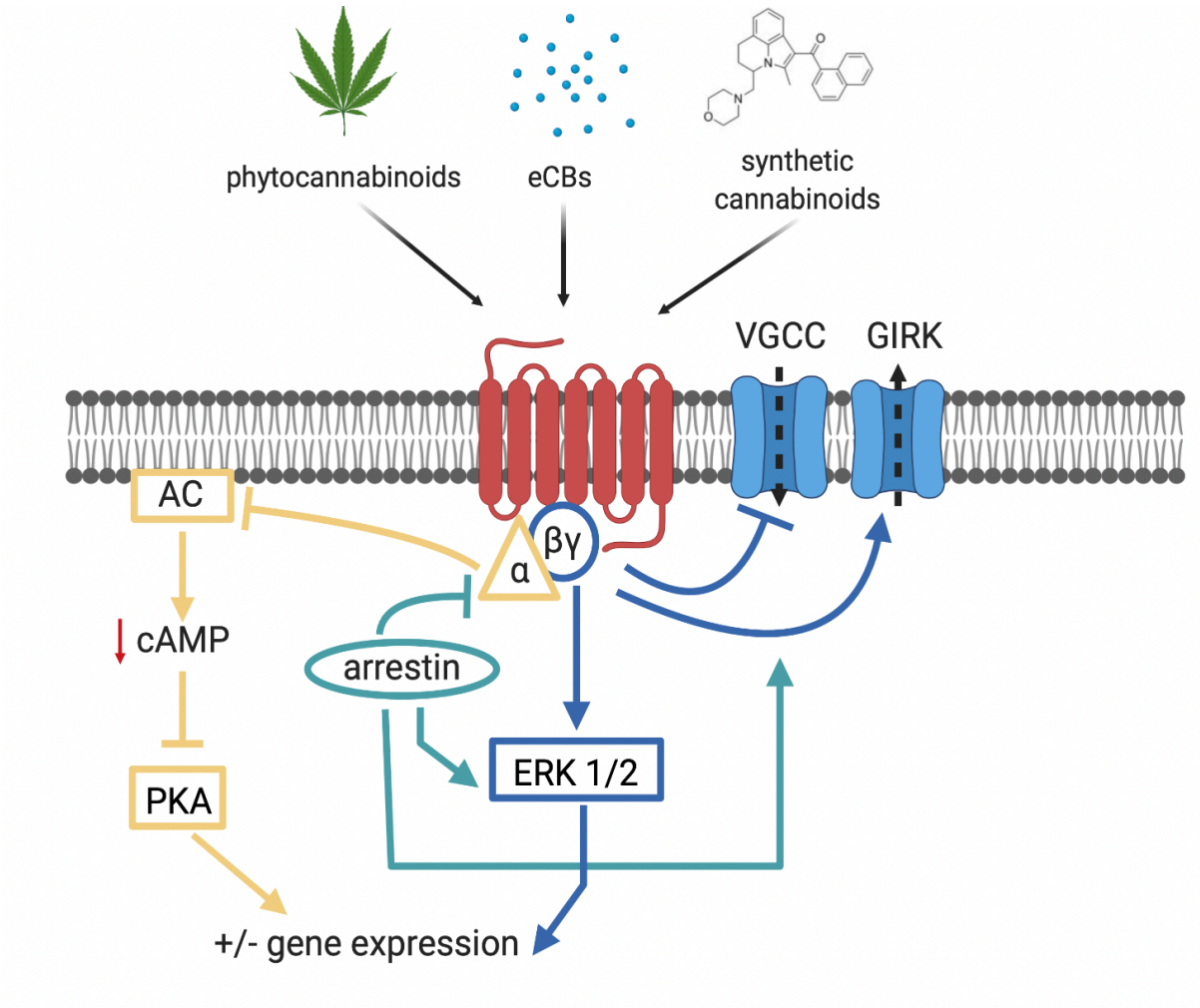

Figure 1.2. Overview of the major CBR signaling pathways. Activation of G_i-coupled CBRs via plant-derived phytocannabinoids, endocannabinoids (eCBs) or other synthetic ligands elicits activation of a range of signaling pathways. Adenylyl cyclase (AC) is inhibited via G_i-protein-dependent mechanisms, which results in a reduction of cyclic adenosine monophosphate (cAMP) and subsequent changes to gene expression. Uncoupling of the ßγ subunit upon ligand binding activates extracellular signal-regulated kinase (ERK1/2) which similarly induces gene expression changes. ßγ subunits can also directly influence ion channel function. Arrestin recruitment leads to receptor internalization and/or activation of other pathways, including ion channel coupling and ERK 1/2 signaling. VGCC; voltage-gated calcium channel, GIRK; g-protein coupled inwardly rectifying potassium channel.

1.3.3 Cannabinoid Receptor Function

The activation of CBR signaling pathways influences a number of complex neurological functions (Zou and Kumar, 2018). In this section, I will outline the functional consequences of CB1R and CB2R activation with a focus on brain-expressed CB2Rs. Given the complexity of the pathways involved in CBR signaling, the functional consequences are numerous. As such, I limit the focus here to neuronal excitability and immune regulation, two processes which are particularly relevant for the development of epilepsy therapeutics.

1.3.3.1 Cannabinoid 1 Receptors

The best described role for CB1Rs in the CNS is in regulating synaptic strength as an 'on-demand' retrograde neuromodulatory system (Wilson and Nicoll, 2001). Upon postsynaptic increases in intracellular calcium concomitant with increased neuronal activity, eCBs are synthesized and released into the extracellular space to act on CB1Rs expressed at the presynaptic terminal (Kano et al., 2009; Ohno-Shosaku and Kano, 2014). Consequently, CB1Rs prevent neurotransmitter release through the inhibition of calcium channels and other AC-dependent pathways (Araque et al., 2017; Mátyás et al., 2008). CB1R-dependent self-inhibition of postsynaptic neurons has also been described (Marinelli et al., 2008; Marinelli et al., 2009).

1.3.3.2 Cannabinoid 2 Receptors

The role of CB2Rs in the CNS is best established on microglia, where they regulate neuroinflammation. Microglia express a functional ECS, and release eCBs under basal and inflammatory conditions in vitro (Carrier et al., 2004; Liu et al., 2006). When microglia are cultured under inflammatory conditions, eCBs mediate a shift from a pro-inflammatory to anti-

inflammatory phenotype, which is reversed by blockade of CB2Rs (Mecha et al., 2015). Moreover, cultured microglia from CB2R knockout mice exhibit diminished phagocytic capacity, reduced expression of anti-inflammatory markers, and altered morphology (Mecha et al., 2015). Similarly, CB2R activation following an inflammatory challenge in microglial culture is associated with decreased expression of pro-inflammatory cytokines and markers of oxidative stress (Ehrhart et al., 2005). CB2Rs are also densely expressed on immune cells in the periphery where they play an influential role in regulating the inflammatory state of T-cells and macrophages (Turcotte et al., 2016).

Although the function of CB2Rs on neurons is not as well characterized, recent studies have demonstrated that activation of neuronally-expressed CB2Rs influences excitability. CB2R agonists inhibit neuronal firing in the ventral tegmental area by enhancing potassium currents, leading to cell hyperpolarization (Ma et al., 2019). In the prefrontal cortex (PFC), CB2Rs mediate the opening of chloride channels in response to increased levels of calcium, and pharmacological activation of CB2Rs reduces PFC neuronal firing rates by almost half (den Boon et al., 2012; den Boon et al., 2014). CB2R activation also reduces neuronal hyperexcitability via activation of GIRK channels in cortical pyramidal neurons (Stumpf et al., 2018). Moreover, action potential-induced release of 2-AG elicits a cell-intrinsic hyperpolarization that is dependent on neuronally expressed CB2Rs in the hippocampus (Stempel et al., 2016).

1.4 Cannabinoid-based therapies for neurological disorders

Studies implicating the ECS in important brain processes such as neuronal excitability and neuroinflammation have heightened interest in the use of cannabinoid-based therapeutics for neurological disorders (Cristino et al., 2020; Mecha et al., 2016). Moreover, the development of highly selective synthetic cannabinoid ligands has enabled the study of how specific pharmacological interventions at CBRs influence outcomes across a range of disorders (Pertwee, 2006). Below, I summarize the evidence in support of cannabinoid -based therapeutics for the treatment of CNS disorders, focusing primarily on CB2Rs.

1.4.1 Neurodegenerative disease, TBI, and stroke

Many studies have now evaluated cannabinoid-based therapeutics for the treatment of neurological disorders which share pathological features with epilepsy, including neurodegenerative disease (ND), TBI, and stroke. Expression of CB1Rs is altered in patients and mouse models of neurological disease; however, it remains unclear whether increased or decreased CB1R activity is concomitant with protection (Ceccarini et al., 2019; Cristino et al., 2020). Protective effects of both CB1R agonists and antagonists have been observed in mouse models of ND, TBI, and stroke (reviewed in Cristino et al., 2020).

CB2R expression is increased in ND, TBI, and stroke (Donat et al., 2014; Gómez-Gálvez et al., 2016; Ramírez et al., 2005). Moreover, CB2R activation is consistently associated with improved outcomes, including prevention of blood brain barrier (BBB) breakdown and peripheral immune cell infiltration, reduced microglial activation, and attenuated neuron loss (Amenta et al., 2012; Chung et al., 2016; Jayant et al., 2016; Shi et al., 2017; Zarruk et al., 2012; Zhang et al., 2007). Furthermore, CB2R agonists normalized hippocampal plasticity and

glutamate signaling in an Alzheimer's disease (AD) model, and reduced glutamate-mediated neurodegeneration of cortical neurons in a stroke model, indicating that CB2Rs can prevent excitotoxicity (Wu et al., 2017; Yu et al., 2015). These cellular changes are often associated with enhanced functional recovery, including improved memory performance in mouse models of AD, as well as improved motor recovery and neurological scores in stroke models (Lou et al., 2017; Wu et al., 2017; Yu et al., 2015). Studies evaluating the effects of CB2R agonists in models of ND, stroke, and TBI are summarized in **Table 1.1**.

1.4.2 Epilepsy

The identification of Δ^9 THC and CBD, alongside emerging case reports describing the anti-convulsant effects of cannabis have heightened interest in the study of medical marijuana for epilepsy. The use of cannabis for seizures was most recently popularized following the case of Charlotte Fiji, whose seizures were reduced from 50 per day to 2-3 per month with administration of a cannabis oil mixture (Maa and Figi, 2014). While similar effects have been described, some patients report that cannabis is ineffective at reducing seizure burden (Consroe et al., 1975; Mortati et al., 2007). For example, one report of 75 patients indicated that only 57% of individuals experienced any reduction in seizures, with 13% experiencing an increase in seizure incidence (Press et al., 2015). Importantly, there is a wide range of responses to medicinal marijuana reported in the literature, which can likely be attributed to differences in epilepsy subtypes and/or the diversity in chemical composition of the cannabis product being used. Additionally, the potential neurotoxic, behavioral, and neurodevelopmental impacts of cannabis use have prevented the widespread application of medicinal marijuana for epilepsy (Dow-Edwards and Silva, 2017; Huestis, 2007). It is thought that these negative effects are likely

a result of Δ^9 THC exposure, and Δ^9 THC administration in mouse models is associated with both anti-convulsant and pro-convulsant effects (Chesher and Jackson, 1974; de Salas-Quiroga et al., 2015; Wallace et al., 2001).

In contrast, CBD exhibits greater safety and a lower side effect profile, and several preclinical studies have demonstrated robust seizure protection in rodents treated with CBD (Friedman et al., 2019; Kaplan et al., 2017; Klein et al., 2017; Patra et al., 2019). The anti-epileptic effects of CBD have been corroborated by several clinical trials, leading to FDA approval of CBD for the treatment of two forms of pediatric epilepsy (Devinsky et al., 2017; Devinsky et al., 2018; Thiele et al., 2018). However, CBD is accompanied by some side effects, and shows drug interactions with some AEDs (Bergamaschi et al., 2011; Lozano, 1997). Additionally, the anti-epileptic mechanism of CBD remains largely unknown, and its pharmacological targets in the brain are likely numerous.

Upregulation of 2-AG and AEA have been observed following induced seizures in rodent models, indicating that enhancement of endogenous cannabinoid signaling could also be leveraged as a therapeutic approach (Marsicano et al., 2003; Wallace et al., 2002). Several studies have utilized pharmacological or genetic blockade of eCB degradation to evaluate whether elevated eCB levels confer seizure resistance. While increased seizure resistance with such approaches has been seen, some studies observed elevated seizure susceptibility (Clement et al., 2003; Ma et al., 2014; Mikheeva et al., 2017; Naderi et al., 2012; Sugaya et al., 2016; von Rüden et al., 2015). eCBs can elicit effects at a number of other receptors in addition to CBRs, which might explain the mixed effects observed in seizure models (Nazıroğlu et al., 2019). Thus, while these studies indicate the potential for increased eCB levels to reduce seizure occurrence, this approach is complicated by the promiscuous nature of endogenous cannabinoid signaling.

The development of highly specific ligands and CBR knockout mice has enabled the evaluation of the specific effects of CBR modulation on seizure phenotypes, bypassing the potential promiscuity observed with endogenous or plant-derived cannabinoid signaling. Given their dense expression on neurons throughout the brain, CB1Rs have been well-studied in the context of epilepsy. Pharmacological and genetic manipulations to CB1Rs generally indicate that enhancing CB1R activity is anti-convulsive (Andres-Mach et al., 2012; Guggenhuber et al., 2010; Rudenko et al., 2012; Sugaya et al., 2016) and anti-epileptogenic (Di Maio et al., 2015; Wendt et al., 2011), although proconvulsive effects of CB1R activation have also been observed (Rudenko et al., 2012; Vilela et al., 2013; Chen et al., 2007; Chen et al., 2003). Evidence also suggests that the effects of CB1Rs on seizure phenotypes are cell-type dependent (Coiret et al., 2012; von Rüden et al., 2015), highlighting the potentially opposing effects of broad acting CB1R therapeutics.

Fewer studies have directly examined the impact of CB2R modulation on seizure susceptibility. Neither CB1R or CB2R knockout mice exhibit spontaneous seizures, however; CB1R/CB2R double knockout mice exhibit both handling-induced and spontaneous seizures, suggesting that loss of CB2Rs contributes to increased seizure susceptibility (Rowley et al., 2017). The few studies that have evaluated the effects of CB2R agonists on seizure susceptibility yielded inconsistent results. Systemic administration of the CB2R agonist, beta caryophyllene, provided modest protection against induced seizures in mice; however, another CB2R agonist, AM1241, increased seizure severity in rats (de Carvalho et al., 2016; de Oliveira et al., 2016; Tchekalarova et al., 2018). Additionally, the CB2R agonist HU-308 had no effect on induced seizures in young rats (Huizenga et al., 2017). Furthermore, the CB2R antagonist, AM630, increased seizure susceptibility in CB1R knockout mice, but not wild-type mice (Sugaya et al.,

2016). Thus, while there is evidence that modulating CB2R activity can affect acute seizure susceptibility, the relationship remains unclear.

More recent studies have implicated a role for CB2Rs in chronic epilepsy models. One study observed an increase in neuronal CB2R expression following status epilepticus (SE) in a rat model of epilepsy (Wu and Wang, 2018). A subsequent study revealed that pretreatment with a CB2R agonist reduced the severity of SE, decreased expression of apoptotic markers, and increased expression of autophagic markers, indicating that CB2Rs mediate a response to neuronal damage following brain injury (Wu et al., 2020). Most recently, treatment with the CB2R inverse agonist, SMM-189, reduced neuroinflammation and improved functional recovery following SE, an effect proposed to be mediated by a shift in microglial inflammatory phenotypes (Yu et al., 2020b). However, the effects of CB2R treatment on the full range of phenotypes observed in epilepsy models has yet to be evaluated. Studies evaluating the effects of targeting the rodent ECS in acute seizure models and chronic epilepsy are summarized in Tables **1.2** and **1.3**.

1.5 Potential advantages of CB2Rs in epilepsy

As outlined above, a large body of evidence suggests that cannabinoid based therapies can modulate disease outcomes in a number of neurological disorders, including epilepsy. Although the well-established role for CB1Rs in neuronal processes has made them an obvious target for therapeutics, there are several limitations to their clinical application. The dense expression of CB1Rs on multiple neuronal and glial subtypes, together with reports of promiscuous G-protein coupling, implies that broad-acting CB1R therapeutics could elicit both inhibitory and excitatory effects. Importantly, compounds that directly target CB1Rs are accompanied by unwanted

psychotropic effects (Volkow et al., 2014). In fact, although Δ^9 THC is a nonspecific CBR agonist, the psychoactive effects of Δ^9 THC are mediated via activity at CB1Rs. Together, this limits the therapeutic utility of CB1R-targeted drugs (Howlett et al., 2002).

In contrast, CB2Rs exhibit distinct advantages, making them well-suited to address some of the current roadblocks to effective epilepsy treatment. First, CB2R activation has functional consequences on both neuronal excitability and neuroinflammation (Jordan and Xi, 2019). While neuronal hyperexcitability has long been recognized as a hallmark of epilepsy, both central and peripheral inflammation have recently emerged as contributors to the development and maintenance of epileptic seizures, indicating that targeting inflammatory processes should also be a component of newly developed AEDs (Rana and Musto, 2018). Unfortunately, most traditional AEDs target neuronal excitability but do not reduce inflammation. In addition, newer drugs designed to specifically target inflammation are sometimes accompanied by adverse side effects (Patrono, 2016; Radu et al., 2017). Thus, CB2R-targeted treatments have the potential to combine the advantages of traditional AEDs and newer anti-inflammatory drugs.

Second, ligands targeting CB2Rs do not appear to be accompanied by psychotropic or unwanted side effects (Deng et al., 2015). This may, in part, be due to the relatively low abundance of CB2Rs on neurons at baseline conditions. The upregulation of CB2Rs that is observed in injured brain regions in models of several neurological disorders suggests that there is context-dependent activation of CB2Rs in a disease state (Jordan and Xi, 2019). This could reduce side effects resulting from unwanted receptor activation in unaffected brain regions or cell types.

Third, CB2Rs may be able to ameliorate some of the behavioral comorbidities which occur with epilepsy. As discussed above, CB2R agonists are associated with improved memory

function and functional recovery in rodent models of neurological disease. Moreover, overexpression of CB2Rs confers resistance to anxiogenic and stress inducing stimuli (García-Gutiérrez et al., 2010; Li and Kim, 2017). Accordingly, CB2R-knockout mice are more vulnerable to stressful stimuli (Ortega-Alvaro et al., 2011). Importantly, memory deficits and anxiety are two behavioral phenotypes which are often observed in epilepsy patients and mouse models.

Lastly, high levels of neuroinflammation, BBB breakdown, and neuron loss often precede the development of seizures in acquired forms of epilepsy, and are thought to contribute to their development (Rana and Musto, 2018). Furthermore, TBI and stroke are among the most common causes of acquired epilepsy in adults, with epilepsy arising from secondary injury including neuroinflammation and excitotoxicity (Webster et al., 2017; Yang et al., 2018). That CB2Rs can target these pathological features in models of acquired brain injury suggests that CB2Rs could be leveraged as an anti-epileptogenic treatment target following brain injury, an indication for which there are currently no approved drugs.

Altogether, CB2Rs have the potential to 1) provide a novel multi-modal mechanism to treat seizures; 2) exhibit a reduced side-effect profile compared to currently available AEDs; 3) ameliorate behavioral comorbidities and 4) provide a potential anti-epileptogenic target to prevent epilepsy development following brain injury.

1.6 Summary and Goals of this dissertation

In summary, CB2R-targeted therapies may be a promising approach to overcome some of the current barriers to effective epilepsy treatment, thereby providing a novel therapeutic strategy for pharmacoresistant patients. As such, the overall goal of my dissertation research was to evaluate CB2R modulation as a possible treatment in mouse models of epilepsy. This was accomplished through the use of pharmacological and genetic manipulations of CB2Rs in mouse models of both genetic and acquired epilepsy.

In chapter 2, I provide evidence that both genetic and pharmacological reduction of CB2R activity increases seizure susceptibility in mice. In chapter 3, I demonstrate that a novel positive allosteric modulator (PAM) acting at CB2Rs confers robust seizure protection in wild-type and *Scn1a* mutant mice without side effects and with a promising pharmacokinetic profile. Finally, in Chapter 4, I provide a discussion of these findings and the future work that will be necessary in order to fully evaluate CB2Rs as a treatment target for epilepsy.

Table 1.1 CB2R agonists in rodent models of ND, TBI, and stroke

Disease	Agonist	Effect	References
PD	JWH-133	↓ BBB breakdown ↓ peripheral immune cell invasion ↓ microglial activation ↓ DA neuron loss	(Chung et al., 2016)
	AM1241	↑ DA neuron regeneration	(Shi et al., 2017)
	THCV	↓ motor deficits ↓ neuron loss	(García et al., 2011)
	HU-308	↓ cytokine expression ↓ neuron loss	(Gomez-Galvez et al., 2016)
	JWH-015	↓ microglial activation ↓ neuron loss	(Price et al., 2009)
	BCP	↓ cytokine expression ↓ oxidative stress ↓ DA neuron loss	(Javed et al., 2016)
AD	MDA7	↑ memory performance normalized glutamate signaling	(Wu et al., 2017)
	JWH-133	↓ microglial activation ↓ cytokine release ↓ cognitive impairment	(Aso et al., 2013; Martín-Moreno et al., 2012)
	1-phenylisatin	improved MWM performance	(Jayant et al., 2016)
	beta-caryophyllene	improved MWM performance ↑ cerebral blood flow	(Lou et al., 2017)

Disease	Agonist	Effect	References
Stroke	AM1241	↓ infarct size improved neurological scores ↓ neurodegeneration	(Chung et al., 2016) (Yu et al., 2015)
	JWH-133	↓ infarct size ↓ neurological impairment ↓ microgliosis ↓ inflammatory cytokines	(Zarruk et al., 2012)
	O-1966	↓ infarct size ↑ motor function	(Zhang et al., 2007)
TBI	O-1966	↓ BBB breakdown ↓ neuron loss ↑ motor function ↓ microgliosis	(Amenta et al., 2012; Elliott et al., 2011)
	JWH-133	↓ inflammatory cytokines ↓ oxidative stress	(Amenta et al., 2014)
	GP1a	↓ pro-inflammatory markers ↑ anti-inflammatory markers ↑ cerebral blood flow ↑ neurological scores	(Braun et al., 2018)
	HU-910	↑ neurological scores ↓ pro-inflammatory markers	(Magid et al., 2019)

Abbreviations: PD; Parkinson's disease, BBB; blood brain barrier, DA; dopamine, AD; Alzheimer's disease, MWM; morris water maze, TBI; traumatic brain injury, BCP; beta caryophyllene

Table 1.2 Cannabinoid modulation in rodent models of acute seizures

Target	Intervention	Model	Effect	References
eCBs	AEA	MES	protects against MES	Wallace et al., 2002
	FAAH Inhibitors	MES, PTZ,	protects against MES ↑ PTZ seizure threshold	Naderi et al., 2012; Naderi et al., 2008; Vilela et al., 2013
		PTZ	↑ seizure incidence	Manna et al., 2012
	FAAH-KO	KA	↓ seizure latencies	Clement et al., 2003
	DAGL-KO	KA	↑ seizure severity	Sugaya et al., 2016
	MAGL inhibitors	PTZ	↓ seizure incidence ↑ seizure latencies	Naderi et al., 2011; Naydenov et al., 2014
		MES, PTZ	No effect	Griebel et al., 2015
CB1R	global CB1R-KO	KA	↑ seizure severity	Marsicano et al., 2003; Sugaya et al., 2016
	conditional CB1R-KO	kindling (acute)	↓ seizure progression w/ inhibitory neuron KO, ↑ seizure progression w/excitatory neuron KO	von Rüden et al., 2015
	viral CB1R overexpression	KA	↓ seizure severity	Guggenhuber et al., 2010

Target	Intervention	Model	Effect	References
CB1R	CB1R agonists	KA, PTZ	↓ PTZ seizure threshold ↓ KA seizure severity	Andres-Mach et al., 2012; Naderi et al.,2012; Rudenko et al., 2012
		PTZ, KA	↑ seizure severity	Rudenko et al., 2012; Vilela et al., 2013
		PTZ	↑ seizure susceptibility	Huizenga et al., 2017
	CB1R antagonists	FS	↓ neuronal hyperexcitability	Chen et al., 2007; Chen et al., 2003
CB2R	agonist (BCP)	MES, KA, PTZ	↓ seizure severity KA protects against MES ↓ seizure latency PTZ	de Oliveira et al., 2016; Tchekalarova et al., 2018
	agonist (AM1241)	PTZ	↑ seizure severity ↓ seizure latency	de Carvalho et al., 2016
	agonist (HU308)	PTZ	no effect	Huizenga et al., 2017
	antagonist (AM630)	PTZ	↑ seizure susceptibility in CB1R-KO mice	Sugaya et al., 2016
	agonist (JWH-133), antagonist (SR144528), CB2R-KO	PTZ	No effect of agonist ↓ seizure latency w/ antagonist ↓ seizure latency w/ KO	Shapiro et al., 2019
	PAM (Ec21a)	PTZ, 6 Hz, flurothyl	↓ seizure susceptibility	Shapiro et al., 2021

Abbreviations: MES; maximal electroshock, PTZ; pentylenetetrazol, KA; kainic acid, FS; febrile seizures, BCP; beta-caryophyllene, KO; knockout, PAM, positive allosteric modulator

Table 1.3 Cannabinoid modulation in rodent models of chronic epilepsy

Target	Intervention	Model	Effect	References
eCBs	DAGL-KO	KA	↑ spontaneous seizure frequency	Sugaya et al., 2016
	MAGL inhibitors	kindling	↑ latency to and ↓ frequency of spontaneous seizures	Griebel et al., 2015; von Rüden et al., 2015
		pilocarpine	↑ spontaneous seizure frequency and duration	Ma et al., 2014
	FAAH inhibitors	KA	↓ SE-induced neuronal loss	Mikheeva et al., 2017
CB1Rs	CB1R agonist	kindling	↑ latency to spontaneous seizures	Vonogradova et al., 2015
		pilocarpine	anti-epileptogenic neuroprotective	Di Maio et al., 2015
		KA	↓ spontaneous seizure frequency and severity	Wallace et al., 2002
CB2Rs	CB2R agonist	pilocarpine	↓ SE severity ↓ apoptotic markers ↑ autophagic markers	Wu et al., 2020
	CB2R inverse agonist	pilocarpine	↑ functional recovery ↓ neuroinflammation	Yu et al., 2020b

Abbreviations:, KO; knockout, SE; status epilepticus

CHAPTER 2

REDUCED CANNABINOID 2 RECEPTOR ACTIVITY INCREASES SUSCEPTIBILITY TO INDUCED SEIZURES IN MICE

Adapted with permission from:

Shapiro, L., Wong, J. C., Escayg, A. (2019) Reduced cannabinoid 2 receptor activity increases susceptibility to induced seizures in mice. *Epilepsia* 60, 2359-2369. doi: 10.1111/epi.16388

2.1 Summary

Despite the promising evidence that CB2Rs can modulate physiological processes involved in epilepsy, few studies had investigated the relationship between CB2R modulation and seizure thresholds at the time I began my dissertation research. Thus, in the experiments outlined in this chapter, I describe the consequences of reduced CB2R activity on susceptibility to induced seizures in mice.

We found that CB2R knockout mice were seizure susceptible, an effect which was recapitulated pharmacologically with the CB2R antagonist, SR144528. Together, this data established our first link between CB2R modulation and seizures. Given these results, we were prompted to evaluate whether administration of a CB2R agonist might increase seizure resistance; however, we did not observe any effect. While unexpected, this result opened the door for future studies evaluating how different classes of CB2R-targeted drugs might differentially impact seizure phenotypes. Finally, to evaluate whether CB2R modulation may also be impactful in a mouse model of epilepsy, we examined seizure susceptibility in *Cnr2* mutants harboring the human *SCN1A* R1648H (RH) epilepsy mutation. In offspring from a cross between the *Cnr2* x RH lines, seizure susceptibility was not significantly increased in mutants expressing both mutations. No spontaneous seizures were observed in either RH or *Cnr2/*RH mutants during 336-504 hours of continuous EEG recordings.

Overall, this study increased our knowledge of the relationship between CB2R modulation and seizure susceptibility, an area which had been understudied at the time of publication. The manuscript resulting from this work is reproduced below with minor alterations.

2.2 Introduction

Over 30% of individuals with epilepsy are refractory to currently available medications, highlighting the need to develop more efficacious treatments (French, 2007). The endocannabinoid system (ECS), comprised of cannabinoid receptors, endocannabinoids (eCBs), and regulatory enzymes is currently under investigation as a potential therapeutic target for the treatment of several neurological disorders, including epilepsy (Brodie and Ben-Menachem, 2018; Kaur et al., 2016; O'Connell et al., 2017). Cannabidiol (CBD), a phytocannabinoid derived from the *Cannabis sativa* plant, recently received FDA approval for the treatment of Dravet syndrome (DS), a catastrophic, early-onset epilepsy (Devinsky et al., 2017; Devinsky et al., 2016). DS is most often caused by mutations in the *SCN1A* gene, encoding the voltage-gated sodium channel (VGSC) $Na_v1.1$. VGSCs play an important role in regulating neuronal excitability, and *SCN1A* mutations have been associated with a variety of seizure disorders (Escayg et al., 2000; Fujiwara, 2006; Mulley et al., 2005). The recent success of the CBD clinical trials in DS suggests a potential role for cannabinoids in other forms of epilepsy (Devinsky et al., 2017). However, CBD treatment can be associated with adverse effects, and is not effective in all patients (Devinsky et al., 2016; Iffland and Grotenhermen, 2017). As such, more specific therapies may serve to improve seizure control while reducing off-target side effects.

Although it is well documented that modulation of cannabinoid 1 receptors (CB1Rs) can improve seizure outcomes, activation of CB1Rs can be accompanied by several psychotropic effects, limiting their potential as a therapeutic target (Blair et al., 2006; Kow et al., 2014; Luszczki et al., 2006; Pertwee, 2012; van Amsterdam et al., 2015). In contrast, cannabinoid 2 receptors (CB2Rs) have received less attention for their role in the CNS. However, several recent studies have described CB2R expression throughout the brain, including regions associated with

seizure generation, such as the hippocampus, cortex, and cerebellum (Chen et al., 2017; Li and Kim, 2015; Liu et al., 2009; Onaivi et al., 2006). Although the cell-specific pattern of CB2R expression in the brain remains controversial, both microglial and neuronal expression have been described (Li and Kim, 2015; Lopez et al., 2018; Schmole et al., 2015; Stempel et al., 2016). Furthermore, CB2Rs are highly upregulated in response to brain injury in several mouse models (Concannon et al., 2016; Yu et al., 2015), including a rat model of epilepsy, suggesting that CB2Rs may play a protective role following injury (Wu and Wang, 2018).

To elucidate the effect of modulating CB2R activity on seizure susceptibility, we examined susceptibility to induced seizures in a CB2R knockout mouse line *(Cnr2*$^{-/-}$) using several seizure induction paradigms. We also administered a CB2R agonist or antagonist to wild-type mice prior to seizure induction in order to determine whether pharmacological manipulation of CB2Rs could similarly alter seizure susceptibility. Furthermore, in order to better understand the potential role of CB2Rs in *SCN1A*-derived epilepsy, we examined seizure susceptibility in the offspring from a cross between *Cnr2* mutant mice and a mouse line expressing the human *SCN1A* R1648H (RH) mutation. The RH mutation was previously identified in a family with genetic epilepsy with febrile seizures plus (GEFS+) (Escayg et al., 2000; Martin et al., 2010). Patients with GEFS+ exhibit a wide range of seizure phenotypes, including absence seizures, myoclonic seizures, and both febrile and afebrile seizures (Escayg et al., 2000). Previous work from our laboratory demonstrated that heterozygous *Scn1a*$^{RH/+}$ mice are susceptible to induced seizures and exhibit infrequent spontaneous seizures (Martin et al., 2010). The results of this study demonstrate that reducing CB2R activity increases seizure susceptibility and support a potential role for CB2Rs as a therapeutic target for the development of anti-epileptic drugs.

2.3 Materials and Methods

Animals

CB2R knockout mice (*Cnr2*$^{-/-}$) were purchased (Jackson Laboratories, Stock No.005786) and backcrossed to the C57BL/6J background (Jackson Laboratories, Stock No. 000664) for one generation. Resulting heterozygous mice from this cross were then mated to generate the experimental generation. Heterozygous *Scn1a*$^{RH/+}$ mice harboring the human *SCN1A* R1648H mutation were generated as previously described and maintained on a C57BL/6J background (Martin et al., 2010). To generate *Cnr2*$^{-/-}$ and *Scn1a*$^{RH/+}$ double-mutants, *Scn1a*$^{RH/+}$ mice were first bred to *Cnr2*$^{-/-}$ mutants. Heterozygous *Cnr2*$^{+/-}$ offspring that also expressed the RH mutation were then crossed to *Cnr2*$^{+/-}$ heterozygous mutants to generate six possible genotypes: *Cnr2*$^{+/+}$/*Scn1a*$^{+/+}$ (WT), *Cnr2*$^{+/+}$/ *Scn1a*$^{RH/+}$ (RH), *Cnr2*$^{+/-}$/*Scn1a*$^{+/+}$/ (*Cnr2*$^{+/-}$), *Cnr2*$^{+/-}$/*Scn1a*$^{RH/+}$ (*Cnr2*$^{+/-}$/RH), *Cnr2*$^{-/-}$/*Scn1a*$^{+/+}$ (*Cnr2*$^{-/-}$), and *Cnr2*$^{-/-}$/*Scn1a*$^{RH/+}$ (*Cnr2*$^{-/-}$/RH). Mice were housed in groups of 3-5 on a 12-hour light/dark cycle with standard laboratory rodent chow (Lab Diet, #5001) and water available *ad libitum*. Animal housing facilities were maintained at 69-72 °F and 30-70% humidity. All experiments were performed in accordance with the Emory University Institutional Animal Care and Use Committee guidelines. Furthermore, the principles outlined in the ARRIVE guidelines and the Basel declaration were implemented in the planning of all experiments (Kilkenny et al., 2010). Unless otherwise stated, adult 8-12 week old mutant animals and wild-type (WT) littermates (20-25 g) were used for all experiments. For all experiments, animals were randomly distributed to groups, and experimenters were blinded to treatment group during data analysis.

Genotyping

Genotyping was performed prior to all experiments. To screen for *Cnr2*$^{-/-}$ mutants, tail DNA was amplified with the following primers: Mutant Forward-GGGGATCGATCCGTCCTGTAAGTCT, WT Forward-GGAGTTCAACCCCATGAAGGAGTAC, and Reverse-GACTAGAGCTTTGTAGGTAGGCGGG. Amplification was performed for 35 cycles of: 94 °C for 30 s, 60 °C for 60 s, and 72 °C for 30 s. Wild-type animals yielded a 385 bp PCR product, heterozygous animals (*Cnr2*$^{+/-}$) yielded 385 bp and 550 bp PCR products, and homozygous animals (*Cnr2*$^{-/-}$) generated a single 550 bp PCR band.

To screen for the *Scn1a*$^{RH/+}$ mutation, tail DNA was amplified using the following primers: Forward- TTGATGACTTCTTCACTGATTGAT, Reverse-AGAGGCTCTGCACTTTCTTC. Amplification was performed for 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. The PCR product was digested with EcoRI to distinguish between wild-type (591 bp) and mutant (461 bp).

Pentylenetetrazole (PTZ)-Induced Seizures

Seizures were induced using the chemiconvulsant pentylenetetrazole (PTZ) as previously described (Loscher et al., 1991). Mice were administered PTZ subcutaneously (100 mg/kg, Sigma-Aldrich) and placed in a plexiglass chamber. The latency to the first myoclonic jerk (MJ) and generalized tonic-clonic seizure (GTCS) were recorded over a 30-minute observation period. N= 8-12/group.

6 Hz-Induced Seizures

Seizures induced by the 6 Hz paradigm were conducted as previously described (Wong et al., 2016; Wong et al., 2019). Thirty minutes prior to seizure induction, the topical anesthetic proparacaine hydrochloride ophthalmic solution (Patterson Veterinary) was applied to each eye. Mice were subjected to corneal electrostimulation (6 Hz, 2 ms pulse width, 3 s duration) at 18 mA using an ECT unit (Ugo Basile; Comerio, Italy). Behavioral seizures were scored using a modified Racine Scale: 0- no seizure, 1- staring/immobile > 3 s, 2- forelimb clonus, and 3- rearing and falling. N= 16-30/group.

Flurothyl-Induced Seizures

Thresholds to flurothyl induced seizures were determined as previously described (Dutton et al., 2017). Briefly, mice were placed in a plexiglass chamber (13.5 x 8 x 6 inches), and exposed to flurothyl (Bis(2,2,2-trifluoroethyl) ether, Sigma Aldrich) at a rate of 20 µL/minute. Latencies to the first MJ and GTCS were recorded. N= 8-12/group.

Kainic Acid (KA)-Induced Seizures

Mice were administered kainic acid (intraperitoneal, i.p. 25 mg/kg, Sigma-Aldrich) and behavioral seizures were recorded over a 2-hour period. Seizures were scored on a modified Racine Scale: 0 – no behavior, 1 – freezing/staring, 2- head nodding, 3 – tail clonus, 4 – forelimb clonus, 5 – rearing and falling, and 6 – death. N= 8/group.

Hyperthermia-Induced Seizures (a model of febrile seizure susceptibility)

Susceptibility to hyperthermia-induced seizures was determined as previously described (Oakley et al., 2009; Wong et al., 2016). Temperature was monitored using a rectal temperature probe connected to a heating lamp and temperature controller (TCAT 2DF, Physitemp, Clifton, NJ, USA). Mice aged P14 or P21 were placed in a clear cylinder, and the core body temperature of each mouse was increased by 0.5 ºC every two minutes until either a GTCS occurred or 42.5 ºC was reached. The temperature at which each mouse exhibited a GTCS was recorded. Behavioral seizures were scored on a modified Racine Scale: 1 – staring, 2 – head nodding, 3 – unilateral forelimb clonus, 4 – bilateral forelimb clonus, 5 – GTCS. N= 8/group.

Drug Treatment

For pharmacological studies, the CB2R antagonist, SR144528 (3 mg/kg, Tocris), or vehicle (0.9% saline with 5% tween 80) was administered by i.p. injection 30 minutes prior to seizure induction. The CB2R agonist, JWH-133 (3 mg/kg, Tocris), or vehicle (0.9% saline with 5% tween 80) was administered by i.p. injection 30 minutes prior to seizure induction.

EEG Surgery and Analysis

Electroencephalogram (EEG) electrodes were surgically implanted in adult RH and *Cnr2*$^{-/-}$/RH mice as previously described (Lamar et al., 2017; Wong et al., 2018). All mice were administered meloxicam immediately prior to the surgical procedure (5 mg/kg, Patterson Veterinary). Anesthesia was maintained with approximately 1.5% isoflurane (Patterson Veterinary) throughout the surgical procedure. Four bipolar stainless-steel screws (Vintage Machine Supplies) were implanted at the following coordinates relative to Bregma: anterior-

poster (AP) + 0.5 mm and medial-lateral (ML) -2.2 mm; AP + 2.0 mm and ML + 1.2 mm; AP – 3.5 mm and ML – 2.2 mm; AP – 1.5 mm and ML + 1.2 mm. Mice were allowed to recover from surgery for one week prior to beginning EEG recordings. Two to three continuous weeks of electrographic recordings were obtained for each mouse. EEG signals were analyzed with Stellate Harmonie EEG software using a high pass filter of 5 Hz and a low pass filter of 35 Hz. Seizure activity was defined as synchronous discharges of increased amplitude at least twice the background and ≥ 3 seconds in duration. N= 4/group.

Data Analysis

All data was analyzed using GraphPad Prism version 6 or 7 (GraphPad Software). Latencies to PTZ and flurothyl-induced seizures in *Cnr2* mutant mice were analyzed using 1-way ANOVA followed by Tukey's *post-hoc* comparison. Analysis of the percent of mice reaching a GTCS following PTZ administration was performed using the Mantel-Cox log-rank test. A 2-way rANOVA followed by a Bonferroni *post-hoc* comparison was used to analyze the time course of KA-induced seizure severity in *Cnr2* mutant mice. Behavioral seizures in the 6 Hz paradigm were analyzed with a Kruskall-Wallis test followed by Dunn's multiple comparisons. A 2-way ANOVA with Tukey's post-hoc comparison was used to compare the effect of agonist or antagonist treatment and genotype on seizure susceptibility, as well as differences between seizure susceptibility in *Cnr2*/RH double mutants. Differences between groups were considered to be significant if $P < 0.05$.

2.4 Results

2.4.1 *Cnr2* mutant mice exhibit increased susceptibility to induced seizures

We first compared seizure susceptibility between male *Cnr2* mutants and WT littermates using the PTZ seizure induction paradigm. $Cnr2^{+/-}$ and $Cnr2^{-/-}$ mutants exhibited significantly lower latencies to the first MJ ($P < 0.001$, **Fig 2.1 A**) and GTCS ($Cnr2^{+/-}$, $P < 0.01$; $Cnr2^{-/-}$, $P < 0.001$, **Fig 2.1 B**) compared to WT littermates. The percent of mice reaching GTCS over time in $Cnr2^{+/-}$ and $Cnr2^{-/-}$ mutants was also significantly different from WT littermates ($P < 0.001$, **Fig 2.1 C**). Seizure latencies did not significantly differ between $Cnr2^{+/-}$ and $Cnr2^{-/-}$ mutants (**Fig 2.1 1A-C**).

We then examined seizure susceptibility using the 6 Hz seizure induction paradigm. Male $Cnr2^{-/-}$ mice exhibited a higher average Racine score compared to WT littermates following an 18 mA stimulus, indicating a more severe seizure response ($P < 0.05$, **Fig 2.1 D)**. Heterozygous mutants did not differ significantly from WT littermates or homozygous mutants.

Susceptibility to seizures induced by flurothyl (**Fig 2.2 A-B**) and KA (**Fig 2.2 C**) were comparable between *Cnr2* mutant mice and WT littermates. To determine whether *Cnr2* mutant mice were susceptible to febrile seizures, we investigated the effect of hyperthermia on seizure generation in mutants and WT littermates at P14 and P21. Neither WT nor mutant mice exhibited hyperthermia-induced seizures at either age.

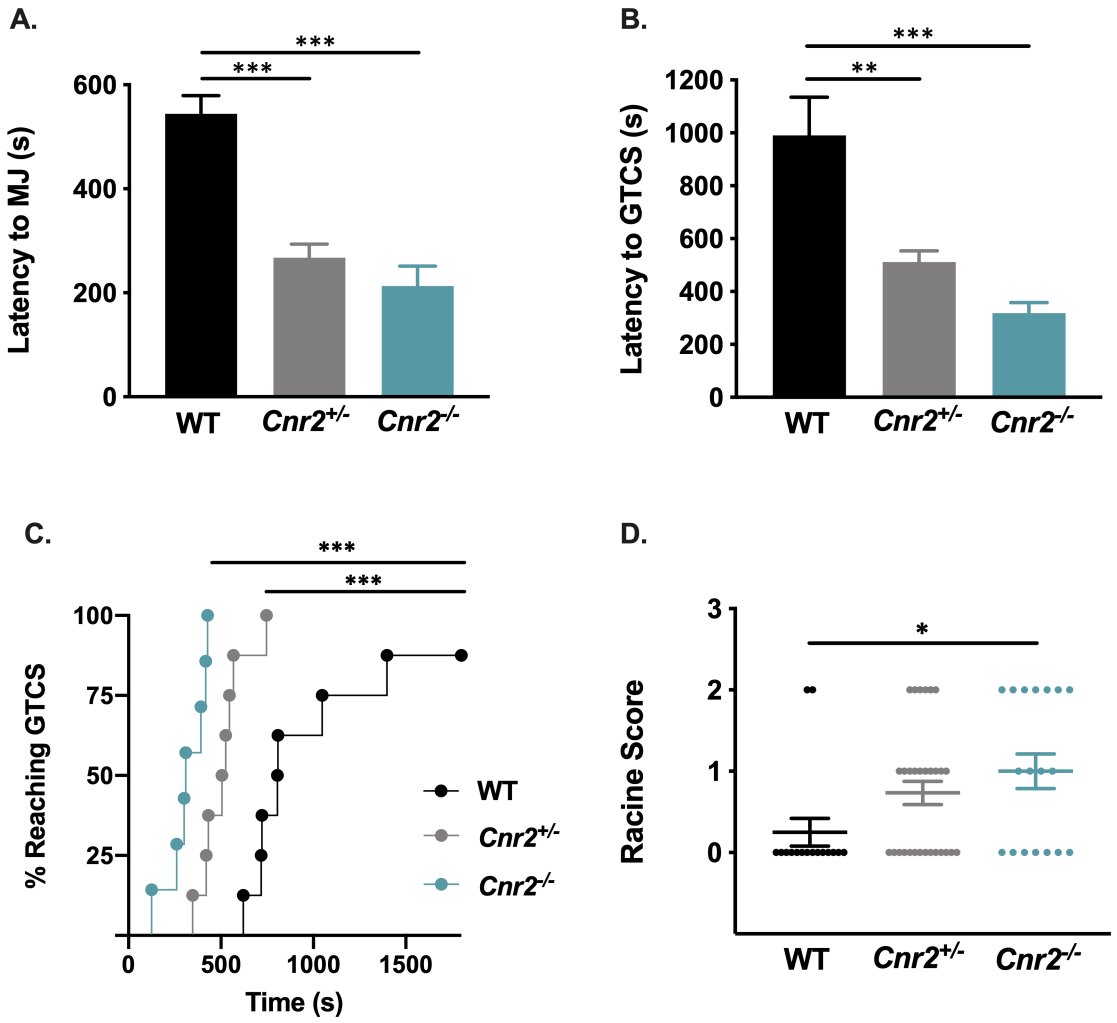

Figure 2.1. *Cnr2* mutant mice exhibit increased susceptibility to induced seizures. **A.** *Cnr2*$^{+/-}$ and *Cnr2*$^{-/-}$ mice exhibit significantly reduced latencies to the first MJ (**A**) and GTCS (**B**) following PTZ administration compared to WT littermates. 1-way ANOVA followed by Tukey's post-hoc comparison, n= 8/group. **C.** Percent of *Cnr2*$^{+/-}$ and *Cnr2*$^{-/-}$ mutants reaching GTCS, and latency to GTCS, are significantly different from WT littermates. Mantel-Cox log-rank test. **D.** *Cnr2*$^{-/-}$ mice exhibit higher average Racine scores when compared to WT littermates following an 18 mA electrical stimulus. Each symbol represents one mouse. Kruskall-Wallis Test with Dunn's multiple comparisons. WT, n=16; *Cnr2*$^{+/-}$, n=30; *Cnr2*$^{-/-}$, n=18. For **A-D**, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. All error bars represent mean +/- SEM.

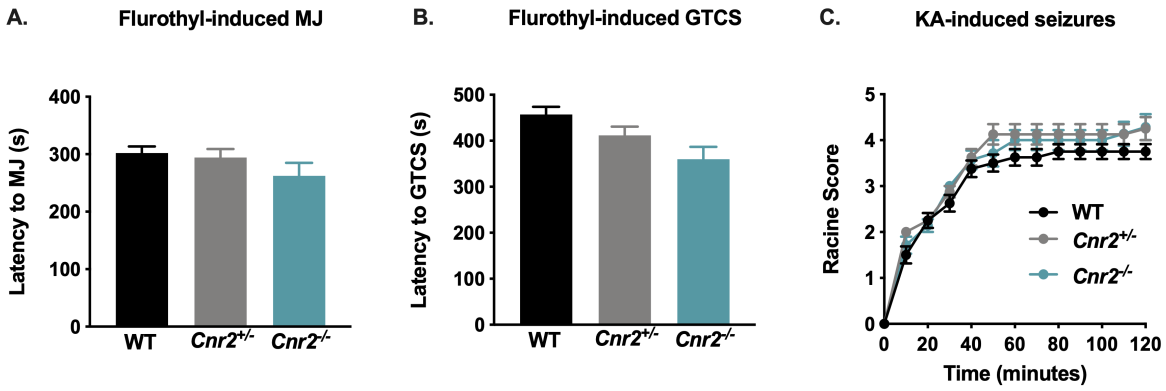

Figure 2.2. *Cnr2* mutant mice are not susceptible to flurothyl- or kainic acid-induced seizures. Latency to the first MJ (**A**) and GTCS (**B**) was comparable between genotypes following flurothyl exposure. 1- way ANOVA with Tukey's post-hoc comparison, n=8/group. **C.** The response of *Cnr2* mutant mice to KA was similar to WT littermates. 2-way rANOVA with Bonferroni post-hoc comparison, n= 8/group. All error bars represent mean +/- SEM.

2.4.2 The CB2R specific antagonist, SR144528, increases seizure susceptibility in WT mice

To determine whether the results observed with the *Cnr2* mutants could be recapitulated with pharmacological methods, we administered the CB2R antagonist, SR144528, to male *Cnr2*$^{-/-}$ and WT littermates 30 minutes prior to PTZ administration. Latencies to the first MJ and GTCS were significantly lower in WT mice treated with SR144528 compared to vehicle-treated mice (MJ, $P < 0.001$; GTCS, $P < 0.05$, **Fig 2.3 A-B**). SR144528 treatment had no effect on latency to the MJ or GTCS in *Cnr2*$^{-/-}$ mice, indicating that the effects of SR144528 are CB2R-specific (**Fig 2.3 A-B**). Vehicle-treated *Cnr2*$^{-/-}$ mutants exhibited decreased latencies to both the MJ and GTCS when compared to vehicle-treated WT littermates ($P < 0.001$, **Fig 2.3 A-B**), consistent with the data shown in **Fig 2.1 A-B**.

We also tested whether SR144528 could impact seizure severity using the 6 Hz seizure induction paradigm. Consistent with the results observed following PTZ administration, WT mice administered SR144528 exhibited significantly higher Racine scores compared to vehicle-treated WT mice ($P < 0.05$, **Fig 2.3 C**). SR144528 had no effect on susceptibility to 6 Hz-induced seizures in *Cnr2*$^{-/-}$ mice (**Fig 2.3 C**).

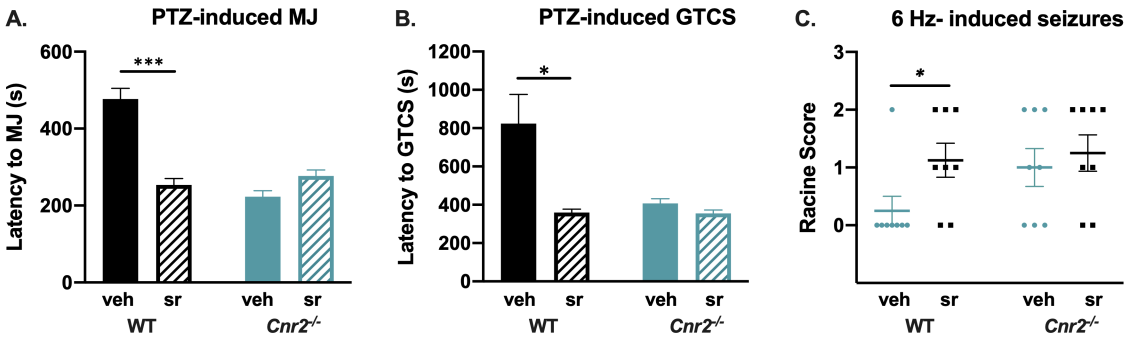

Figure 2.3. The CB2R specific antagonist, SR144528, increases seizure susceptibility. A. SR144528 significantly reduced latencies to the first MJ following PTZ administration in WT mice compared to vehicle-treated controls. **B.** WT mice treated with SR144528 exhibit significantly reduced latencies to the first GTCS following PTZ administration compared to vehicle-treated WT mice. SR144528 treatment did not affect latencies to the first MJ or GTCS in *Cnr2*$^{-/-}$ mutants. 2-way ANOVA with Tukey's post-hoc comparison, n=8/group. **C.** WT mice treated with SR144528 exhibit significantly higher average Racine scores when compared to vehicle-treated WT mice. SR144528 treatment did not affect susceptibility to 6 Hz-induced seizures in *Cnr2*$^{-/-}$ mutants. Each symbol represents one mouse. 2-way ANOVA with Tukey's post hoc comparison, n= 8/group. $*P < 0.05$, $***P < 0.001$. Veh, vehicle; sr, SR144528. All error bars represent mean +/- SEM.

2.4.3 The CB2R specific agonist, JWH-133, does not increase resistance to PTZ-induced seizures

To determine whether increasing CB2R activity could be seizure protective, we administered the CB2R selective agonist, JWH-133, to WT, *Cnr2*$^{+/-}$, and *Cnr2*$^{-/-}$ mice 30 minutes prior to inducing seizures with PTZ. As expected, vehicle-treated *Cnr2*$^{-/-}$ and *Cnr2*$^{+/-}$ mutants show reduced latencies to both the MJ and GTCS when compared to vehicle-treated WT littermates; however, JWH-133 did not have any effect on seizure susceptibility regardless of genotype (**Fig 2.4**).

2.4.4 Deletion of CB2Rs does not worsen seizure phenotypes in *SCN1A* mutant mice

We next examined whether decreasing CB2R expression would influence seizure susceptibility in a mouse model of *SCN1A* derived epilepsy. To do so, we crossed *Cnr2* mutant mice to a mouse line harboring the human R1648H *SCN1A* mutation, and subsequently evaluated susceptibility to PTZ-induced seizures in male and female offspring of each genotype. Male and female mutants of all genotypes exhibited significantly lower average latencies to the first MJ and GTCS when compared to WT littermates (**Fig 2.5**). Further, male *Cnr2*$^{-/-}$/RH mutant mice exhibited significantly reduced latencies to the MJ compared to RH mutant mice ($P < 0.001$, **Fig 2.5 A**), although average latency to the first GTCS was not significantly different (**Fig 2.5 B**). Female *Cnr2*$^{-/-}$/RH mice did not show significantly altered latencies to either the MJ or GTCS when compared to female RH mutants (**Fig 2.5 C-D**). These results indicate that although reduced *Cnr2* expression and the *SCN1A* R1648H mutation both result in increased seizure susceptibility, the absence of *Cnr2* does not exacerbate seizure susceptibility in *Scn1a* mutants.

To determine whether the results observed with PTZ were paradigm specific, we also examined the effect of co-expression of both mutations on susceptibility to flurothyl-induced seizures. Male *Cnr2*$^{+/-}$/RH mutants showed significantly reduced latencies to the MJ when compared to RH mutants ($P < 0.05$, **Fig 2.6 A**), but did not show significantly different latencies to the GTCS (**Fig 2.6 B**). *Cnr2*$^{-/-}$/RH male mice did not show any significant differences in seizure latencies when compared to RH-only mutants (**Fig 2.6 A-B**). Female *Cnr2*$^{-/-}$/RH mutants showed significantly reduced latencies to the MJ when compared to RH mutants ($P < 0.05$, **Fig 2.6 C**), but did not show significant differences in latency to the GTCS (**Fig 2.6 D**). Similar to the observations with PTZ, these results indicate that the absence of *Cnr2* does not consistently alter susceptibility to flurothyl-induced seizures in *SCN1A* mutant mice.

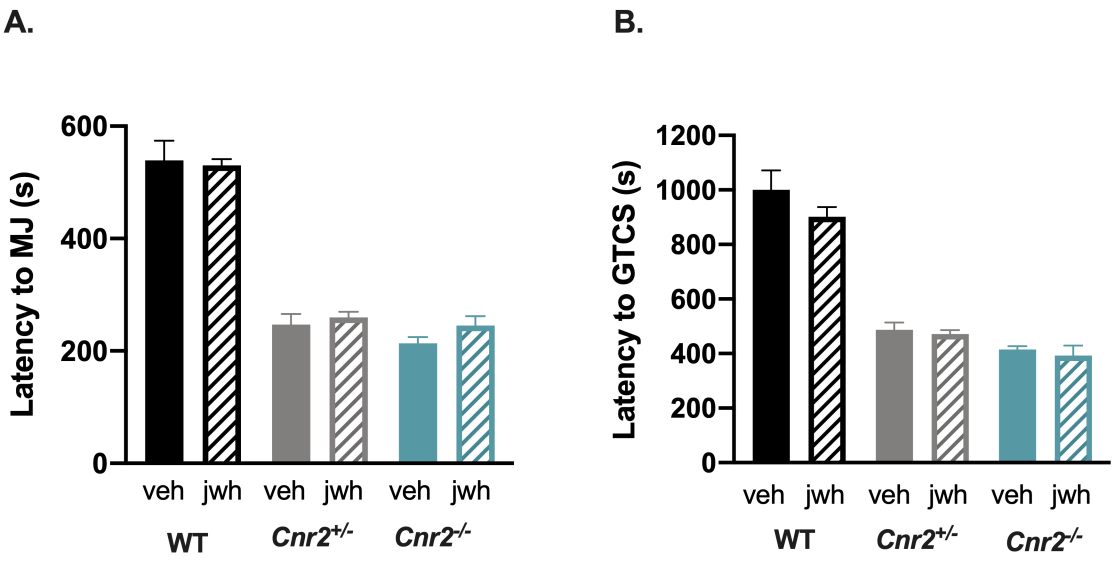

Figure 2.4. The CB2R specific agonist, JWH-133, does not alter susceptibility to PTZ-induced seizures. The CB2R agonist JWH-133 did not significantly alter latencies to the first MJ (**A**) or GTCS (**B**) in WT, *Cnr2*$^{+/-}$ or *Cnr2*$^{-/-}$ mutant mice. 2-way ANOVA with Tukey's post-hoc comparison, n=8/group. Veh, vehicle; jwh, JWH-133. All error bars represent mean +/- SEM.

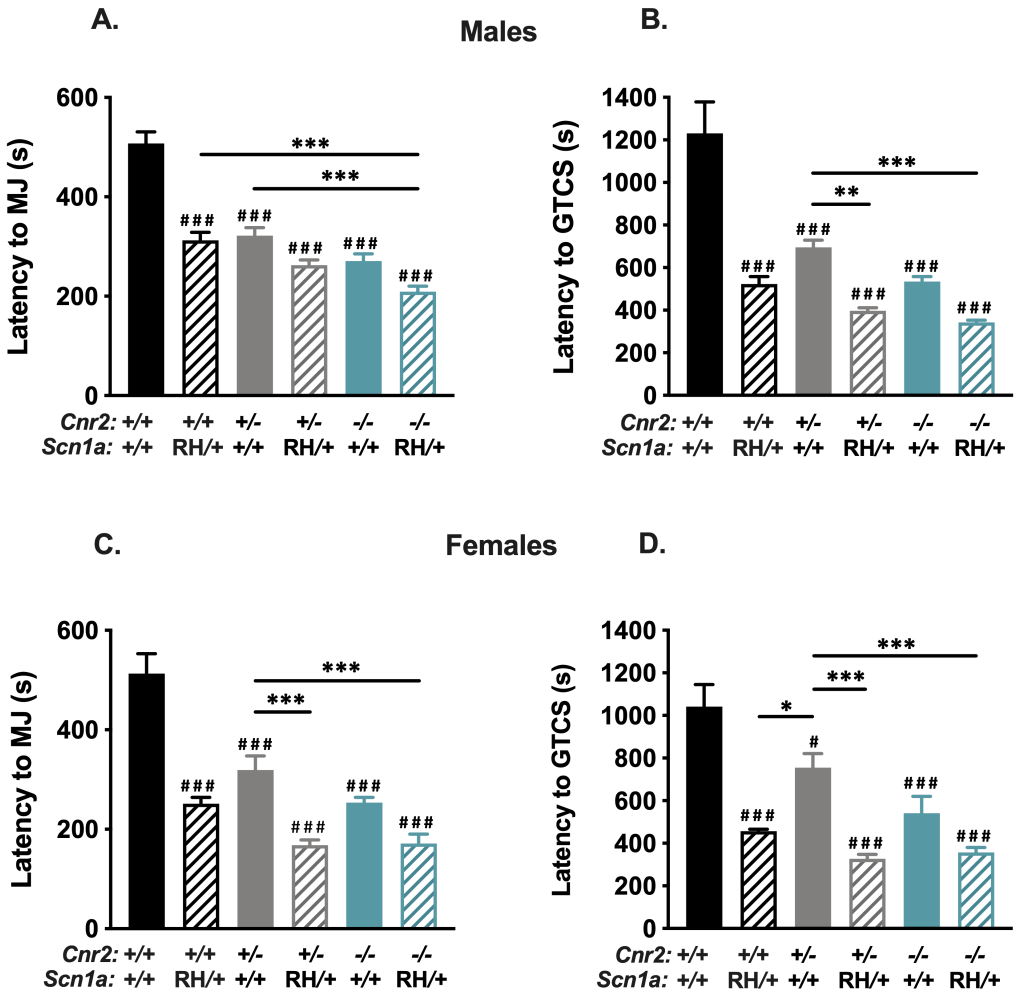

Figure 2.5. Deletion of CB2Rs does not worsen PTZ-induced seizures in *Scn1a*$^{RH/+}$ mutant mice. All groups of mutant male (**A-B**) and female (**C-D**) mice exhibited reduced average latencies to both the MJ and GTCS compared to WT. **A.** Male *Cnr2*$^{-/-}$/RH mutants show decreased latency to the MJ compared to both *Cnr2*$^{+/-}$ and RH. **B.** Both *Cnr2*$^{+/-}$/RH and *Cnr2*$^{-/-}$/RH male mice exhibit decreased latency to GTCS when compared to *Cnr2*$^{+/-}$ mice. **C.** Female *Cnr2*$^{+/-}$/RH and *Cnr2*$^{-/-}$/RH mice exhibit decreased latencies to the MJ when compared to *Cnr2*$^{+/-}$ mutant mice. **D.** Female *Cnr2*$^{+/-}$/RH and *Cnr2*$^{-/-}$/RH mice exhibit decreased latencies to the GTCS when compared to *Cnr2*$^{+/-}$ mutant mice. RH female mice exhibit decreased latency GTCS when compared to *Cnr2*$^{+/-}$ mutant mice. 2-way ANOVA with Tukey's post-hoc test, n=8-12/group. # denotes statistical significance from WT. $^{*/\#}P < 0.05$, $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$. All error bars represent mean +/- SEM.

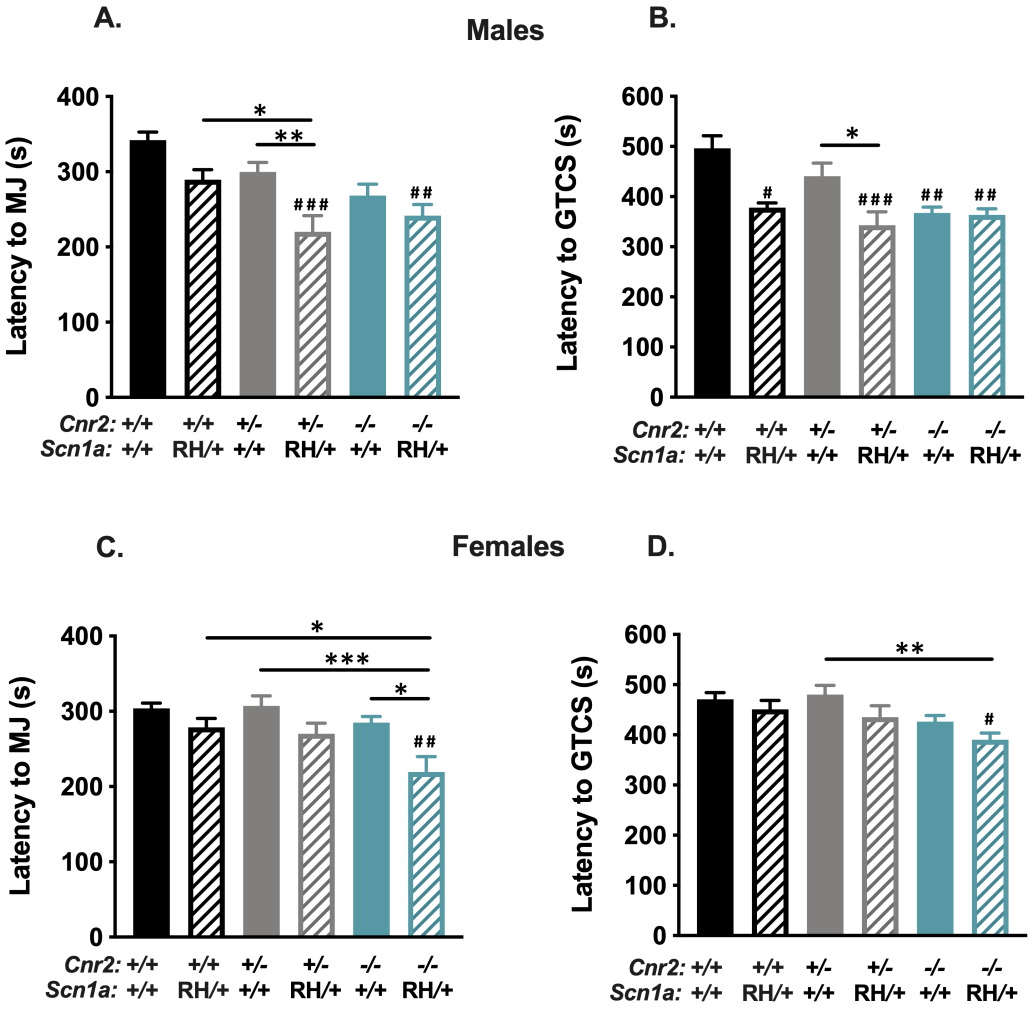

Figure 2.6. Deletion of CB2Rs does not worsen flurothyl-induced seizures in *Scn1a*$^{RH/+}$ mutant mice. **A.** Male *Cnr2*$^{-/-}$/RH and *Cnr2*$^{+/-}$/RH mutants exhibit decreased latency to the MJ when compared to WT. *Cnr2*$^{+/-}$/RH mutants also show decreased latency to the MJ when compared to RH and *Cnr2*$^{+/-}$. **B.** When compared to WT, RH, *Cnr2*$^{+/-}$/RH, *Cnr2*$^{-/-}$ and *Cnr2*$^{-/-}$ /RH male mutants exhibit significantly decreased latencies to GTCS. *Cnr2*$^{+/-}$/RH mutants exhibit significantly decreased latency to the GTCS compared to *Cnr2*$^{+/-}$ mice. **C.** *Cnr2*$^{-/-}$ /RH female mice show significantly decreased latency to the MJ when compared to WT, RH, *Cnr2*$^{+/-}$, and *Cnr2*$^{-/-}$ mice. **D.** *Cnr2*$^{-/-}$ /RH female mice exhibit decreased latency to the GTCS when compared to WT and *Cnr2*$^{+/-}$ mutant mice. 2-way ANOVA with Tukey's post hoc comparison, n=8-12/group. # denotes statistical significance from WT. $^{*/\#}P < 0.05$, $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$. Error bars represent mean +/- SEM.

2.4.5 Deletion of CB2Rs does not increase spontaneous seizure frequency in mice expressing the *SCN1A* R1648H mutation

We previously demonstrated that heterozygous RH mutant mice exhibit infrequent spontaneous seizures (Martin et al., 2010). In order to test whether the lack of CB2Rs could increase spontaneous seizure frequency, length, or severity in RH mice, we performed continuous EEG recordings for 336-504 hours in RH and *Cnr2*$^{-/-}$/RH mutant mice. Spontaneous seizures were not detected in any mouse during the recording period (data not shown. n= 4/genotype).

2.5 Discussion

We show that the loss of CB2Rs results in increased susceptibility to PTZ- and 6 Hz-induced seizures, and administration of the CB2R antagonist, SR144528, in WT mice increases seizure susceptibility, suggesting that increasing CB2R expression or activity may confer seizure protection. Interestingly, while both heterozygous (*Cnr2*$^{+/-}$) and homozygous (*Cnr2*$^{-/-}$) mutants exhibited increased susceptibility to PTZ-induced seizures, there was no significant difference between *Cnr2*$^{+/-}$ and *Cnr2*$^{-/-}$ mutants, indicating that partial reduction of CB2R activity is sufficient to increase seizure susceptibility. While *Cnr2*$^{+/-}$ mutants presumably have 50% loss of CB2R protein compared to WT littermates, the lack of reliable CB2R antibodies makes it difficult to quantify differences in protein levels between genotypes (Baek et al., 2013; Zhang et al., 2019).

Studies which have previously examined the effects of CB2R compounds on seizure phenotypes have yielded inconsistent results (de Carvalho et al., 2016; de Oliveira et al., 2016; Huizenga et al., 2017; Sugaya et al., 2016; Tchekalarova et al., 2018). Although some studies

reported that the CB2R agonist beta caryophyllene can be seizure protective (de Oliveira et al., 2016; Tchekalarova et al., 2018), other studies observed either no effect of a CB2R agonist (HU308) (Huizenga et al., 2017), or greater seizure severity following administration of the CB2R agonist AM1241 (de Carvalho et al., 2016). We found no effect of the CB2R agonist, JWH-133, on PTZ-induced seizures (**Fig 2.4**). These inconsistencies may be due to differences in study design, including seizure induction paradigm, species, and the specific agonist or antagonist tested. We selected JWH-133 and SR144528 for our studies because they are highly potent and CB2R-specific, exhibiting few off-target effects in vitro (Huffman, 2005; Rinaldi-Carmona et al., 1998; Soethoudt et al., 2017b). Furthermore, at doses similar to those used in our study, JWH-133 has shown therapeutic effects in the brain including attenuation of stress-induced neuroinflammation (Zoppi et al., 2014), and reduction of post-surgical cognitive impairment and associated neuroinflammation (Sun et al., 2017). In addition, administration of SR144528 at doses comparable to that in the current study was associated with detrimental effects in the brain, including increased infarct size and delayed motor recovery following stroke (Bravo-Ferrer et al., 2017; Zarruk et al., 2012). Thus, both drugs have been shown to be effective at doses similar to those used in our study. The inclusion of *Cnr2*$^{-/-}$ mice in our study also served as a control for drug specificity, as drugs acting at CB2Rs would not be expected to have any effect on mice lacking functional receptors.

It is possible that we did not observe any effects of JWH-133 on seizure susceptibility because endogenous endocannabinoids were already eliciting maximal receptor occupancy. It is well-accepted that elevated neuronal activity, including seizures, can cause transient release of endocannabinoids (Marinelli et al., 2009; Marsicano et al., 2003; Stempel et al., 2016). This increase in endocannabinoids could potentially act on and occupy CB2Rs yielding a “ceiling

effect," thereby limiting the potential for JWH-133 to elicit an additional response at CB2Rs. A similar effect has been described for CB1Rs, in which treatment with a CB1R agonist prior to pilocarpine-induced seizures had no effect on seizure severity, yet CB1R knockout mice and CB1R-specific antagonists exacerbated the effects of pilocarpine (Kow et al., 2014). This raises the possibility that antagonism of CB2Rs may have a greater effect on seizure susceptibility than agonism of CB2Rs.

While the results of this study indicate a role for CB2Rs in modulating seizure susceptibility, it is unclear whether these effects might be mediated via neuronal or glial mechanisms. CB2R expression is observed on both neurons and glial cells (Li and Kim, 2015; Lopez et al., 2018; Schmole et al., 2015; Stempel et al., 2016) and recent studies have shown that CB2Rs play a role in regulating neuronal excitability (den Boon et al., 2012; Li and Kim, 2015; Stempel et al., 2016). For example, application of JWH-133 reduces the excitability of cortical pyramidal neurons in brain slices from WT mice (den Boon et al., 2012). More recently, Stempel *et. al* observed that pyramidal neurons in the CA2 and CA3 regions of the hippocampus become hyperpolarized in response to a train of action potentials, and that this hyperpolarization is dependent on CB2Rs, but not CB1Rs (Stempel et al., 2016). Thus, it appears that CB2Rs can facilitate inhibition of excitatory cells, thereby reducing excitability of the hippocampus in response to excessive neuronal activity. An increase in CB2R expression on hippocampal neurons has been observed following status epilepticus in a rat model of epilepsy (Wu and Wang, 2018). This upregulation could be involved in a neuroprotective mechanism which reduces excitability following excessive neuronal firing, raising the possibility that targeting CB2Rs may be therapeutic in epilepsy.

Several studies indicate that CB2R-mediated effects in the brain might also be due to their effects on microglial function. A significant increase in expression of CB2Rs have been observed on activated microglia compared to microglia in the resting state (Maresz et al., 2005), and stimulation of CB2Rs has been shown to suppress microglia activation and subsequently reduce microglia-mediated release of pro-inflammatory cytokines and phagocytosis of cells (Ehrhart et al., 2005). Given the potential roles for CB2R on neurons and microglia, future experiments should be designed to evaluate the distinct roles of neuronal and non-neuronal CB2Rs on modulating seizure susceptibility.

Although both *Cnr2*$^{-/-}$ and RH mutant mice were susceptible to PTZ-induced seizures, there was no additive effect of the mutations on seizure susceptibility. We previously reported a low frequency of spontaneous seizures in RH mice (Martin et al., 2010); however, we did not observe spontaneous seizures in either RH or *Cnr2*$^{-/-}$/RH mice in the current study. While preclinical studies examining the role of the ECS in *SCN1A* derived epilepsy are limited, a previous study showed that CBD is protective against hyperthermia-induced seizures as well as spontaneous seizures and sociability deficits in heterozygous *Scn1a* knockout mice, which serve as a model of DS (Kaplan et al., 2017). Despite the fact that CBD has shown success in a mouse model of DS and patients with *SCN1A*-derived epilepsy, accumulating evidence suggests that its anti-epileptic effects are not likely through CB1R- or CB2R- mediated mechanisms (Devinsky et al., 2014; Kaplan et al., 2017). This is consistent with our observation that the loss of CB2R did not worsen seizure phenotypes in RH mutant mice.

CB2R-targeted treatment may also be indicated for other forms of epilepsy which are not *SCN1A*-derived. CB2Rs play a well-documented role in reducing neuroinflammation in mouse models of neurological disease. Specifically, treatment with CB2R agonists have been shown to

reduce levels of reactive gliosis and pro-inflammatory cytokines following neuronal injury in models of Parkinson's disease, Alzheimer's disease, and traumatic brain injury (Amenta et al., 2012; Aso et al., 2013; Gomez-Galvez et al., 2016). This reduction in neuroinflammation has been associated with improved outcomes in these models (Aso et al., 2013; Elliott et al., 2011; Malfitano et al., 2014). Several forms of drug-resistant epilepsy, such as mesial temporal lobe epilepsy (MTLE), are characterized by high levels of neuroinflammation (Gales and Prayson, 2017; Tezer et al., 2018). Given the reduction in inflammation that is observed following CB2R activation, treatments targeting CB2R might prove to be particularly efficacious in epilepsy disorders like MTLE.

The results of this study indicate that the loss of CB2R activity increases seizure susceptibility in mice. Although modulation of CB2R activity did not alter seizure phenotypes in a mouse model of *SCN1A*-derived epilepsy, treatments that target CB2Rs may still be indicated for other forms of epilepsy, particularly those which are characterized by extensive neuroinflammation.

2.1 Acknowledgements

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CHAPTER 3

ALLOSTERIC MODULATION OF THE CANNABINOID 2 RECEPTOR CONFERS SEIZURE RESISTANCE IN MICE

Adapted with permission from:

Shapiro L, Gado F, Manera C, Escayg A. (2021) Allosteric modulation of the cannabinoid 2 receptor confers seizure resistance in mice. *Neuropharmacology* 188:108448. doi: 10.1016/j.neuropharm.2021.108448

3.1 Summary

The data in **Chapter 2** demonstrated that reduced CB2R activity increases seizure susceptibility, but a CB2R agonist was unable to provide seizure protection. To determine how different classes of CB2R-acting drugs might influence seizure thresholds, we evaluated the therapeutic potential of the CB2R positive allosteric modulator, Ec21a, against induced seizures in mice. The pharmacokinetic profile of Ec21 demonstrated a similar distribution in brain and plasma, with detection up to 12 hours following injection. Ec21a increased resistance to induced seizures in CF1 wild-type mice and mice harboring the *SCN1A* R1648H human epilepsy mutation. A rotarod test provided evidence that Ec21a does not cause neurotoxicity-induced motor deficits at its therapeutic dose, and seizure protection was maintained with repeated drug administration. The selectivity of Ec21a for CB2R was supported by the ability of the CB2R antagonist AM630, but not the CB1R antagonist AM251, to block Ec21a-conferred seizure protection in mice, and a lack of significant binding of Ec21a to 34 brain-expressed receptors and transporters in vitro.

Overall, these results identify allosteric modulation of CB2Rs as a promising therapeutic approach for the treatment of epilepsy. The manuscript resulting from this work is reproduced below with minor alterations.

3.2 Introduction

The endocannabinoid system (ECS) consists of G-protein-coupled cannabinoid receptor subtypes 1 (CB1R) and 2 (CB2R), their endogenous ligands, and regulatory enzymes (Lu and Mackie, 2016). The activation of CBRs mediates the effects of endogenous endocannabinoids, plant-derived phytocannabinoids, and other synthetic ligands in the central nervous system (CNS) (Howlett et al., 2002; Mechoulam et al., 1995). There is ample evidence suggesting that modulation of the ECS may be therapeutic in models of several neurological diseases (Basavarajappa et al., 2017; Donvito et al., 2018; Kolb et al., 2019; Pacher and Kunos, 2013).

Although CB1Rs are densely expressed on neurons throughout the brain, where they play a well-established role in regulating neuronal excitability, drugs that target these receptors can be accompanied by adverse and psychoactive side effects, limiting their therapeutic potential (Mackie, 2005; Pertwee, 2012; van Amsterdam et al., 2015). In contrast, CB2Rs are expressed predominately on microglia, and at low levels on neurons in several regions of the brain, and their modulation is not accompanied by psychotropic side effects (Chen et al., 2017; Li and Kim, 2015; Onaivi et al., 2006). CB2Rs are highly inducible and show strong upregulation in response to neurological injury, a characteristic that could allow for spatial and temporal specificity of receptor activation in a disease state (Concannon et al., 2016; Yu et al., 2015). While a role for CB2Rs in the regulation of neuroinflammation is well established (Braun et al., 2018; Rom and Persidsky, 2013; Sahu et al., 2019; Sun et al., 2017), recent data also suggest that CB2Rs can directly modulate neuronal excitability in brain regions including the cortex and hippocampus (Stempel et al., 2016; Stumpf et al., 2018). Accumulating evidence supports a therapeutic role for CB2R agonists in preclinical models of neurological diseases, including neuropathic pain (Aly et al.,

2019; Sheng et al., 2019), neurodegenerative disease (Aso et al., 2013; Basavarajappa et al., 2017; Cassano et al., 2017), traumatic brain injury (Amenta et al., 2012), and stroke (Yu et al., 2015).

There is some evidence that CB2R-targeted therapeutics might also be beneficial in the treatment of epilepsy. We previously observed that CB2R knockout mice were susceptible to pentylenetetrazol (PTZ)-induced seizures, and that administration of the CB2R antagonist, SR144528, increased seizure susceptibility, suggesting that an increase in CB2R activity may confer seizure resistance (Shapiro et al., 2019). However, we saw no change in susceptibility to PTZ-induced seizures following the administration of the CB2R agonist, JWH-133 (Shapiro et al., 2019). Furthermore, other studies investigating the effects of CB2R agonists on seizure susceptibility have yielded inconsistent results (de Carvalho et al., 2016; de Oliveira et al., 2016; Huizenga et al., 2017; Tchekalarova et al., 2018).

Recently, Ec21a, the first selective positive allosteric modulator (PAM) of CB2Rs, was developed and shown to be protective in a mouse model of neuropathic pain (Gado et al., 2019). Allosteric modulators may exhibit distinct advantages over orthosteric ligands for clinical development, including greater target specificity, temporal and spatial selectivity, and resistance to receptor desensitization, which could enhance therapeutic action and reduce side-effect profiles (Foster and Conn, 2017; Wold et al., 2019).

In the present study, we determined the pharmacokinetic profile of Ec21a and evaluated its ability to increase resistance to induced seizures in CF1 wild-type mice, as well as in a mouse line harboring the human *SCN1A* R1648H epilepsy mutation (Escayg et al., 2000; Martin et al., 2010). We also evaluated the specificity for Ec21a at CB2Rs using both *in vivo* and *in vitro* methods. These results provide insight into the therapeutic potential of CB2R-targeted PAMs for the treatment of epilepsy.

3.3 Materials and methods

Animals

Heterozygous mice expressing the human *SCN1A* R1648H epilepsy mutation (RH mutants) on a C57BL/6J background were generated and genotyped as previously described (Martin et al., 2010). Male RH mutant mice and wild-type (WT) littermates or CF1 WT mice (Charles River Laboratories, Wilmington, MA) aged 2-4 months were used for all experiments. Mice were housed on a 12-hour light/dark cycle with standard laboratory chow (Lab Diet, St. Louis, MO) and water available *ad libitum.* All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Emory University Institutional Animal Care and Use Committee guidelines.

Drugs

For the dose-response curve, Ec21a (Provided by Clementina Manera, University of Pisa, or Tocris, Bristol, UK) was dissolved in a vehicle of corn oil with 5% ethanol to allow for solubility of high doses. For all other experiments, Ec21a (10 mg/kg) was dissolved in 0.9% sterile saline with 5% DMSO and 5% Tween 20. For acute experiments, Ec21a or vehicle was administered via intraperitoneal (i.p.) injection in 10 mL/kg injection volume 45 minutes prior to seizure induction or rotarod task, unless otherwise stated. The CB2R antagonist AM630 or CB1R antagonist AM251 (2 mg/kg, 0.9% sterile saline with 5% DMSO and 5% Tween 20, Cayman Chemical, Ann Arbor, MI) were administered via i.p. injection 15 minutes prior to Ec21a administration. For repeated drug administration, Ec21a or vehicle was administered three times daily for four days. On day five, mice received a single dose of Ec21a or vehicle 45 minutes prior to seizure induction. All vehicle-treated control mice received 5% DMSO and 5% Tween 20 in 0.9% saline.

Pharmacokinetic analysis

Blood and brain samples were collected from male C57BL/6 mice at the following time points following a single i.p. injection of Ec21a (10 mg/kg): 0.25, 0.5, 1, 2, 4, 8, 12, and 24 hours. Plasma was isolated from whole blood. Brain samples were homogenized in 3x volume of phosphate-buffered saline. Concentrations of Ec21a in plasma and brain were determined by an LC/MS/MS method. The Non-Compartmental Analysis module in Phoenix WinNonlin software version 7.0 (Certara, Princeton, NJ) was used to analyze pharmacokinetic data (Sai Life Sciences Limited, Hinjewadi, India).

6 Hz-induced seizures

6 Hz seizure induction was performed as previously described (Shapiro et al., 2019; Wong et al., 2016). Thirty minutes prior to seizure induction, mice were administered the topical anesthetic proparacaine hydrochloride ophthalmic solution (Patterson Veterinary, Devens, MA) to each eye. Mice were subjected to corneal electrostimulation (6-Hz, 2-ms pulse width, 3-s duration) at 22 mA (CF1) or 24 mA (RH) using an ECT unit (Ugo Basile, Comerio, Italy). Mice were immediately evaluated for the occurrence of a behavioral seizure characterized by an initial stun followed by rearing, forelimb clonus, vibrissae twitching, and Straub tail as previously described (Barton et al., 2001). Mice were considered protected in the absence of these seizure behaviors.

Pentylenetetrazol (PTZ)-Induced seizures

Seizures were induced using the chemoconvulsant PTZ as previously described (Loscher et al., 1991; Shapiro et al., 2019; Wong et al., 2019). PTZ (Sigma Aldrich, St. Louis, MO) dissolved in sterile saline was injected subcutaneously into CF1 mice (85 mg/kg) or RH mutants

and WT littermates (115 mg/kg). Mice were placed in a plexiglass chamber, and the latency to the first generalized tonic-clonic seizure (GTCS) was recorded during a 30-minute observation period.

Flurothyl-Induced seizures

Seizures were induced using the inhaled chemoconvulsant, Bis(2,2,2-trifluoroethyl) ether (flurothyl, Santa Cruz, Dallas, TX) as previously described (Prichard et al., 1969; Shapiro et al., 2019). Mice were placed in a plexiglass chamber (13.5 x 8 x 6 inches) and exposed to flurothyl at a rate of 20 µL/minute. Latencies to the first GTCS were recorded.

Rotarod

Mice were placed on a rotarod (Columbus Instruments, Columbus, OH) for three consecutive trials. Trials 1 and 2 were training trials during which the rotarod moved at a constant speed of 5 RPM for 60 seconds. During Trial 3, the rotarod accelerated at a rate of 0.1 RPM/second over a 5-minute period to a max speed of 40 RPM. Latency to fall was recorded for each mouse.

Primary binding screen

A primary binding screen of 32 brain-expressed GPCRs and two neurotransmitter transporters was performed by the National Institute of Health Psychoactive Drug Screening Program (NIH PDSP, Chapel Hill, NC) (Besnard et al., 2012). Briefly, Ec21a (10µM) was added to wells containing either HEK293 or CHO cells transfected with a human recombinant GPCR and the appropriate radiolabeled orthosteric ligand. Radioactivity was used to determine displacement of each ligand in the presence of Ec21a. Any target at which Ec21a elicited > 50%

or < -20% displacement of the orthosteric ligand was marked for secondary analysis. Further details on methodology and analysis can be found at https://pdsp.unc.edu/pdspweb/.

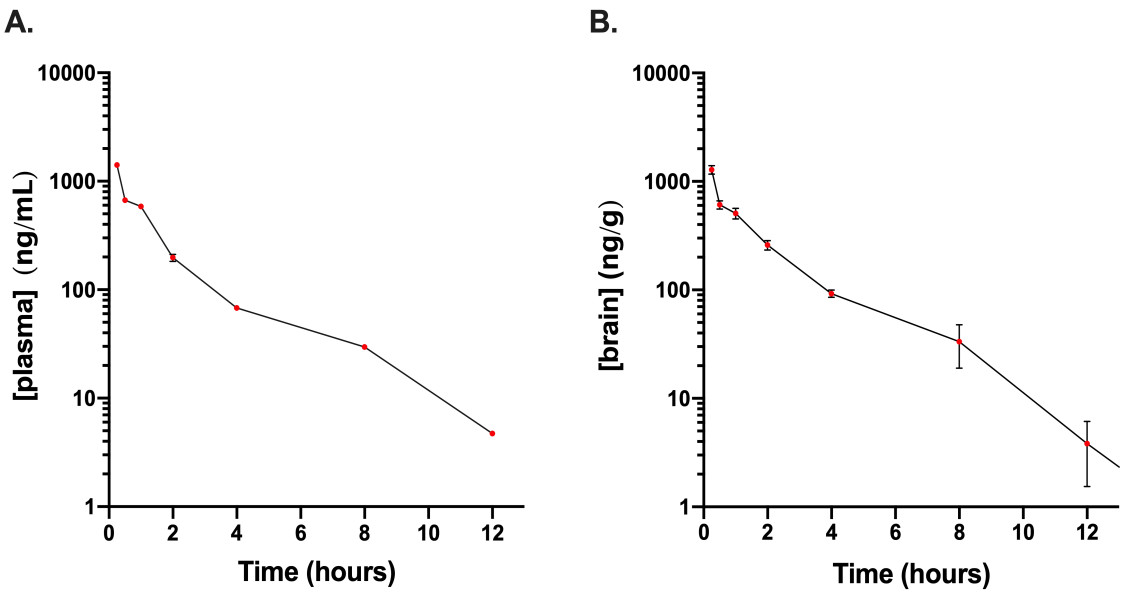
Data Analysis

Data were analyzed using GraphPad Prism version 7 (GraphPad, San Diego, CA). 6 Hz data were analyzed using a Fisher's exact comparison. Latencies to PTZ-induced GTCS were analyzed using the Mantel-Cox log-rank test. Latencies to fall from the rotarod and to flurothyl-induced GTCS in CF1 mice were analyzed with an unpaired t-test. Effects of antagonist pretreatment were analyzed with 1-way ANOVA followed by Tukey's post hoc comparison. Differences between groups were considered to be significant if $P < 0.05$. For all figures, * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, and **** indicates $P < 0.0001$. Experimenters were blinded to genotype and drug treatment during all analyses.

3.4 Results

3.4.1 Ec21a pharmacokinetic profile

Pharmacokinetic analysis was performed to determine the plasma and brain distribution of Ec21a following a single i.p. injection (10 mg/kg). Maximum concentrations of Ec21a (T_{max}) were observed at 15 minutes following administration in both plasma and brain, with measurable concentrations up to 12 hours. Ec21a was not detected at 24 hours following injection. The half-life of Ec21a ($T_{1/2}$) was found to be approximately two hours in both plasma and brain. Total drug exposure in plasma and brain were comparable, as indicated by a brain:plasma ratio ranging from 0.87-1.53 across time points (**Fig 3.1 1A-C**, n = 3/time point).



C.

Matrix	T_{max} (hr)	C_{max} (ng/mL)	$T_{1/2}$ (hr)	AUC_{last} (hr*ng/mL)	AUC_{inf} (hr*ng/mL)	Cl_F (mL/min/kg)	Vz_F (L/kg)
Plasma	.25	1410.75	2.08	1669.36	1686.14	98.85	17.81
Brain	.25	1282.99	1.99	1791.64	1810.33	92.06	15.89

Figure 3.1. **Ec21a pharmacokinetic profile.** Ec21a concentration in plasma (**A**) and brain (**B**) over 12 hours. Ec21a could not be detected in brain or plasma at 24 hours. **C)** Summary of pharmacokinetic parameters in plasma and brain. T_{max} = time to maximum concentration; C_{max} = maximum concentration; $T_{1/2}$ = half-life; AUC_{last} = area under concentration curve to 24 hours; AUC_{inf} = area under concentration curve to infinity; Cl_F = rate of drug clearance; Vz_F = volume of distribution. n=3/time point. All error bars represent mean +/- SEM.

3.4.2 Acute effects of Ec21a in CF1 WT mice

3.4.2.1 Ec21a increases resistance to induced seizures in CF1 mice

To evaluate the seizure protective effects of Ec21a, we first generated a dose-response curve (0, 5.6, 10, 32, and 56 mg/kg) using the 6 Hz seizure induction paradigm and a 45-minute pretreatment time. We observed a maximum seizure protective effect at 10 mg/kg, with 60% of mice protected from a seizure. A Fisher's exact test revealed that mice treated with 10 mg/kg exhibited significantly lower seizure occurrence when compared to vehicle-treated mice (P= 0.01, **Fig 3.2 A**, n=10/group). With doses higher than 10 mg/kg seizure protection began to diminish, with 50% of mice protected at 32 mg/kg, and only 20% protected at 56 mg/kg. However, mice treated with 32 mg/kg were still significantly protected compared to vehicle-treated mice (P= 0.03, **Fig 3.2 A**, n= 10/group). As such, a dose of 10 mg/kg was used for all subsequent experiments. We next evaluated the ability of Ec21a to confer protection in other seizure induction paradigms. When tested using PTZ, Ec21a treatment 45 minutes prior to seizure induction significantly increased latencies to the first GTCS when compared to vehicle treatment [χ^2 (1) = 16.5, P< 0.0001]. Furthermore, 3 out of 14 Ec21a-treated mice did not exhibit a GTCS during the observation period (n = 12-14/group, **Fig 3.2 B**). Mice administered Ec21a 45 minutes prior to seizure induction also exhibited significantly increased latencies to the first flurothyl-induced GTCS compared to vehicle-treated mice [t(18) = 2.93, P=0.009]. (**Fig 3.2 C**, n=10/group). Together, these results illustrate that Ec21a can increase seizure resistance in CF1 WT mice.

Although a 45-minute pretreatment time achieved seizure protection in several paradigms (**Fig 3.2 A-C**), we observed peak concentrations of Ec21a in the brain at 15 minutes post-administration (**Fig 3.1**). As such, we compared the efficacy of Ec21a in the PTZ paradigm with 15- or 45-minute pretreatment times. Latency to the first GTCS was significantly increased in both

15- [χ^2 (1) = 11.5, P = 0.0007] and 45-minute pretreatment groups [χ^2 (1) = 10.7, P = 0.001] compared to the vehicle-treated group. Two mice in each Ec21a pretreatment group did not exhibit a GTCS during the observation period. While no significant differences were observed between 15- and 45-minute pretreatment groups, the average latency to the first GTCS was slightly increased in the 45-minute group. As such, a 45-minute pretreatment time was used for subsequent experiments (**Fig 3.2 D,** n = 8-9/group).

3.4.2.2 CB2Rs, but not CB1Rs, mediate the seizure protection conferred by Ec21a

To determine whether Ec21a-mediated seizure protection is CB2R-specific, CF1 mice were pretreated with either a CB1R (AM251) or CB2R (AM630) antagonist prior to Ec21a administration and PTZ-seizure induction. One-way ANOVA indicated a main effect of treatment on latency to the first GTCS [F(5, 90) = 14.0, P < 0.0001]. Post hoc analyses revealed that neither AM251 nor AM630 alone altered seizure responses (**Fig 3.2 E**, grey and red bars *vs.* black bar), consistent with previous findings at these doses (Gobira et al., 2015; Huizenga et al., 2017; Suemaru et al., 2018; Vilela et al., 2017). As we previously observed, Ec21a significantly increased latencies to the first GTCS compared to vehicle-treated mice (**Fig 3.2 E**, black striped *vs.* black bar; P < 0.0001). Mice pretreated with AM251 prior to Ec21a also exhibited increased latencies to the first GTCS compared to vehicle-treated mice (**Fig 3.2 E**, grey striped *vs.* black bar; P = 0.004), and did not significantly differ from mice treated with Ec21a only (**Fig 3.2 E**, grey striped *vs.* black striped bar; P = 0.14), indicating that CB1R blockade does not significantly influence the efficacy of Ec21a. In contrast, the seizure-protective effect of Ec21a was abolished with AM630 pretreatment (**Fig 3.2 E**, red striped *vs.* black striped; P < 0.0001), demonstrating that the ability of Ec21a to confer seizure resistance is mediated via CB2Rs (**Fig 3.2 E,** n = 16/group).

In the original report by Gado *et al.,* Ec21a was shown to have functional activity at CB2Rs, but not CB1Rs in an *in vitro* $GTP_{\gamma}S$ assay (Gado et al., 2019); however, Ec21a was not tested for potential off-target activity against a larger panel of CNS targets. To further investigate the specificity of Ec21a for CB2Rs, a primary binding screen of Ec21a against 32 brain-expressed GPCRs and 2 neurotransmitter transporters was performed by the NIMH PDSP program (Besnard et al., 2012). No significant binding to any of the targets was observed, providing further evidence of CB2R specificity (**Table 3.1**).

3.4.2.3 Ec21a does not cause neurotoxicity-induced motor deficits

To evaluate whether the therapeutic dose of Ec21a may elicit neurotoxic effects, we administered Ec21a (10 mg/kg) or vehicle prior to a rotarod motor coordination test. Mice in the vehicle- and Ec21a-treated groups did not exhibit any differences in performance during either training trial (data not shown), and no significant differences in latency to fall from the accelerating rotarod was seen during Trial 3 [$t(18) = 1.07$, NS], indicating that Ec21a does not cause neurotoxicity-induced motor deficits at the seizure-protective dose (**Fig 3.2 F**, n = 10/group).

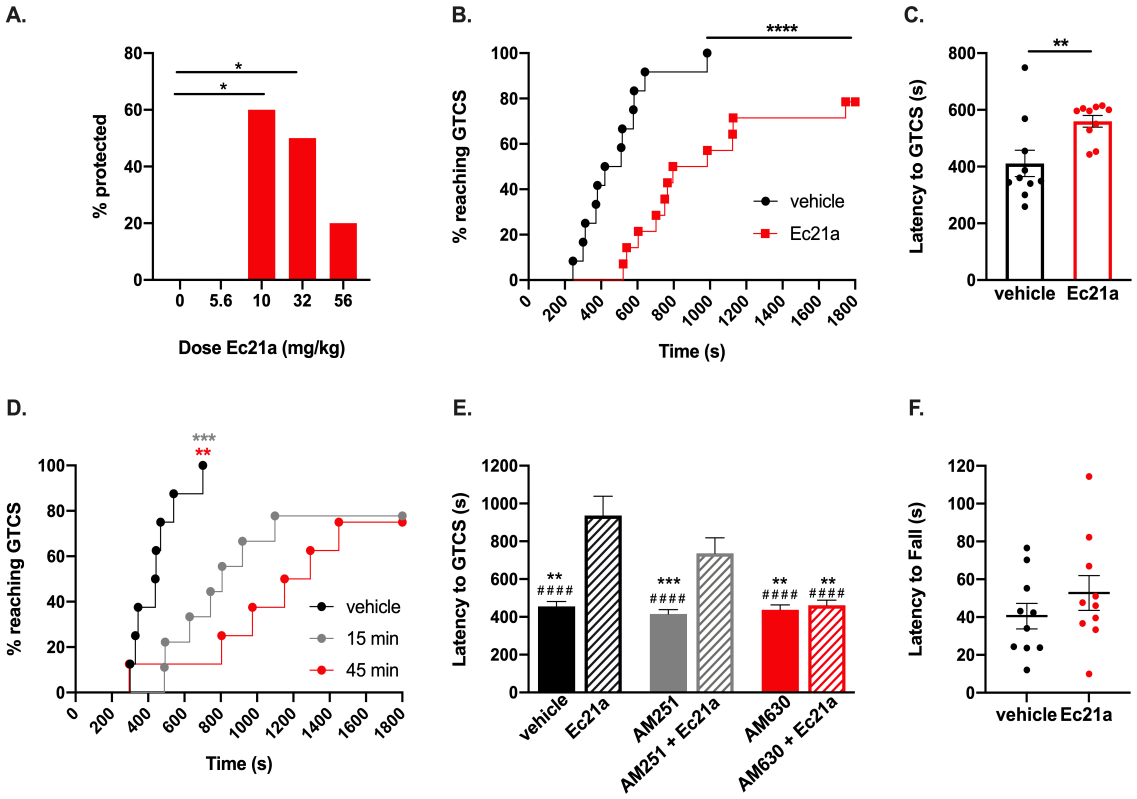


Figure 3.2. Acute effects of Ec21a in CF1 mice. A) Mice treated with either 10 or 32 mg/kg Ec21a exhibited reduced incidence of 6-Hz induced seizures compared to vehicle-treated mice. Fisher's exact test, n = 10/group. **B)** Ec21a-treatment increased latencies to the first PTZ-induced GTCS compared to vehicle-treatment. Mantel-Cox log-rank test, n = 12-14/group. **C)** Ec21a treatment increased latencies to the first flurothyl-induced GTCS compared to vehicle-treatment. Unpaired t-test, n = 10/group. **D)** Mice in both 15- and 45-minute Ec21a pretreatment groups exhibited increased latencies to the first PTZ-induced GTCS compared to vehicle-treated mice. Mantel-Cox log-rank test, n = 8-9/group. **E)** Ec21a-treated mice showed longer latencies to the first PTZ-induced GTCS compared to vehicle, AM251-only, AM630-only, and AM630/Ec21a-treated mice. AM251 pretreatment prior to Ec21a increased latencies to the first GTCS compared to vehicle, AM251-only, AM630-only, and AM630/Ec21a-treatment. # -significance from Ec21a-only, * -significance from AM251/Ec21a. 1-way ANOVA with Tukey's post hoc comparison, n = 16/group. **F)** Ec21a did not affect latency to fall from the rotarod compared to vehicle-treatment. Unpaired t-test, n = 10/group. All error bars represent mean +/- SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Table 3.1. Summary of results of *in vitro* primary binding screen.

Receptor	% Inhibition	Secondary (Ki, nM)
Histamine receptor 1 (H1)	3.92	-
Histamine receptor 2 (H2)	11.42	-
Histamine receptor 3 (H3)	29.53	-
Histamine receptor 4 (H4)	-11.50	-
Muscarinic receptor 1 (M1)	-4.70	-
Muscarinic receptor 2 (M2)	-5.87	-
Muscarinic receptor 3 (M3)	14.98	-
Muscarinic receptor 4 (M4)	-4.92	-
Muscarinic receptor 5 (M5)	-2.30	-
Sigma 2 receptor	10.44	-
Delta opioid receptor (DOR)	-4.72	-
Kappa opioid receptor (KOR)	-11.62	-
Mu opioid receptor (MOR)	-2.97	-
Serotonin transporter (SERT)	-16.46	-
Norepinephrine transporter (NET)	-4.15	-
Benzodiazepine binding site (BZP)	-2.49	-
GABAA Receptor	-7.49	-

Receptor	% Inhibition	Secondary (Ki, nM)
Serotonin receptor 1A (5-HT1A)	-16.73	-
Serotonin receptor 1B (5-HT1B)	5.75	-
Serotonin receptor 1D (5-HT1D)	-0.11	-
Serotonin receptor 1E (5-HT1E)	-17.43	-
Serotonin receptor 6 (5-HT6)	-3.43	-
Adrenergic receptor Alpha 1A	-16.86	-
Adrenergic receptor Alpha 1B	-25.64	> 10,000
Adrenergic receptor Alpha 1D	-1.60	-
Adrenergic receptor Alpha 2A	-6.66	-
Adrenergic receptor Alpha 2B	-1.29	-
Adrenergic receptor Beta 1	-4.06	-
Adrenergic receptor Beta 2	0.08	-
Adrenergic receptor Beta 3	5.40	-
Dopamine receptor 2 (D2)	-1.14	-
Dopamine receptor 3 (D3)	-12.29	-
Dopamine receptor 4 (D4)	13.29	-
Dopamine receptor 5 (D5)	-0.34	-

Note: Targets for which % inhibition was between -25 to 50 % inhibition were not marked for secondary analysis. Dash (–) indicates that criteria for secondary screening was not met.

3.4.3 Ec21a increases seizure resistance in RH mutant mice

We next assessed whether Ec21a confers seizure protection in mice heterozygous for the human *SCN1A* R1648H mutation (RH mutants), which was identified in a family with genetic epilepsy with febrile seizures plus (GEFS+) (Escayg et al., 2000). In the 6 Hz paradigm, Ec21a-treated RH mutants exhibited significantly lower seizure occurrence when compared to vehicle-treated mutants (P = 0.03, n = 16-19/group, **Fig 3.3 A**). In the PTZ paradigm, latencies to the first GTCS were significantly longer in Ec21a-treated RH mice [x^2 (1) = 8.0, P = 0.005] and WT littermates [χ^2 (1) = 14.3, P = 0.0002] compared to vehicle-treated mice of the same genotype. One Ec21a-treated WT mouse did not exhibit a GTCS (**Fig 3.3 B**, n=11-13/group). These results demonstrate that Ec21a also confers seizure protection in RH mutants.

3.4.4 Ec21a-mediated seizure protection is maintained with repeated administration in CF1 mice

To evaluate whether seizure protection would be maintained with repeated administration of Ec21a, we administered Ec21a to CF1 mice 3 times daily for 4 days prior to PTZ-seizure induction on day 5. A significant increase in latency to the first GTCS was still observed in the group that received multiple doses of Ec21a when compared to vehicle-treated mice [x^2 (1) = 10.1, P = 0.002], indicating that repeated drug administration does not mitigate the ability of Ec21a to confer seizure protection (n = 10/group, **Fig 3.4**).

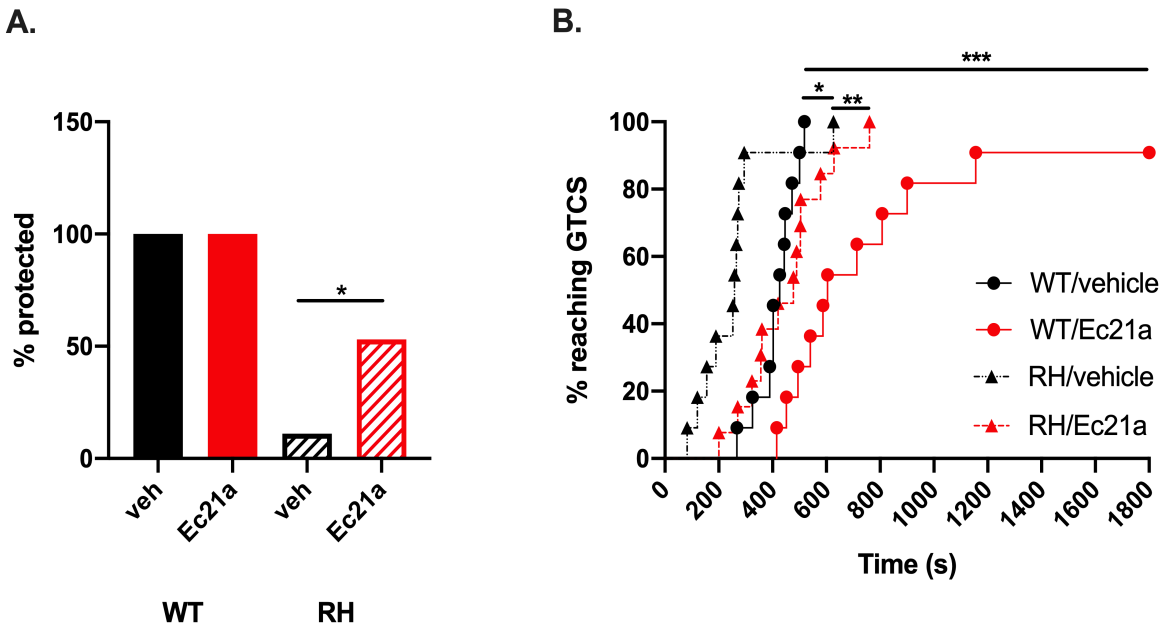


Figure 3.3. Ec21a increases seizure resistance in *Scn1a*$^{RH/+}$ mutant mice. A) Ec21a-treated RH mutants exhibited significantly lower seizure occurrence compared to vehicle-treated mice in the 6 Hz paradigm (P = 0.03). Fisher's exact test, n = 16-19/group. **B)** RH mutant mice exhibited shorter latencies to the first GTCS compared to WT mice (P = 0.03). Ec21a- treated mice exhibited increased latencies to the first GTCS compared to vehicle-treated mice for both WT (P = 0.0002) and RH (P = 0.005). Mantel-Cox log-rank test, n = 11-13/group. * P < 0.05, ** P < 0.01, and *** P < 0.001.

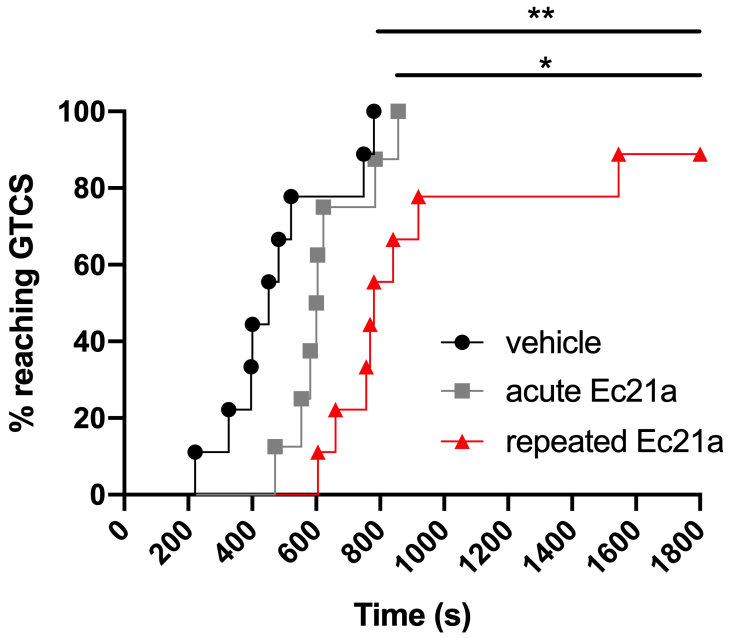


Figure 3.4. Ec21a-mediated seizure protection is maintained with repeated administration. Latency to the first GTCS was increased in mice that were repeatedly treated with Ec21a compared to the vehicle treated group (P = 0.002). Mantel-Cox log-rank test, n = 10/group. * P < 0.05, ** P < 0.01.

3.5 Discussion

We previously reported that reduced CB2R activity is associated with increased seizure susceptibility in mice (Shapiro et al., 2019). In the current study, we show that a positive allosteric modulator at CB2Rs can increase seizure resistance in both WT mice and mice harboring the human *SCN1A* R1648H epilepsy mutation.

While our results indicate that Ec21a can impact seizure phenotypes, the mechanisms by which modulation of CB2Rs confer seizure protection remain unknown. One possibility is that CB2Rs exert seizure protection through their immunomodulatory effects. CB2Rs play a well-established role in reducing inflammation, including the reduction of reactive gliosis and inflammatory cytokine release in models of neurological disease (Braun et al., 2018; Rom and Persidsky, 2013; Sahu et al., 2019; Sun et al., 2017). However, given the acute nature of the seizure induction paradigms used in this study, it is unlikely that there would have been sufficient time for an inflammatory response. A more likely possibility is that CB2Rs are able to confer seizure resistance by directly regulating neuronal excitability. In fact, recent reports have demonstrated CB2R expression on neurons, and that modulation of CB2Rs can have a direct impact on excitability. For example, Stempel *et al.* provided electrophysiological evidence that CB2Rs mediate self-inhibition of hippocampal primary neurons in response to neuronal activity, a result recapitulated in cortical primary neurons (Stempel et al., 2016; Stumpf et al., 2018). It is thus feasible that Ec21a is able to enhance endocannabinoid-mediated self-inhibition of excitatory neurons in order to dampen the acute seizure response. Given the ability of CB2Rs to influence both neuronal excitability and neuroinflammation, there may be a particular advantage to CB2R-targeted therapies in forms of epilepsy with a significant inflammatory component, such as mesial temporal lobe epilepsy (MTLE), which is characterized by robust neuroinflammation in addition

to neuronal hyperexcitability (Gales and Prayson, 2017; Leal et al., 2017; Uludag et al., 2015). Indeed, an increase in CB2R expression has been observed following status epilepticus (SE) in two rodent models of MTLE, and a recent report indicates that treatment with the CB2R inverse agonist, SMM-189, is able to reduce neuroinflammation and neuron loss in a mouse MTLE model (Wu and Wang, 2018; Yu et al., 2020b).

Despite the promising data suggesting that CB2Rs can modulate processes involved in seizure generation, studies that have investigated the effects of CB2R agonists on seizure susceptibility have yielded inconsistent results. While two studies did reveal modest seizure protection following the administration of beta caryophyllene in mice (de Oliveira et al., 2016; Tchekalarova et al., 2018), another study observed reduced latency to seizure onset and increased seizure severity with administration of AM1241 in a rat PTZ model (de Carvalho et al., 2016), while HU-308 did not have any effect on seizure susceptibility in another study (Huizenga et al., 2017). Furthermore, we previously demonstrated that the CB2R agonist, JWH-133, did not provide protection against induced seizures in mice (Shapiro et al., 2019). This body of literature raises the question of how differences between allosteric and orthosteric modulation of CB2Rs might differentially impact seizure susceptibility. One possibility is that Ec21a is able to bypass a potential ceiling that is reached through receptor occupation by endocannabinoids. It is well established that increased neuronal activity, as occurs during a seizure, causes high levels of on-demand endocannabinoid release (Marinelli et al., 2009; Marsicano et al., 2003). Endogenous endocannabinoids might thus saturate the receptor, causing the addition of an orthosteric agonist to have a limited effect. In contrast, Ec21a may bind to a different site on the receptor to further enhance endogenous endocannabinoid activity. Consistent with this, Gado *et al.* reported that Ec21a increases $GTP_{\gamma}S$ binding to CB2Rs in the presence of both the synthetic agonist CP55,940,

as well as the endocannabinoid 2-archidonylglycerol (2-AG), illustrating that Ec21a is able to increase functional output at CB2Rs under conditions in which the orthosteric ligand is bound (Gado et al., 2019).

Another possible explanation for the protection observed with Ec21a, but not CB2R agonists, is that Ec21a activates different signaling pathways upon binding to the receptor. A recent comprehensive profile of several CB2R agonists revealed that JWH-133, HU-310, AM1241, and 2-AG activate different CB2R signaling targets, including cAMP, beta arrestin, ERK, and G protein-coupled inwardly rectifying potassium (GIRK) channels (Soethoudt et al., 2017a). Likewise, a previous report by Atwood *et al.* demonstrated differential activation of MAPK pathways, inhibition of voltage-gated calcium channels (VGCCs), and receptor internalization, dependent on which CB2R ligand was applied *in vitro* (Atwood et al., 2012). Thus, differences in signaling bias may, in part, explain the variable effects observed following the *in vivo* administration of different CB2R ligands. Although it is not yet established to what extent different signaling pathways are impacted by Ec21a, it may enhance the normal signaling behavior of endogenous endocannabinoids, thereby conveying a stronger therapeutic effect. Further functional testing will be necessary to establish how Ec21a impacts signaling in comparison to other CB2R ligands, and which signaling pathways are necessary to mediate the observed seizure protection.

In addition to differences in signaling bias, there are other potential therapeutic advantages of allosteric modulation of CB2Rs. We know that the presence of an orthosteric ligand is required for Ec21a activity at CB2Rs *in vitro* (Gado et al., 2019). Because the action of Ec21a would thus rely on engagement of endocannabinoids with CB2Rs *in vivo*, the effects of Ec21a will maintain fidelity to the temporal and spatial characteristics of endogenous signaling. In this way, Ec21a would be predicted to act in specific brain regions and at specific times critical for seizure control,

while avoiding unnecessary receptor activation. This state-dependency may be particularly important during chronic dosing. Constitutive receptor activity caused by long-term treatment with orthosteric ligands can cause receptor desensitization and/or internalization, which are associated with behavioral tolerance, drug dependence, and toxicity (Khurana et al., 2017; May and Christopoulos, 2003). Since the allosteric activity of Ec21a would be tightly controlled by endocannabinoid release, it may result in lower levels of desensitization, and consequentially, drug tolerance. The data presented in Figure 4 suggested that greater seizure protection could be achieved with repeated Ec21a administration when compared to acute treatment. However, latencies to PTZ-induced seizures following repeated Ec21a administration were similar to the latencies that were observed following acute Ec21a administration in several other experiments (see Fig. 3.2B & 3.2D). When the results of all experiments were considered, the effect of repeated Ec21a administration was determined to be similar to that achieved with acute treatment. Importantly, these results demonstrate that seizure protection was maintained following five days of Ec21a administration, suggesting that CB2Rs were not desensitized with repeated drug administration. Longer-term dosing studies will be required to ensure that behavioral tolerance is not observed with continued administration of Ec21a.

In conclusion, we provide the first pharmacokinetic profile of Ec21a and demonstrate that it can increase resistance to induced seizures, an effect mediated by CB2Rs. These results highlight the potential of allosteric modulation of CB2Rs as a treatment for epilepsy.

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CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary

At the beginning of my dissertation project, few studies had examined CB2R modulation as a potential anti-epileptic therapy, possibly due to the prevailing view that CB1Rs are the main brain-expressed CBR. However, a growing body of evidence suggested that CB2Rs are expressed in the brain and could influence processes linked to epilepsy. Additionally, that many epilepsy patients do not achieve seizure control with currently available therapies highlighted an unmet need to identify novel molecular targets for AED development. As such, the overarching goal of my dissertation research was to establish the relationship between CB2R activation and seizure thresholds in order to identify a potential new target for AED development.

Overall, I discovered that there is indeed a relationship between CB2R modulation and seizure resistance in mice, although this relationship is dependent on the mode of receptor modulation. Specifically, in **Chapter 2** I demonstrated that while both genetic deletion of CB2R and pharmacological block of CB2R activation lead to increased seizure susceptibility, administration of a CB2R agonist does not result in greater seizure resistance. In contrast, I demonstrated in **Chapter 3** that the administration of a CB2R-specific PAM, Ec21a, increases seizure thresholds in wild-type mice and exhibits a promising therapeutic profile including adequate brain distribution, lack of neurotoxicity, and the ability to maintain protection with repeated administration. To enhance the clinical relevance of these studies, I also utilized *Scn1a*$^{R1648H/+}$ mutant mice (RH) to determine whether CB2R manipulation influences seizure thresholds in a mouse model of human epilepsy. While knockout of CB2Rs in RH mutants did not further increase seizure susceptibility, Ec21a administration conferred robust seizure resistance, highlighting that CB2R modulation may be a promising approach to treat epilepsy

patients. In this chapter, I will integrate a discussion of these results with potential future directions.

4.2 Potential mechanisms of CB2R-mediated protection

The data presented in this thesis demonstrate that modulation of CB2Rs has a strong influence on susceptibility to induced seizures in mice. By what mechanism does CB2R activation confer seizure resistance? The canonical view is that while CB1Rs are highly expressed on neurons, where they regulate neuronal excitability, CB2Rs are predominant on microglia, where they regulate neuroinflammation. However, more recent advances in the field have enabled the identification of neuronally-expressed CB2Rs, although the physiological consequences of their activation have not been extensively explored (Li and Kim, 2015; Stempel et al., 2016). Given the acute nature of our seizure induction paradigms, it seems unlikely that the observed effects are solely a result of neuroimmune modulation. I instead hypothesize that the observed protection is mediated largely by the response of neuronally-expressed CB2Rs.

Consistent with this hypothesis, recent electrophysiological studies indicate a role for neuronal CB2Rs in regulating excitability in brain regions associated with seizure generation. In particular, action potential-induced release of 2-AG leads to a CB2R-mediated self-inhibition of excitatory cells in the hippocampus. A similar hyperpolarization is also observed in excitatory cells of the cortex (Stempel et al., 2016; Stumpf et al., 2018). When considering that seizures are characterized by excessive action potential firing, these studies support a model in which 2-AG acts at neuronal CB2Rs to control seizure activity during induced seizure paradigms. The cell-intrinsic hyperpolarization observed in previous studies was abolished in CB2R-KO mice and with application of CB2R antagonists. This is consistent with our observation that CB2R-KO

mice exhibit increased seizure susceptibility, an effect that was also observed with administration of a CB2R antagonist (**Chapter 2**). Perhaps the lack of available CB2Rs on which 2-AG can bind prevents eCB-mediated hyperpolarization in important brain regions including cortex and hippocampus, leaving CB2R-KO mice more vulnerable to seizure induction. In contrast, pretreatment with Ec21a may enhance 2-AG action at CB2Rs, providing greater inhibitory tone, thereby increasing seizure resiliance as we observed in **Chapter 3**.

Given the diverse array of signaling pathways involved in CB2R activation, it is unclear which pathways are involved in mediating this regulation of neuronal excitability (Soethoudt et al., 2017a). In the studies described above, the observed hyperpolarization was dependent on the sodium-bicarbonate transporter (NBC) and GIRK channels in the hippocampus and cortex, respectively (Stempel et al., 2016; Stumpf et al., 2018). Both NBCs and GIRKs have been previously implicated in epilepsy and could represent components of pathways through which CB2Rs reduce neuronal excitability (Huang et al., 2018; Park et al., 2019; Signorini et al., 1997). However, CB2Rs also influence the function of other ion channels, including VGCCs and calcium-dependent chloride channels, both of which could have profound impact on neuronal excitability (Atwood et al., 2012; den Boon et al., 2012). It is likely that the mechanisms involved in CB2R-mediated inhibition of seizures are numerous, and may be highly dependent on time point, brain regions, and cell-types involved. Our proposed mechanism of CB2R-mediated regulation of neuronal excitability is illustrated in Fig **4.1**.

Interestingly, the model we propose may distinguish CB2R-mediated regulation of neuronal excitability from that of CB1Rs. While CB1Rs act to predominately inhibit presynaptic neurotransmitter release, CB2Rs could instead provide postsynaptic inhibition through cell-intrinsic mechanisms (Kano et al., 2009; Ohno-Shosaku and Kano, 2014). Furthermore, since

CB2Rs are highly inducible, it is possible these receptors might play more of a protective role following brain injury, in contrast to the high level of CB1R activation in basal conditions (Concannon et al., 2016; Wilson and Nicoll, 2001). In the case of epilepsy, perhaps the self-inhibition of CB2Rs acts as a 'braking system' on postsynaptic neurons in instances of excessive neuronal firing. This is supported by evidence that CB2R inhibition is observed on excitatory neurons of the hippocampus, cortex, and the ventral tegmental area, but not on inhibitory interneurons in the same brain regions (Ma et al., 2019; Stempel et al., 2016; Stumpf et al., 2018). However, given that the subcellular localization of CB2Rs has not been fully explored, it cannot be ruled out that CB2Rs also exert presynaptic effects. A better understanding of how CB2Rs regulate neuronal excitability during a seizure would not only inform our understanding of CB2Rs as a therapeutic target, but would also more broadly serve to distinguish the physiological roles of CB1Rs and CB2Rs on neurons.

It is also possible that alterations to microglial function contribute to the observed effects on seizure thresholds. While microglia-mediated neuroinflammation is tightly linked to chronic forms of epilepsy, the relationship between microglia and acute seizure phenotypes is not well-established (Devinsky et al., 2013). Although the short time course of these paradigms makes a neuroinflammatory mechanism less likely, microglia play other important roles in maintaining cell homeostasis under basal conditions, including synaptic pruning and debris clearance (Nimmerjahn et al., 2005). Moreover, knockout of microglial-specific receptors can alter susceptibility to induced seizures, indicating that changes to basal microglia function are sufficient to modulate acute seizure phenotypes (Eyo et al., 2014; Mirrione et al., 2010). Microglia from CB2R knockout mice exhibit diminished phagocytic capacity and altered morphology, illustrating that basal microglia function is altered in these mice (Mecha et al.,

2015). Thus, it is feasible that the increased seizure susceptibility in CB2R-KO mice may be microglia-dependent.

In order to disentangle the contributions of neurons and microglia to the observed seizure phenotypes, future studies could be performed using conditional CB2R knockout mice in place of the global knockout which was utilized in these studies. A mouse line with a floxed *Cnr2* gene has already been developed and could be crossed with existing *Cx3cr1* or *synapsin1* Cre lines to delete CB2Rs in microglia or neurons, respectively (Stempel et al., 2016). This would enable us to ascertain whether conditional knockout mice are similarly seizure susceptible, and whether the protective actions of Ec21a are mediated via neuronal and/or microglial CB2Rs.

In vitro electrophysiological studies would be a complementary approach to such in vivo experiments and would enable a more specific evaluation of the mechanisms of CB2R-mediated protection. Since it has been established that CB2Rs elicit a 2-AG dependent neuronal hyperpolarization, it would be interesting to evaluate whether the addition of Ec21a enhances the inhibitory potential of endogenous eCB signaling in electrophysiological studies. Such experiments could also be used to probe which ion channels or pathways might be influenced by Ec21a. Lastly, the use of conditional knockout mice described above would also allow us to determine which cell types contribute to any observed effects.

4.3 Allosteric modulation of CB2Rs as an approach to anti-epileptic therapies

When beginning these studies, I hypothesized that I would observe seizure protection with administration of CB2R agonists, which was supported by the robust protective effects of CB2R agonists in related neurological disorders. Surprisingly, we observed no effects on seizure susceptibility following the administration of the CB2R orthosteric agonist, JWH-133 (**Chapter**

2), whereas we observed strong seizure protection with the CB2R PAM, Ec21a (**Chapter 3**). These results highlight another outstanding question resulting from this work: Why might allosteric modulation of CB2Rs be a more successful therapeutic approach than traditional orthosteric ligands? While traditional ligands at CB2Rs will compete with eCBs for binding at the orthosteric site, PAMs bind to a distinct allosteric site on the receptor, inducing conformational changes to enhance endogenous signaling (Christopoulos, 2002). In light of this, there are two possible explanations for the different effects observed with JWH-133 and Ec21a.

First, it is likely that CB2R expression on neurons is low under basal conditions. If eCBs are occupying existing CB2Rs, this may create a ceiling effect, in which the efficacy of an orthosteric ligand such as JWH-133 is limited by low receptor expression and occupancy by endogenous ligands. That CB1R agonists are able to influence acute seizure phenotypes may be explained by the relative abundance of brain-expressed CB1Rs compared to that of CB2Rs. In contrast, Ec21a will not compete with eCB signaling and could thus bypass such a ceiling effect.

Alternatively, the distinct observations with JWH-133 and Ec21a could be explained by the activation of different signaling pathways. An emerging concept in the field of GPCR drug-design is functional selectivity, in which different ligands acting at the same receptor can exert biased activation of different signaling pathways (Atwood et al., 2012; Soethoudt et al., 2017a). This might explain the variable results observed in the few other studies which have examined the effects of CB2R agonists on seizure phenotypes (de Carvalho et al., 2016; Huizenga et al., 2017; Tchekalarova et al., 2018). In our experiments, JWH-133 might activate different signaling pathways than eCBs upon binding to CBRs. In contrast, Ec21a may simply enhance activation of endogenous signaling pathways, explaining the distinct observations between the two drugs. The concept of functional selectivity also raises the possibility that administration of Ec21a

preferentially biases eCB agonism towards signaling pathways that enhance seizure protection, which may not occur with orthosteric agonists. However, little work has been done to evaluate to what extent particular signaling pathways are affected by Ec21a administration. In the initial publication describing Ec21a, it was shown to enhance GTPγS signaling in the presence of both synthetic (CP,55940) and endogenous (2-AG) orthosteric ligands (Gado et al., 2019). A subsequent publication showed a similar effect on inhibition of cAMP signaling and beta arrestin recruitment (Gado et al., 2020). However, the effects of Ec21a on a range of other relevant signaling pathways including modulation of VGCCs, GIRKs, and MAPK pathways have not yet been investigated. Likewise, Ec21a has not yet been directly compared to other CB2R orthosteric ligands which are commonly used in rodent studies. It will be necessary in future studies to identify which downstream signaling pathways are influenced by Ec21a, and to determine which pathways are most important for seizure control. Given the critical importance of attenuating neuronal excitability in the treatment of seizures, I hypothesize that Ec21a preferentially enhances eCB regulation of ion channels important for neuronal control, including GIRKs and VGCCs. Further evaluation of affected signaling pathways will not only enhance our knowledge of how Ec21a confers seizure resistance, but may also inform the development of novel CB2R PAMs which specifically activate therapeutic signaling pathways in the brain.

Because PAMs have no intrinsic activity, their actions are spatially and temporally restricted to the signaling pattern of the endogenous ligand (Christopoulos, 2002). This fidelity to endogenous signaling patterns will likely reduce unintended effects resulting from excessive receptor activation in unaffected cell-types and brain regions. In the context of epilepsy, eCB release is ‘on-demand’ in affected brain regions, indicating that Ec21a will exert strongest effects in seizure susceptible brain regions during times of increased neuronal firing (Marsicano et al.,

2003; Wallace et al., 2002). This is important because while CB2R activation has been shown to cause relatively few side effects compared to CB1R activation, it cannot be ruled out that long-term treatment with broad-acting CB2R agonists could induce immunosuppressive side effects as a result of the broad expression of CB2Rs on microglia and macrophages throughout the body (Deng et al., 2015). The context-dependent activity of PAMs also reduces the likelihood of receptor desensitization that could occur with repeated treatment. This is supported by our observation that Ec21a could still confer seizure protection after 5 days of repeated administration (**Chapter 3**).

While binding motifs at the orthosteric site are often shared between receptors, allosteric sites are thought to be more distinct. Thus, it can be difficult to produce an orthosteric ligand which acts only at the intended target, whereas PAMs are likely more target selective (Dopart et al., 2018; Wold and Zhou, 2018). In the case of CBRs, many orthosteric cannabinoid ligands have some activity at both CB1R and CB2Rs (Pertwee, 1999). Since CB1R activation is associated with psychotropic effects, any unintended targeting of CB1R could limit therapeutic use (Volkow et al., 2014). Our data demonstrates that the seizure protective effects of Ec21a are mediated through CB2Rs, but not CB1Rs. Likewise, our *in vitro* binding screen suggests that Ec21a does not significantly bind to common brain-expressed receptors and transporters, suggesting that Ec21a is highly CB2R-selective.

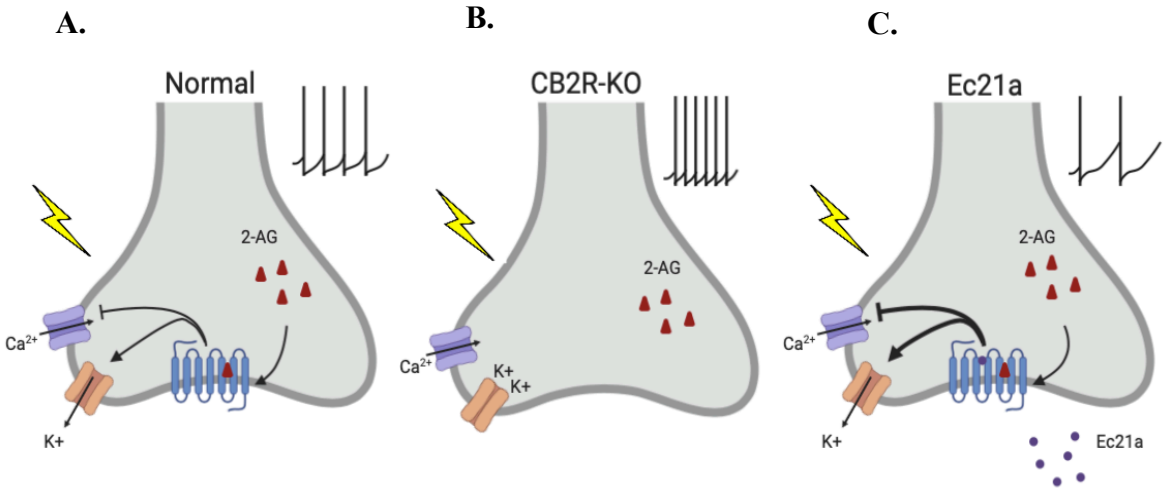

Figure 4.1 Proposed mechanisms of CB2R-mediated seizure protection. **A.** Upon stimulation (indicated by lightning bolt), 2-AG acts upon CB2Rs within the same cell. CB2Rs inhibit voltage-gated calcium channels (VGCCs) and open g-protein inwardly rectifying potassium channels (GIRKs) to hyperpolarize the cell, leading to a reduction in firing. **B.** This process is absent in CB2R knockout mice, leading to increased neuronal firing. **C.** With the addition of Ec21a, this process is strengthened, resulting in a greater decrease in neuronal firing.

4.4 Expanding our understanding of the therapeutic potential of CB2Rs in epilepsy

Perhaps the most important future direction will be to further evaluate the therapeutic potential of Ec21a in chronic forms of epilepsy. While we have begun to address this question by establishing that Ec21a can increase seizure resistance without any apparent neurotoxicity-induced side effects, the studies presented in this dissertation primarily utilized models of induced seizures. However, it is important to note that the mechanisms the underlie induced seizures can differ widely from chronic epilepsy, which is characterized by recurrent spontaneous seizures and is often accompanied by other behavioral comorbidities. Thus, the evaluation of Ec21a in a broader range of clinically-relevant models will be necessary.

In our studies, we utilized the RH mouse model of GEFS+ epilepsy to determine whether CB2R modulation increases seizure resistance in a mouse model of human epilepsy. Although we did not observe a further increase in seizure susceptibility following the knock-out of CB2Rs in RH mice (**Chapter 2**), Ec21a did confer significant seizure protection in RH mutants (**Chapter 3**). Since RH mutants also exhibit infrequent spontaneous seizures and behavioral abnormalities, a natural extension of this work will be to determine whether repeated Ec21a administration attenuates spontaneous seizures and behavioral changes in these mice (Martin et al., 2010; Sawyer et al., 2016).

Furthermore, a remaining question is whether expression of ECS components is altered in *Scn1a* mutants. Addressing this question may provide important insight into the mechanisms of CB2R-mediated protection in *SCN1A*-derived epilepsy. For example, if CB2R expression is reduced in RH mutants, perhaps Ec21a acts at remaining CB2Rs to enhance eCB tone, thereby conferring seizure resistance. Reduced CB2R expression in RH mutants could similarly explain

why knockout of CB2Rs did not further increase seizure susceptibility. Likewise, changes to eCB synthesis or degradation may inform the mechanism by which Ec21 acts in this model.

Future studies could evaluate whether Ec21a is efficacious in other mouse models of *SCN1A* derived epilepsy. Our laboratory maintains *Scn1a*$^{+/-}$ knockout mice, a mouse model of Dravet syndrome (DS). DS is a severe, early-life encephalopathy which is often caused by mutations in *SCN1A*. Heterozygous *Scn1a*$^{+/-}$ knockout mice exhibit early-life spontaneous seizures, behavioral abnormalities, and premature lethality, presenting an overall phenotype which is more severe than that of the RH mutants (Ogiwara et al., 2007; Yu et al., 2006). Thus, it would be informative to evaluate whether Ec21a could prevent both induced and spontaneous seizures in this line, in addition to behavioral comorbidities. The ability for Ec21a to protect *Scn1a*$^{+/-}$ animals may provide additional support for CB2R-based therapies for the use in severe, treatment-resistant forms of epilepsy.

At the onset of this project, I hypothesized that CB2Rs might be particularly effective in forms of epilepsy which are accompanied by significant neuroinflammation. However, given that the *Scn1a* RH mouse model was established in our laboratory, and that CBD had received recent approval for the treatment of *SCN1A*-derived epilepsy, we chose to first pursue the evaluation of CB2Rs in the RH line. However, the close link between CB2Rs and the attenuation of neuroinflammation indicates that CB2R-targeted treatment should be evaluated in models of epilepsy characterized by extensive neuroinflammation. Specifically, MTLE is a common form of epilepsy in which inflammation has been linked to the development of seizures and behavioral changes (Engel, 2001). In **Appendix A**, I further discuss the potential use of CB2R-targeted treatments in a mouse model of MTLE and present preliminary data evaluating the effects of this treatment. A more comprehensive evaluation of Ec21a in an MTLE model will further our

understanding of the potential of CB2R PAMs as novel AEDs, in addition to illuminating their potential as anti-epileptogenic agents following brain injury.

4.5 Overall Conclusions

In conclusion, the data presented in this dissertation suggest that CB2R function directly influences seizure susceptibility in mice. Importantly, this research identified the CB2R PAM, Ec21a, as a potential new therapeutic for epilepsy, thereby directly addressing the important unmet need to develop novel treatments for refractory forms of epilepsy. More broadly, this research provides evidence that allosteric modulation of the ECS may be more therapeutically beneficial than traditional orthosteric approaches, which could be applied to improve treatment across a range of neurological disorders.

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APPENDIX A

ESTABLISHING THE EFFECTS OF CB2R LIGANDS IN A MOUSE MODEL OF MESIAL TEMPORAL LOBE EPILEPSY

A.1 Introduction

After establishing a relationship between CB2Rs and susceptibility to induced seizures (**Chapters 2 & 3**), we were interested to investigate the effects of CB2R modulation in a mouse model of acquired epilepsy. Since CB2Rs attenuate neuroinflammation in models of several neurological disorders, I hypothesized that CB2R compounds might be particularly effective in a model of epilepsy characterized by high levels of inflammation in addition to neuronal hyperexcitability (Amenta et al., 2012; Bisogno et al., 2016; Elliott et al., 2011; Zarruk et al., 2012).

Mesial temporal lobe epilepsy (MTLE) is one of the most common forms of epilepsy in adults, and patients with MTLE are at risk for several clinically challenging phenotypes including treatment-refractory seizures, and impaired social, behavioral, and cognitive function (Cornaggia et al., 2006; Engel, 2001; Nilsson et al., 1999; Suurmeijer et al., 2001). Brain tissue from MTLE patients is characterized by hippocampal sclerosis, reactive gliosis, elevated levels of inflammatory cytokines, and blood brain barrier (BBB) breakdown (Johnson et al., 2016; Leal et al., 2017; Liimatainen et al., 2013; Liu et al., 2012; Strauss and Elisevich, 2016). The pilocarpine mouse model of MTLE recapitulates many of the key features of human MTLE, including a robust neuroinflammatory response that contributes to the subsequent development of spontaneous seizures (Cavalheiro, 1995; Curia et al., 2008). In this set of experiments, I first established the behavioral and neuroinflammatory phenotypes of the mouse pilocarpine model before examining the effects of treatment with CB2R-targeted compounds.

A.2 Methods

A.2.1 Pilocarpine Model

The pilocarpine mouse model of MTLE was utilized for all experiments in this Appendix. To generate the model, male C57BL/6N mice (10-12 weeks) were first administered scopolamine methyl bromide and terbutaline hemisulfate (2 mg/kg each, i.p.) in a single injection in order to prevent peripheral effects of pilocarpine. Twenty minutes later, mice were injected with pilocarpine (280 mg/kg, i.p.), and observed for the onset of status epilepticus (SE), a period of continuous seizures. Following 1 hour of SE, diazepam was administered (10mg/kg) to interrupt seizure activity. In this model, SE is followed by a latent period of approximately 2-3 weeks, during which time a number of epileptogenic processes occur, including extensive neuronal loss and neuroinflammation. These changes contribute to the development of spontaneous, recurrent seizures and behavioral abnormalities during the chronic period (Scorza et al., 2009). An overview of the pilocarpine model is shown in **Fig. A1**. For some experiments, mice were treated with CB2R-specific compounds daily following SE. Details on treatment regime for individual experiments are described in the results section below.

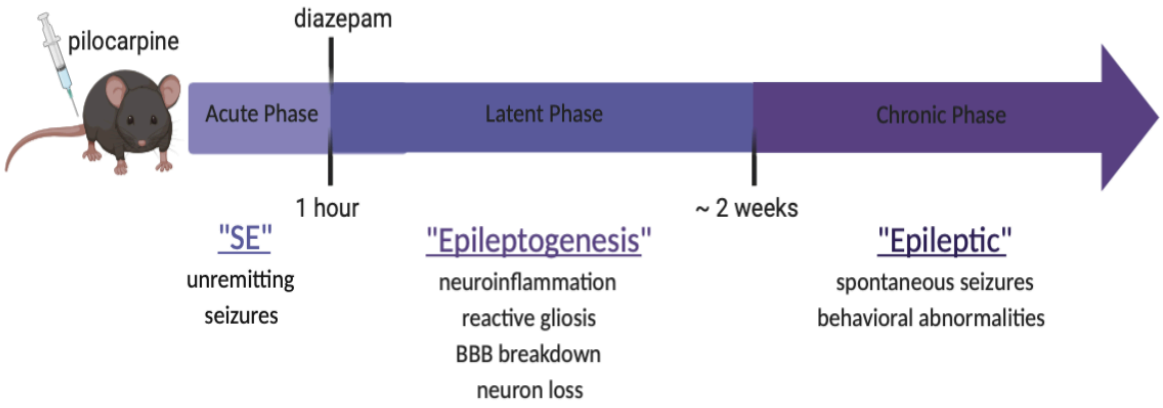

Figure A1 Pilocarpine model of MTLE. Status epilepticus (SE) is induced with pilocarpine (280 mg/kg) and is interrupted after 1 hour with diazepam (10 mg/kg). SE is followed by an epileptogenic latent period of 2-3 weeks during which extensive neuroinflammation, blood brain barrier (BBB) breakdown, and neuronal loss occur, contributing to the development of spontaneous seizures and behavioral changes during the epileptic chronic phase.

A.2.2 Functional Recovery

Mice were monitored daily for functional recovery following SE in one of two ways. In some experiments, animals underwent an Irwin Scoring protocol as previously described (Jiang et al., 2015). The composite score of all features was used as a measure of functional recovery. In other experiments, mice were placed in an open field apparatus and observed for 5 minutes. Distance traveled in the 5 minute period was scored using AnyMaze tracking software.

A.2.3 Gene Expression Analysis

Analysis of inflammatory cytokine and CB2R expression in animals sacrificed 4 days following SE was performed using qPCR. In brief, hippocampal tissue was dissected following transcardial perfusion and RNA extraction was performed (Qiagen Rneasy Lipid Mini kit). RNA was then reverse-transcribed to cDNA and qPCR was performed using standard SYBR green protocols. Data was analyzed using the standard ddCt method. Primer sequences for gene expression analysis are included in **Table A1.**

Table A1 Primer sequences for gene expression analysis.

Target	F Primer	R Primer
CB2R	GGGTCGACTCCAACGCTATC	AGGTAGGCGGGTAACACAGA
Iba1	GGATTTGCAGGGAGGAAAAG	TGGGATCATCGAGGAATTG
IL1ß	TGAGCACCTTCTTTTCCTTCA	TGAGCACCTTCTTTTCCTTCA
GFAP	TGGAGGTGGAGAGGGACAAC	CTTCATCTGCCTCCTGTCTATACG
LCN2	ATTACCCTGTATGGAAGAACC AAGG	ACTCACCACCCATTCAGTTGT
TGF-ß	TCGACATTCGGGAAGCAGT	ACGCCAGGAATTGTTGCTAT
TNF-α	TCTTCTGTCTACTGAACTTCGG	AAGATGATCTGAGTGTGAGGG

A.2.4 Behavioral Analysis

Open field analysis was performed as previously described in our laboratory (Inglis et al., 2020). In brief, animals were placed in an open field apparatus for ten minutes and video recorded. Movement was automatically scored using AnyMaze tracking software. The light/dark box paradigm was also performed as previously described (Wong et al., 2018). Mice were placed in the light side of a light dark box and video recorded for ten minutes. Time spent on either side of the box was automatically recorded using AnyMaze tracking software.

A.2.5 EEG Analysis

EEG surgery and analysis were performed as described in **Chapter 2**. In brief, animals were surgically implanted with 4 cortical electrodes and were monitored for the occurrence of spontaneous seizures characterized by synchronous discharges of increased amplitude at least twice the background and ≥ 3 seconds in duration.

A.3 Results

A.3.1 Establishment of the Pilocarpine Model

I first established the pilocarpine model in our laboratory using C57BL/6N male mice. In order to determine whether 1 hour of SE was sufficient to produce the neuroinflammation which is characteristic of the latent phase, mice were sacrificed at 4 days following SE and qPCR was performed on dissected hippocampal tissue to determine the levels of inflammatory cytokines. We observed robust increases in inflammation at 4-days post-SE (**Fig A2**), similar to previous reports (Jiang et al., 2015; Li et al., 2011). Interestingly, we also observed an increase in CB2R gene expression at 4 days post-SE, consistent with previous reports that CB2R expression is

elevated following SE and other brain injuries (Wu and Wang, 2018; Yu et al., 2015; Yu et al., 2020b) (**Fig A2**).

Next, I examined whether 1 hour of SE was sufficient to elicit the spontaneous seizures and behavioral abnormalities that are typically observed during the chronic phase of the pilocarpine model (Cavalheiro, 1995; Müller et al., 2009). Animals underwent SE and were equipped with an EEG headcap to monitor spontaneous seizure development. We observed that mice experiencing 1 hour of SE exhibited spontaneous seizures at 3 week post-SE. Likewise, we also observed spontaneous seizures in a separate cohort of animals at 10 weeks post-SE, indicating that the model reliably produces spontaneous seizures during the chronic phase. A representative EEG trace of a spontaneous seizure is provided in **Fig A3**.

Lastly, I evaluated whether pilocarpine-treated animals exhibit behavioral abnormalities which are typical in this model. To do so, we generated a cohort of animals undergoing pilocarpine treatment and tested the mice for behavioral deficits at 2 weeks post-SE and again at 8 weeks post-SE. At 2 weeks, we observed normal behavior in the open field and light/dark box paradigms. In contrast, when tested again at 8 weeks post-SE, pilocarpine-treated animals spent less time in the center of the open field and a greater amount of time on the dark side of the apparatus during the light/dark box paradigm. Together, these results indicate that post-SE animals exhibit anxiety-like behaviors in the chronic phase (**Fig A4**).

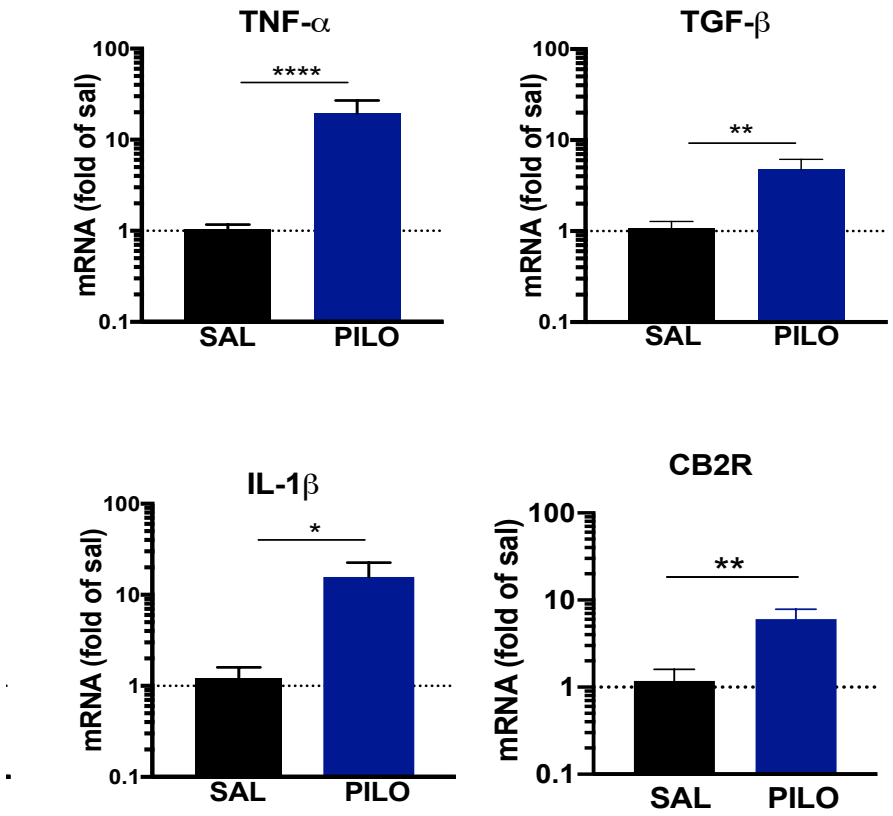

Figure A2 Gene expression in the pilocarpine model. Gene expression of inflammatory cytokines TNF-α, TGF-β, and IL-1β are significantly increased in pilocarpine-treated animals 4-days following SE. Likewise, CB2R gene expression is significantly increased at 4-days post-SE. Y axis represents fold change from saline-treated control animals (sal). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$

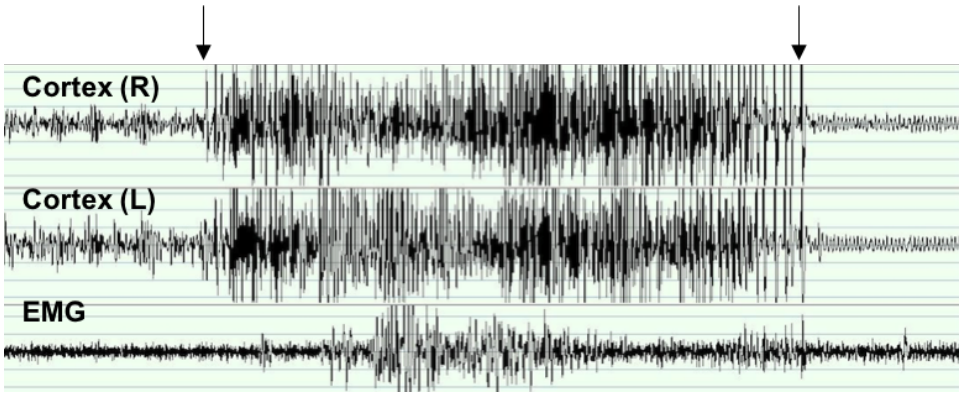

Figure A3. Representative spontaneous seizure in pilocarpine model. R; right side, L; left side, EMG; electromyography. Arrows indicate start and end of seizure (30 seconds).

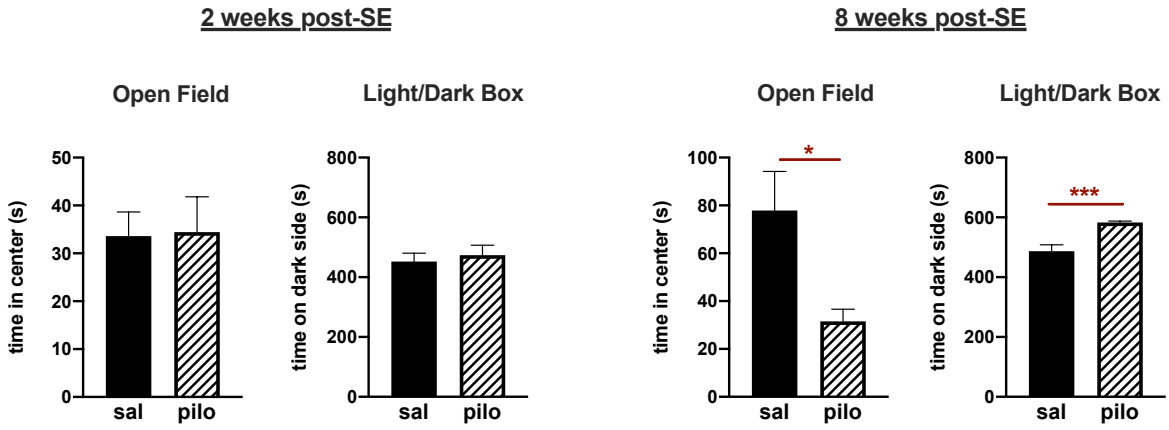

Figure A4. Behavioral phenotypes following SE. Pilocarpine-treated mice do not exhibit abnormalities in open field or light/dark box paradigms at 2 weeks post-SE. At 8 weeks post-SE pilocarpine-treated animals spent less time in the center of the open field and more time on the dark side of the light/dark box. *p<.05**,** ***p< .001. Unpaired t-test, Mean +/- SEM. N= 4/group.

A.3.2 Effects of CB2R-specific compounds in the pilocarpine model

We next assessed whether treatment with CB2R-specific compounds might reduce the pathology observed following SE. We generated the pilocarpine model and treated animals with the CB2R-specific agonist, JWH-133 (3 mg/kg) 3x daily for four days following SE. We measured the weight of the animals daily and monitored their functional recovery, as measured by a modified Irwin score (Jiang et al., 2015). We also evaluated whether JWH-133 treatment could reduce levels of inflammatory cytokines in the hippocampus, as has been observed in mouse models of other neurological disorders. However, we did not observe any effect of JWH-133 on weight, functional recovery, or inflammatory cytokine expression (**Fig A5**).

Given the robust acute seizure protection we observed with Ec21a administration, we performed a similar experiment to evaluate the effects of Ec21a treatment following SE. We administered Ec21a twice daily (10 mg/kg) following SE and similarly evaluated the effects on weight, functional recovery, and neuroinflammation. Both vehicle and Ec21a treated groups differed significantly in weight recovery from saline-treated animals across all time points. By day 4, Ec21a-treated animals showed significantly greater weight recovery compared to vehicle-treated controls. When evaluating functional recovery, Ec21a-treated animals were significantly more active compared to both vehicle-treated mice and saline controls that did not receive pilocarpine on day 4. We observed no effect of Ec21a on neuroinflammatory markers compared to vehicle-treated control mice (**Fig A6**).

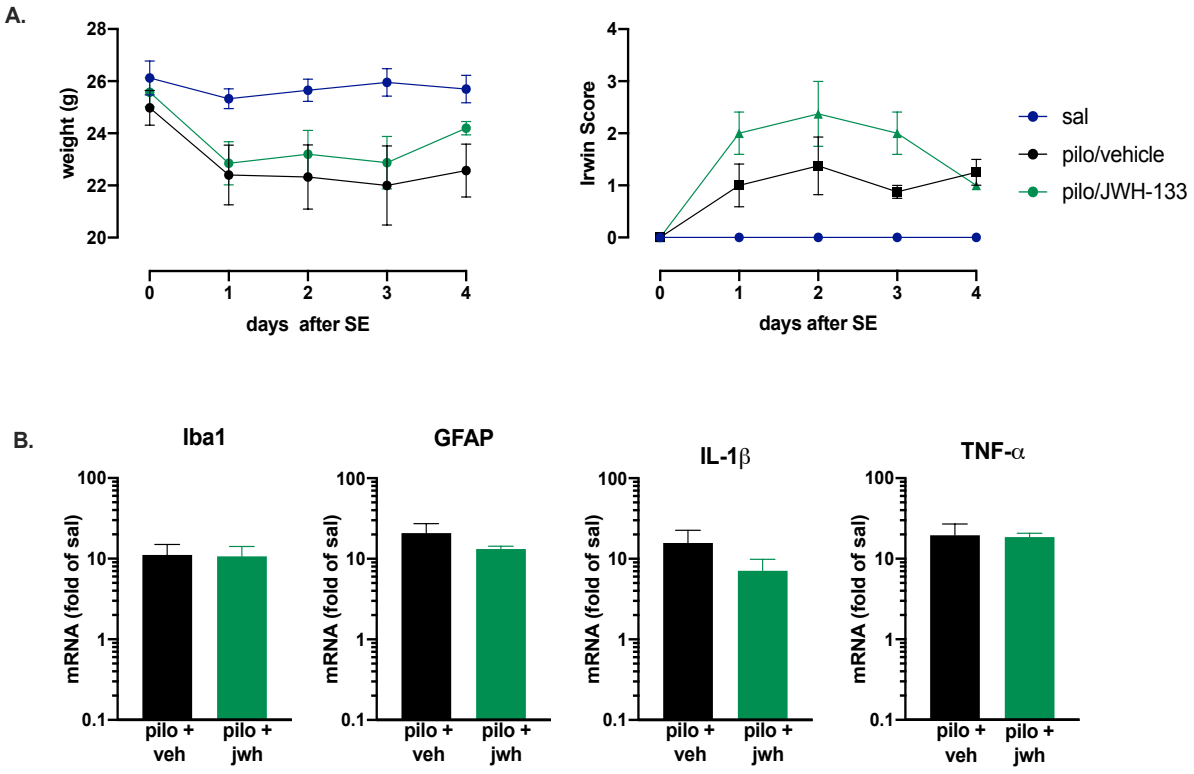

Figure A5. Effects of JWH-133 in the pilocarpine model. A) JWH-133 did not significantly impact weight or functional recovery following SE. **B)** JWH-133 did not significantly impact inflammatory cytokine expression 4 days post-SE. n=4-5/group.

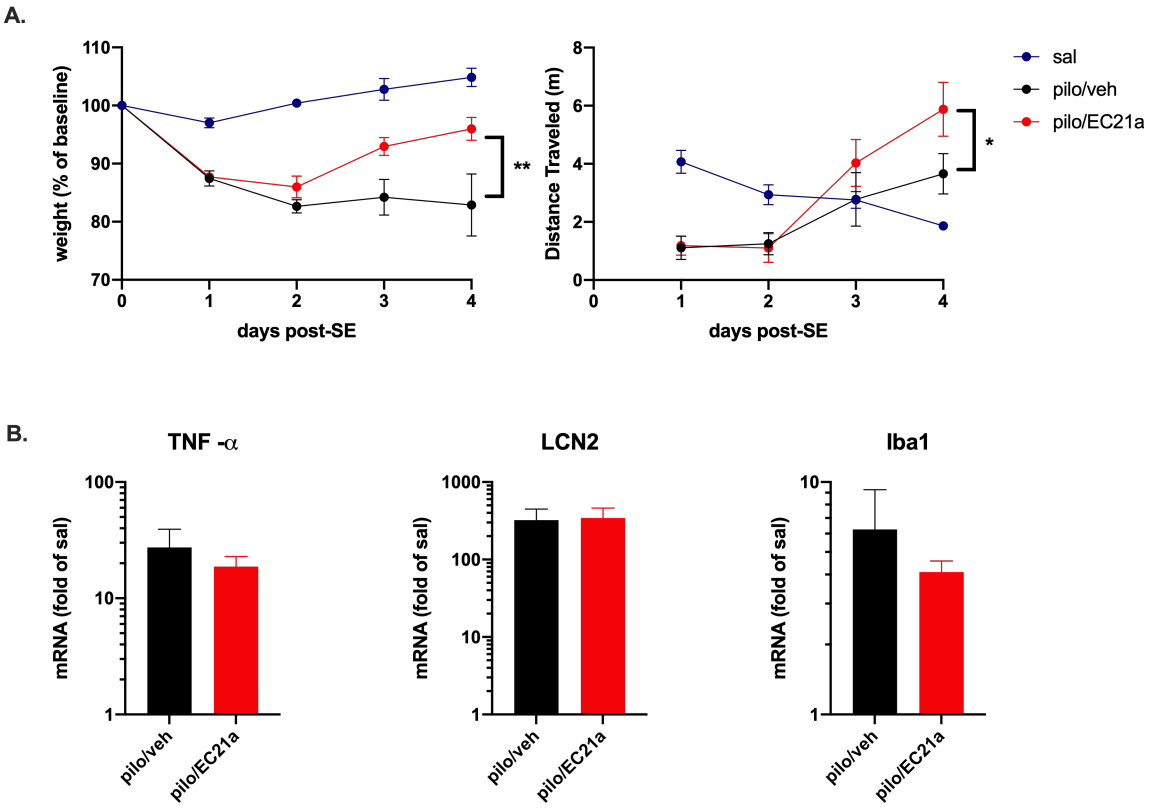

Figure A6. Effects of Ec21a in the pilocarpine model. A) Ec21a-treated animals show significant weight recovery compared to vehicle-treated mice at 4 days post-SE. Ec21a-treated animals traveled a greater distance in the open field compared to both saline and pilo/vehicle mice at 4 days post-SE. * $P < 0.05$, ** $P < 0.01$. 2-way ANOVA. **B)** Ec21a did not significantly impact inflammatory cytokine expression 4 days post-SE. n= 5-6/group.

A.4 Summary

Our results suggest that Ec21a treatment may have accelerated functional recovery following SE. However, that Ec21a-treated animals were significantly more active compared to animals that did not undergo SE could suggest hyperactivity, a behavioral abnormality sometimes observed in epilepsy models. Our interpretation of these results is limited by the small sample sizes. Further studies will be necessary to definitively establish the effect of Ec21a on functional recovery in this model.

Although it was disappointing that we did not observe any effects of JWH-133 or Ec21a on neuroinflammation, it is possible that there are other phenotypes which could have been impacted by drug treatment which we did not measure. For example, in models of other neurological diseases, CB2R agonists protected against neuron loss and BBB breakdown. Thus, future studies can assess whether treatment with these compounds impact other phenotypes observed in the model. Likewise, we have not yet investigated whether long-term treatment with CB2R compounds might ameliorate the development of spontaneous seizures and behavioral abnormalities in the chronic phase of the model. Moreover, our laboratory is actively evaluating the effects of Ec21a treatment on a panel of inflammatory markers in a primary microglia culture assay, in order to ascertain whether Ec21a treatment may influence other inflammatory targets which we have not yet examined in vivo. Overall, further studies will be necessary to determine the full extent of CB2R-mediated effects in the pilocarpine model.

A.5 Acknowledgements

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