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## Reduced Cannabinoid 2 Receptor Activity Increases Susceptibility to Induced Seizures in Mice

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### SUMMARY

**Objective**—The endocannabinoid system (ECS) is comprised of cannabinoid receptors 1 and 2 (CB1R and CB2R), endogenous ligands, and regulatory enzymes, and serves to regulate several important physiological functions throughout the brain and body. Recent evidence suggests that the ECS may be a promising target for the treatment of epilepsy, including epilepsy subtypes that arise from mutations in the voltage-gated sodium channel *SCN1A*. The objective of this study was to explore the effects of modulating CB2R activity on seizure susceptibility.

**Methods**—We examined susceptibility to induced seizures using a number of paradigms in CB2R knockout mice (*Cnr2*<sup>-/-</sup>), and determined the effects of the CB2R agonist, JWH-133, and the CB2R antagonist, SR144528, on seizure susceptibility in wild-type mice. We also examined seizure susceptibility in *Cnr2* mutants harboring the human *SCN1A* R1648H (RH) epilepsy mutation and performed EEG analysis to determine whether the loss of CB2Rs would increase spontaneous seizure frequency in *Scn1a* R1648H mutant mice.

**Results**—Both heterozygous (*Cnr2*<sup>+/-</sup>) and homozygous (*Cnr2*<sup>-/-</sup>) knockout mice exhibited increased susceptibility to PTZ-induced seizures. The CB2R agonist JWH-133 did not significantly alter seizure susceptibility in wild-type mice; however, administration of the CB2R antagonist SR144528 resulted in increased susceptibility to PTZ-induced seizures. In offspring from a cross between the *Cnr2* x RH lines, both *Cnr2* and RH mutants were susceptible to PTZ-induced seizures; however, 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.

**Significance**—Our results demonstrate that reduced CB2R activity is associated with increased seizure susceptibility. CB2Rs might therefore provide a therapeutic target for the treatment of some forms of epilepsy.

### Keywords

Cannabinoid 2 Receptor; *SCN1A*; seizure susceptibility; CB2R antagonist; CB2R agonist

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### DISCLOSURE OF CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

## INTRODUCTION

Over 30% of individuals with epilepsy are refractory to currently available medications, highlighting the need to develop more efficacious treatments.<sup>1</sup> The endocannabinoid system (ECS), comprised of cannabinoid receptors, endogenous ligands, and regulatory enzymes, is currently under investigation as a potential therapeutic target for the treatment of several neurological disorders, including epilepsy.<sup>2-4</sup>

Several studies have demonstrated that drugs targeting the ECS can modulate seizure susceptibility via activity at cannabinoid receptors.<sup>5-7</sup> 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.<sup>8; 9</sup> DS is most often caused by mutations in the *SCN1A* gene, encoding the voltage-gated sodium channel (VGSC) Na<sub>v</sub>1.1. VGSCs play an important role in regulating neuronal excitability, and *SCN1A* mutations have been associated with a variety of seizure disorders.<sup>10-12</sup> The recent success of the CBD clinical trials in DS suggests a potential role for cannabinoids in other forms of epilepsy.<sup>8</sup> However, CBD treatment can be associated with adverse effects, and is not effective in all patients.<sup>9; 13</sup> 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.<sup>14-18</sup> In contrast, cannabinoid 2 receptors (CB2Rs) have received less attention for their role in the central nervous system (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.<sup>19-22</sup> Although the cell-specific pattern of CB2R expression in the brain remains controversial, both microglial and neuronal expression have been described.<sup>20; 23-25</sup> Furthermore, CB2Rs are highly upregulated in response to brain injury in several mouse models,<sup>26; 27</sup> including a rat model of epilepsy in which CB2R expression was upregulated on hippocampal neurons following status epilepticus, suggesting that CB2Rs may play a protective role following injury.<sup>28</sup>

The few studies that have directly examined the effect of modulating CB2R activity on seizure susceptibility have yielded inconsistent results. Systemic administration of the CB2R agonist, beta caryophyllene, was shown to protect against acutely induced seizures in mice;<sup>29,30</sup> however, another study reported that direct brain injection of another CB2R agonist, AM1241, increased seizure severity in rats.<sup>31</sup> Additionally, the CB2R agonist HU-308 had no effect on PTZ-induced seizures in young rats.<sup>32</sup> Furthermore, the CB2R antagonist, AM630, increased seizure susceptibility in CB1R knockout mice.<sup>33</sup> 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 may contribute to increased seizure susceptibility.<sup>34</sup> Thus, while there is evidence that modulating CB2R activity can affect seizure susceptibility, this relationship remains unclear, highlighting the impact of differences in study design, including species, seizure model, and dosing.

To elucidate the effect of modulating CB2R activity on seizure susceptibility, we examined susceptibility to induced seizures in a CB2R knockout mouse line (*Cnr2*<sup>-/-</sup>) 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+).<sup>10; 35</sup> Patients with GEFS+ exhibit a wide range of seizure phenotypes, including absence seizures, myoclonic seizures, and both febrile and afebrile seizures.<sup>10</sup> Previous work from our laboratory demonstrated that heterozygous *Scn1a*<sup>RH/+</sup> mice are susceptible to induced seizures and exhibit infrequent spontaneous seizures.<sup>35</sup>

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.

## MATERIALS AND METHODS

### Animals

Two CB2R knockout mice (*Cnr2*<sup>-/-</sup>) 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*<sup>RH/+</sup> mice harboring the human *SCN1A* R1648H mutation were generated as previously described and maintained on a C57BL/6J background.<sup>35</sup> To generate *Cnr2*<sup>-/-</sup> and *Scn1a*<sup>RH/+</sup> double-mutants, *Scn1a*<sup>RH/+</sup> mice were first bred to *Cnr2*<sup>-/-</sup> mutants. Heterozygous *Cnr2*<sup>+/-</sup> offspring that also expressed the RH mutation were then crossed to *Cnr2*<sup>+/-</sup> heterozygous mutants to generate six possible genotypes: *Cnr2*<sup>+/+</sup>/*Scn1a*<sup>+/+</sup> (WT), *Cnr2*<sup>+/+</sup>/*Scn1a*<sup>RH/+</sup> (RH), *Cnr2*<sup>+/-</sup>/*Scn1a*<sup>+/+</sup> (*Cnr2*<sup>+/-</sup>), *Cnr2*<sup>+/-</sup>/*Scn1a*<sup>RH/+</sup> (*Cnr2*<sup>+/-</sup>/RH), *Cnr2*<sup>-/-</sup>/*Scn1a*<sup>+/+</sup> (*Cnr2*<sup>-/-</sup>), and *Cnr2*<sup>-/-</sup>/*Scn1a*<sup>RH/+</sup> (*Cnr2*<sup>-/-</sup>/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.<sup>36</sup> 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*<sup>-/-</sup> 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*<sup>+/-</sup>) yielded 385 bp and 550 bp PCR products, and homozygous animals (*Cnr2*<sup>-/-</sup>) generated a single 550 bp PCR band.

To screen for the *Scn1a*<sup>RH/+</sup> 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.<sup>37</sup> 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.<sup>38; 39</sup> 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.<sup>40</sup> Briefly, mice were placed in a plexiglass chamber (13.5 × 8 × 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.<sup>38; 41</sup> 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 °C every two minutes until either a GTCS occurred or 42.5 °C 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*<sup>-/-</sup>/RH mice as previously described.<sup>42; 43</sup> 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 *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 Kruskal-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  $P < 0.05$ .

## RESULTS

### ***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*<sup>+/-</sup> and *Cnr2*<sup>-/-</sup> mutants exhibited significantly lower latencies to the first MJ ( $P < 0.001$ , Fig 1A) and GTCS (*Cnr2*<sup>+/-</sup>,  $P < 0.01$ ; *Cnr2*<sup>-/-</sup>,  $P < 0.001$ , Fig 1B) compared to WT littermates. The percent of mice reaching GTCS over time in *Cnr2*<sup>+/-</sup> and *Cnr2*<sup>-/-</sup> mutants was also significantly different from WT littermates ( $P < 0.001$ , Fig 1C). Seizure latencies did not significantly differ between *Cnr2*<sup>+/-</sup> and *Cnr2*<sup>-/-</sup> mutants (Fig 1A–C).

We then examined seizure susceptibility using the 6 Hz seizure induction paradigm. Male *Cnr2*<sup>-/-</sup> 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 1D). Heterozygous mutants did not differ significantly from WT littermates or homozygous mutants.

Susceptibility to seizures induced by flurothyl (Fig 2A–B) and KA (Fig 2C) 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.

### **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*<sup>-/-</sup> 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 3A–B). SR144528 treatment had no effect on latency to the MJ or GTCS in *Cnr2*<sup>-/-</sup> mice, indicating that the effects of SR144528 are CB2R-specific (Fig 3A–B). Vehicle-treated *Cnr2*<sup>-/-</sup> mutants exhibited decreased latencies to both the MJ and GTCS when compared to vehicle-treated WT littermates ( $P < 0.001$ , Fig 3A–B), consistent with the data shown in Fig 1A–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 3C). SR144528 had no effect on susceptibility to 6 Hz-induced seizures in *Cnr2*<sup>-/-</sup> mice (Fig 3C).

### **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*<sup>+/-</sup>, and *Cnr2*<sup>-/-</sup> mice 30 minutes prior to inducing seizures with PTZ. As expected, vehicle-treated *Cnr2*<sup>-/-</sup> and *Cnr2*<sup>+/-</sup> 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 4).

### **Deletion of CB2Rs does not worsen seizure phenotypes in mice expressing the *SCN1A* R1648H mutation**

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 5). Further, male *Cnr2*<sup>-/-</sup>/RH mutant mice exhibited significantly reduced latencies to the MJ compared to RH mutant mice ( $P < 0.001$ , Fig 5A), although average latency to the first GTCS was not significantly different (Fig 5B). Female *Cnr2*<sup>-/-</sup>/RH mice did not show significantly altered latencies to either the MJ or GTCS when compared to female RH mutants (Fig 5C–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* mutant mice.

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*<sup>+/-</sup>/RH mutants showed significantly reduced latencies to the MJ when compared to RH mutants ( $P < 0.05$ , Fig 6A), but did not show significantly different latencies to the GTCS (Fig 6B). *Cnr2*<sup>-/-</sup>/RH male mice did not show any significant differences in seizure latencies when compared to RH-only mutants (Fig 6A–B). Female *Cnr2*<sup>-/-</sup>/RH mutants showed significantly reduced latencies to the MJ when compared to RH mutants ( $P < 0.05$ , Fig 6C), but did not show significant differences in latency to the GTCS (Fig 6D). 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.

### **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.<sup>35</sup> 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*<sup>-/-</sup>/RH mutant mice. Spontaneous seizures were not detected in any mouse during the recording period ( $n = 4/\text{genotype}$ ).

## **DISCUSSION**

We show that the loss of CB2Rs (*Cnr2*<sup>-/-</sup> mutants) 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*<sup>+/-</sup>) and homozygous (*Cnr2*<sup>-/-</sup>) mutants exhibited increased susceptibility to PTZ-induced seizures, there was no significant difference between *Cnr2*<sup>+/-</sup> and *Cnr2*<sup>-/-</sup> mutants, indicating that partial reduction of CB2R activity is sufficient to increase seizure susceptibility. While *Cnr2*<sup>+/-</sup> mutants presumably have 50% loss of CB2R protein compared to WT littermates, the lack of reliable CB2R antibodies<sup>44; 45</sup> makes it difficult to quantify differences in protein levels between genotypes.

Studies which have previously examined the effects of CB2R compounds on seizure phenotypes have yielded inconsistent results.<sup>29-33</sup> Although some studies reported that the CB2R agonist beta caryophyllene can be seizure protective,<sup>29; 30</sup> other studies observed either no effect of a CB2R agonist (HU308),<sup>32</sup> or greater seizure severity following administration of the CB2R agonist AM1241.<sup>31</sup> We found no effect of the CB2R agonist, JWH-133, on PTZ-induced seizures (Fig 4). These inconsistencies may be due to differences in study design, including seizure induction paradigm, species, and the specific agonist or antagonist tested.<sup>29-33</sup> We selected JWH-133 and SR144528 for our studies because they are highly potent and CB2R-specific, exhibiting few off-target effects in vitro.<sup>46-48</sup> 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<sup>49</sup> and reduction of post-surgical cognitive impairment and associated neuroinflammation.<sup>50</sup> 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.<sup>51; 52</sup> Thus, both drugs have been shown to be effective at doses similar to those used in our study. The inclusion of *Cnr2*<sup>-/-</sup> 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.<sup>23; 53; 54</sup> 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.<sup>14</sup> 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,<sup>20; 23-25</sup> and recent studies have shown that CB2Rs play a role in regulating neuronal excitability.<sup>20; 23; 55</sup> For example, application of JWH-133 reduces the excitability of cortical pyramidal neurons in brain slices from WT mice.<sup>55</sup> 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.<sup>23</sup> 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.<sup>28</sup> 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,<sup>56</sup> 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.<sup>57</sup> 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*<sup>-/-</sup> 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<sup>35</sup>; however, we did not observe spontaneous seizures in either RH or *Cnr2*<sup>-/-</sup>/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 cannabidiol (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 Dravet syndrome.<sup>58</sup> 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.<sup>58; 59</sup> 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.<sup>60-62</sup> This reduction in neuroinflammation has been associated with improved outcomes in these models.<sup>62-64</sup> Several forms of drug-resistant epilepsy, such as mesial temporal lobe epilepsy (MTLE), are characterized by high levels of neuroinflammation.<sup>65; 66</sup> 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.

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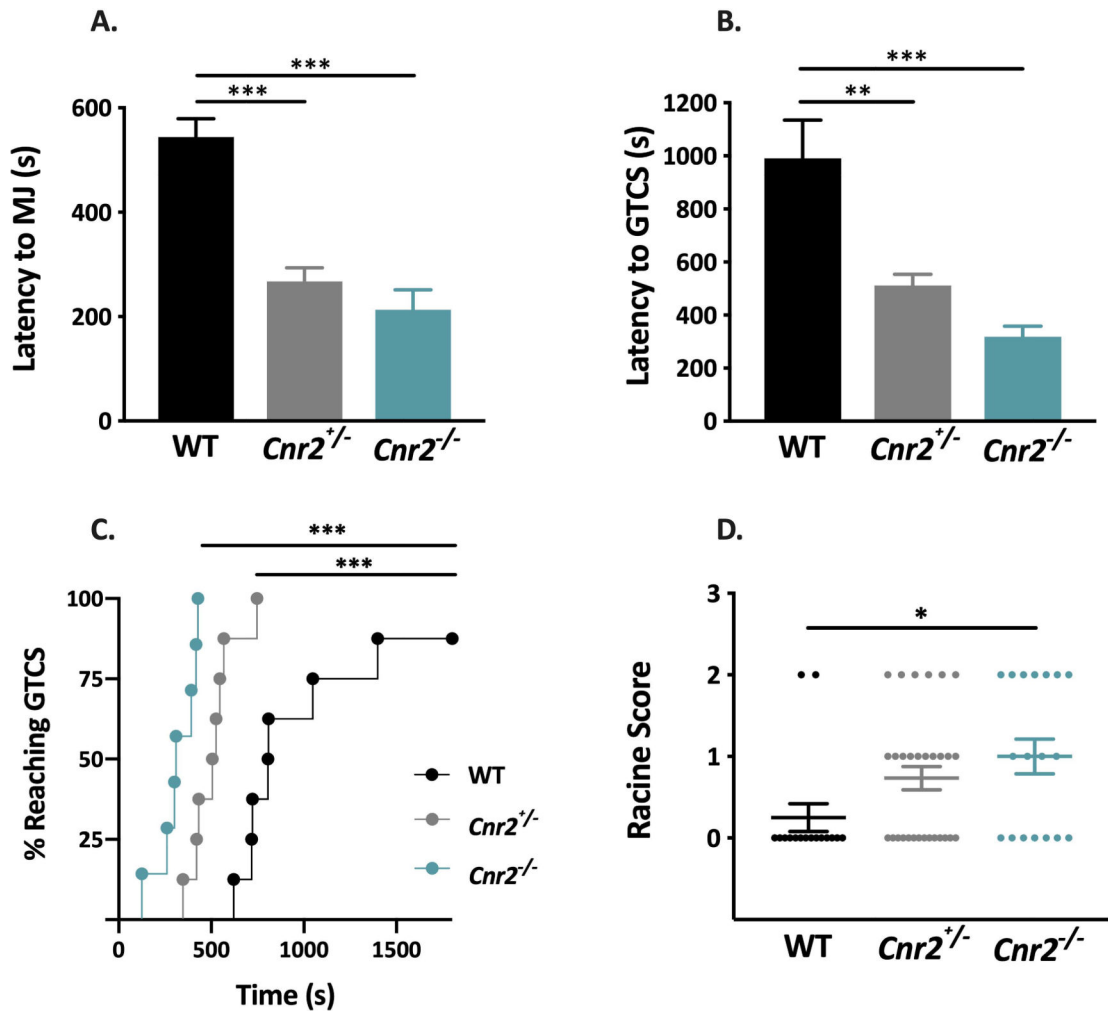
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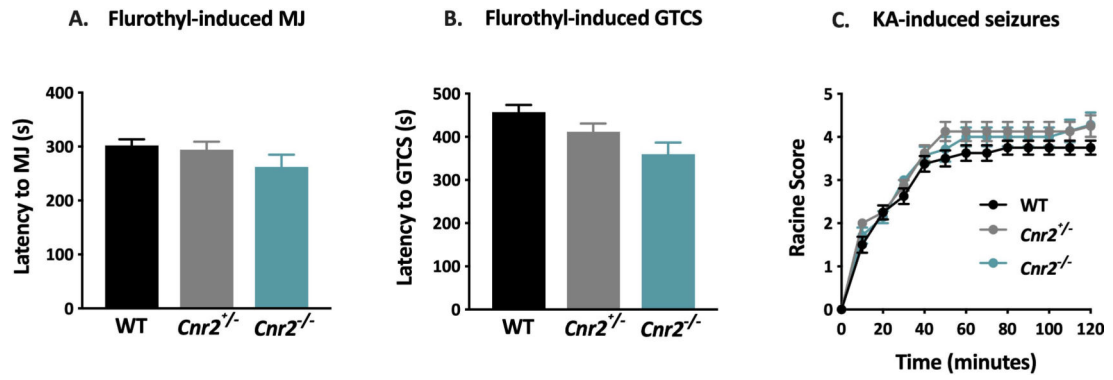
**KEY POINTS**

- Genetic deletion of the cannabinoid 2 receptor (CB2R) increases seizure susceptibility in mice.
- The CB2R antagonist, SR144528, increases seizure susceptibility in wild-type mice.
- Deletion of CB2Rs in a mouse model of human *SCN1A*-derived epilepsy does not further increase seizure susceptibility.
- Spontaneous seizure frequency was not increased in mice expressing both *Cnr2* and *Scn1a* mutations.
- CB2Rs may provide a therapeutic target for the treatment of some forms of epilepsy.



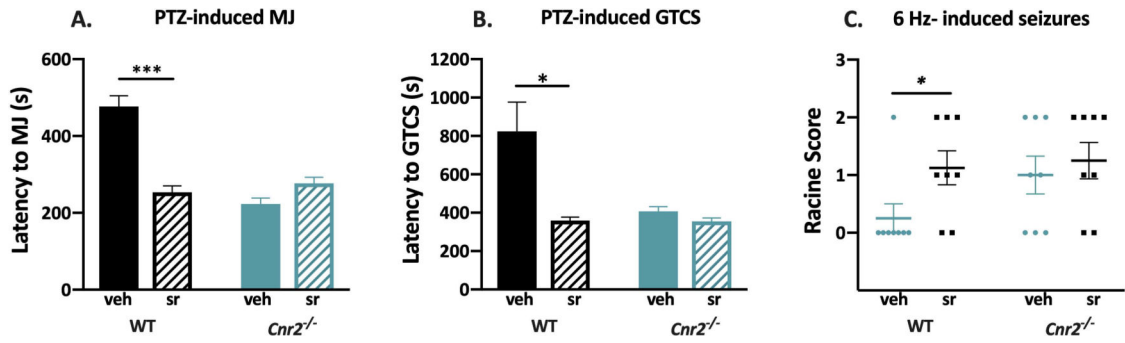
**Figure 1. *Cnr2* mutant mice exhibit increased susceptibility to induced seizures.**

**A.** *Cnr2*<sup>+/-</sup> and *Cnr2*<sup>-/-</sup> 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*<sup>+/-</sup> and *Cnr2*<sup>-/-</sup> mutants reaching GTCS, and latency to GTCS, are significantly different from WT littermates. Mantel-Cox log-rank test. **D.** *Cnr2*<sup>-/-</sup> mice exhibit higher average Racine scores when compared to WT littermates following an 18 mA electrical stimulus. Each symbol represents one mouse. Kruskal-Wallis Test with Dunn's multiple comparisons. WT, n=16; *Cnr2*<sup>+/-</sup>, n=30; *Cnr2*<sup>-/-</sup>, n=18. For **A-D**, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. All error bars represent mean ± SEM.



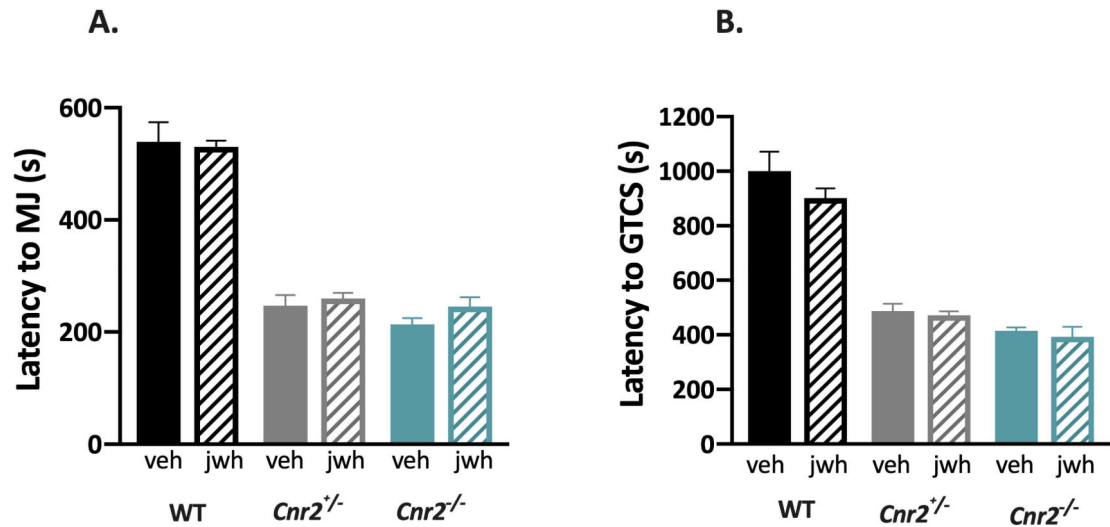
**Figure 2.** *Cnr2* mutant mice are not susceptible to flurothyl- or kainic acid-induced seizures. Latency to the first MJ (A) and GTCS (B) was comparable between genotypes following flurothyl exposure. 1- way ANOVA with Tukey's post-hoc comparison, n=8/group. C. The response of *Cnr2* mutant mice to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





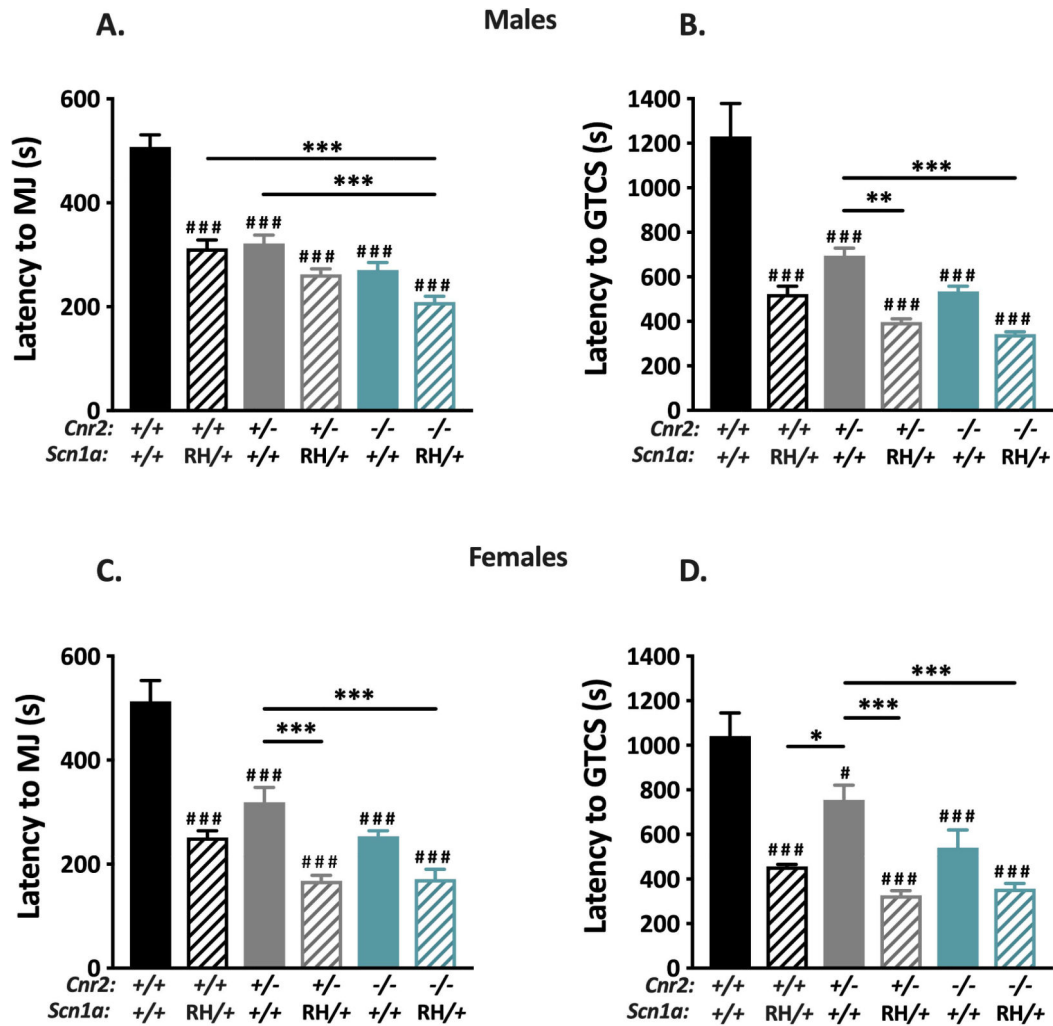
**Figure 3. The CB2R specific antagonist, SR144528, increases seizure susceptibility.**

**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*<sup>-/-</sup> 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*<sup>-/-</sup> 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  $\pm$  SEM.

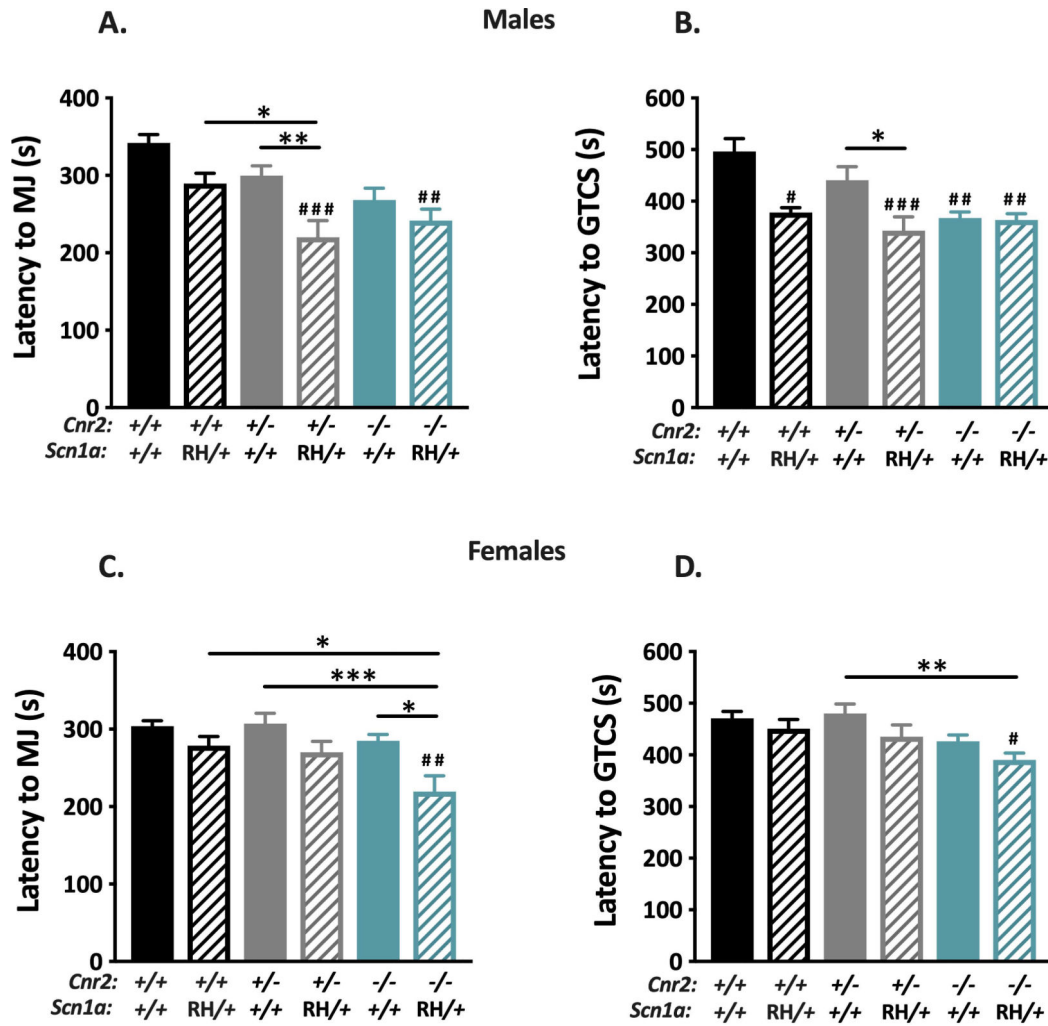


**Figure 4. The CB2R specific agonist, JWH-133, does not alter susceptibility to PTZ-induced seizures.**

The CB2R agonist JWH-133 did not significantly alter latencies to the first MJ (A) or GTCS (B) in WT, *Cnr2*<sup>+/-</sup> or *Cnr2*<sup>-/-</sup> 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 5. Deletion of CB2Rs does not worsen PTZ-induced seizures in *Scn1a*<sup>RH/+</sup> 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 (### $P < 0.001$ , # $P < 0.05$ ). **A.** Male *Cnr2*<sup>-/-</sup>/RH mutants (teal stripe) show decreased latency to the MJ compared to both *Cnr2*<sup>+/-</sup> (gray) and RH (black stripe) (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). **B.** Both *Cnr2*<sup>+/-</sup>/RH (gray stripe) and *Cnr2*<sup>-/-</sup>/RH male mice (teal stripe) exhibit decreased latency to GTCS when compared to *Cnr2*<sup>+/-</sup> mice (gray) (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). **C.** Female *Cnr2*<sup>+/-</sup>/RH (gray stripe) and *Cnr2*<sup>-/-</sup>/RH (teal stripe) mice exhibit decreased latencies to the MJ when compared to *Cnr2*<sup>+/-</sup> (gray) mutant mice (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). **D.** Female *Cnr2*<sup>+/-</sup>/RH (gray stripe) and *Cnr2*<sup>-/-</sup>/RH (teal stripe) mice exhibit decreased latencies to the GTCS when compared to *Cnr2*<sup>+/-</sup> (gray) mutant mice (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). RH female mice (black stripe) exhibit decreased latency GTCS when compared to *Cnr2*<sup>+/-</sup> (gray) mutant mice (\* $P < 0.05$ ). 2-way ANOVA with Tukey's post-hoc test, n=8-12/group. Symbols above individual bars denote statistical significance from WT. All error bars represent mean  $\pm$  SEM.



**Figure 6. Deletion of CB2Rs does not worsen flurothyl-induced seizures in *Scn1a*<sup>RH/+</sup> mutant mice.**

**A.** Male *Cnr2*<sup>-/-</sup>/RH (teal stripe) and *Cnr2*<sup>+/-</sup>/RH (gray stripe) mutants exhibit decreased latency to the MJ when compared to WT (black) (<sup>##</sup>*P* < 0.01, <sup>###</sup>*P* < 0.001). *Cnr2*<sup>+/-</sup>/RH mutants (gray stripe) also show decreased latency to the MJ when compared to RH (black stripe) (<sup>\*</sup>*P* < 0.05) and *Cnr2*<sup>+/-</sup> (gray) (<sup>\*\*</sup>*P* < 0.001). **B.** When compared to WT, RH (black stripe) (<sup>#</sup>*P* < 0.05), *Cnr2*<sup>+/-</sup>/RH (gray stripe) (<sup>###</sup>*P* < 0.001), *Cnr2*<sup>-/-</sup> (teal) (<sup>##</sup>*P* < 0.01), and *Cnr2*<sup>-/-</sup>/RH (teal stripe) male mutants (<sup>###</sup>*P* < 0.01) exhibit significantly decreased latencies to GTCS. *Cnr2*<sup>+/-</sup>/RH mutants (gray stripe) exhibit significantly decreased latency to the GTCS compared to *Cnr2*<sup>+/-</sup> mice (gray) (<sup>\*</sup>*P* < 0.05). **C.** *Cnr2*<sup>-/-</sup>/RH female mice (teal stripe) show significantly decreased latency to the MJ when compared to WT (black) (<sup>##</sup>*P* < 0.01), RH (black stripe) (<sup>\*</sup>*P* < 0.05), *Cnr2*<sup>+/-</sup> (gray) (<sup>\*\*\*</sup>*P* < 0.001), and *Cnr2*<sup>-/-</sup> (teal) (<sup>\*</sup>*P* < 0.05) mice. **D.** *Cnr2*<sup>-/-</sup>/RH female mice (teal stripe) exhibit decreased latency to the GTCS when compared to WT (<sup>#</sup>*P* < 0.05) and *Cnr2*<sup>+/-</sup> mutant mice (gray) (<sup>\*\*</sup>*P* < 0.01). 2-way ANOVA with Tukey's post hoc comparison, n=8–12/group. Symbols above individual bars indicate significance from WT. Error bars represent mean ± SEM