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Cannabinoid receptor 1/2 double-knockout mice develop epilepsy

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Summary

The endocannabinoid system has gained attention as an important modulator of activity in the central nervous system (CNS). Initial studies focused on cannabinoid receptor 1 (CB1), which is widely expressed in the brain, but recent work also implicates cannabinoid receptor 2 (CB2) in modulating neuronal activity. Both receptors are capable of reducing neuronal activity, generating interest in cannabinoid receptor agonists as potential anticonvulsants. CB1 (Cnr1) and CB2 (Cnr2) single-knockout mice have been generated, with the former showing heightened seizure sensitivity, but not overt seizures. Given overlapping and complementary functions of CB1 and CB2 receptors, we queried whether double-knockout mice would show an exacerbated neurological phenotype. Strikingly, 30% of double knockout mice exhibited provoked behavioral seizures, and 80% were found to be epileptic following 24/7 video-EEG monitoring. Single knockout animals did not exhibit seizures. These findings highlight the importance of the endocannabinoid system for maintaining network stability.

Keywords

Cannabinoid receptors; epilepsy; spontaneous seizures

Introduction

Endocannabinoids and their receptors are recognized as important regulators of neuronal activity^{1,2}. Attention has focused primarily on CB1 receptors (encoded by Cnr1), which are

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expressed widely throughout the CNS. The role of CB2 receptors (encoded by *Cnr2*) in epilepsy has received much less attention; mainly because initial studies suggested that CNS expression was limited. CB2 receptors are highly expressed in immune mediators, where they play important roles in regulating immune function. Recent evidence that CB2 receptors are expressed in brain³, however, has raised the possibility that they could also play a direct role in regulating CNS function.

Cnr1^{-/-} mice have reduced seizure threshold in the kainate model of temporal lobe epilepsy⁴. Moreover, a majority of studies indicate that increased CB1 receptor activity is anticonvulsant and decreased activity is proconvulsant⁵. On the other hand, *Cnr2*^{-/-} mice have not been reported to exhibit a seizure phenotype⁶; although neurological deficits – including memory impairment and schizophrenia-like symptoms – have been reported⁷.

CB1 and CB2 receptors are activated by the endogenous cannabinoids anandamide and 2-arachidonoylglycerol (2-AG). They are primarily coupled to the G_{i/o} family of G-proteins. Both receptors can decrease excitatory synaptic transmission in the CNS⁸. Recent studies have identified novel roles for CB2 receptors in inducing hippocampal pyramidal cell hyperpolarization³, and suppressing epileptic seizures⁶. Given these newfound roles for CB2 receptors in brain, we asked whether *Cnr1*^{-/-}/*Cnr2*^{-/-} double-knockout mice would exhibit a neurological phenotype distinct from single-knockouts.

Methods

Animals

Animal procedures were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Hospital Research Foundation and conform to NIH Guidelines. *Cnr1*^{-/-} and *Cnr2*^{-/-} knockout mice were created as described previously^{9,10}. *Cnr1*^{-/-} and *Cnr2*^{-/-} single-knockout mice are maintained on a C57BL/6J background, and in the present study are compared to C57BL/6J wildtype (BL6-WT) mice. *Cnr1*^{-/-}/*Cnr2*^{-/-} double-knockout mice and their respective *Cnr1*^{+/+}/*Cnr2*^{+/+} controls (BL6/CD1-WT) were generated by crossing *Cnr1*^{+/+} and *Cnr2*^{+/+} mice. *Cnr1*^{-/-}/*Cnr2*^{-/-} double-knockout and BL6/CD1-WT mice were maintained by homozygous breeding thereafter on a mixed C57BL/6J; CD-1 background. Premature mortality was assessed in 142 BL6/CD1-WT, 64 *Cnr1*^{-/-}, 21 *Cnr2*^{-/-}, and 170 *Cnr1*^{-/-}/*Cnr2*^{-/-} double-knockout male and female mice. Tail DNA was tested after euthanasia or death to confirm genotype in experimental animals.

Implantation and Video/EEG Monitoring

Four BL6/CD1-WT, four *Cnr1*^{-/-}, four *Cnr2*^{-/-}, and five *Cnr1*^{-/-}/*Cnr2*^{-/-} mice were implanted with EEG electrodes at approximately four months-of-age (120±8.6, 120.4±3.3, 124, and 125±6.4 days, respectively; *p*=0.907, one-way ANOVA). All implanted mice were male. Briefly, mice were anesthetized with isoflurane (induction at 4%, maintenance at 1–1.5%), the skull was exposed, and 1-mm-diameter holes were drilled at positions 1.5 mm anterior to lambda and 1.5 mm lateral to midline over each hemisphere. The dura was left intact. A single wire electrode was positioned in each hole just above the dura. Additional support was provided by setting two skull screws, and the entire assembly was secured with

dental cement. The two-lead wireless transmitter (TA11ETA-F10; Data Sciences International) was placed subcutaneously under the back of the animal. Animals were housed in cages placed on top of EEG receiver plates (RPC1; Data Sciences International). Animal behavior was monitored by video (resolution, 640 × 480 pixels; Axis 221 cameras; Axis Communications). Synchronized video/EEG data was collected continuously for approximately one month or until animal death. Seizures were identified by an investigator blind to animal genotype using Neuroscore software (version 3.4.2; Data Sciences International).

Handling-Induced Seizures

One to two weeks after surgery, all mice implanted with EEG electrodes were suspended by the tail with slight agitation for 10 seconds to assess for provoked seizures. Animals that exhibited an electrographic seizure during tail suspension were not tested again. Animals that did not experience a seizure were re-tested one week later. Additionally, cohorts of non-implanted mice from all groups were tested for behavioral seizures by tail suspension. Testing was done once a week for 3 weeks by tail suspension for 10 seconds. Final numbers for tail suspension testing were: BL6/CD1-WT (13 male, 11 female), BL6-WT (11 male, 11 female), *Cnr1*^{-/-} (13 male, 14 female), *Cnr2*^{-/-} (11 male, 11 female), and *Cnr1*^{-/-}/*Cnr2*^{-/-} (16 male, 13 female). Tail suspension experiments were conducted on adult animals that ranged in age from 3 to 5 months. Age did not differ by group ($p=0.284$). All experiments were conducted with the investigator blind to genotype.

Statistics

One-way ANOVAs were conducted using SigmaPlot version 13.0. Shapiro-Wilk and Brown-Forsythe tests were used to assess normality and equal variance. Fisher's exact tests for association between two categorical variables (groups and seizure) were conducted using SAS software, version 9.3, SAS Institute Inc., Cary, N.C.; RRID:SCR_008567). $P<0.05$ was accepted as significant. Means $\pm$ SEM are reported.

Results

Cnr1^{-/-}/*Cnr2*^{-/-} mice were viable, and appeared healthy at young ages (<8 weeks). Casual observation during routine handling did not reveal obvious differences in behavior between young BL6/CD1-WT, BL6-WT, single or double-knockout animals. Beginning at approximately two months of age, however, *Cnr1*^{-/-}/*Cnr2*^{-/-} mice were observed to exhibit spontaneous seizure-like behavior, particularly during handling for cage changes. Behaviors were consistent with convulsive, clonic-tonic seizures with rearing and falling, suggesting that a significant portion of double-knockout mice develop epilepsy. To quantify these anecdotal observations, a cohort of adult BL6/CD1-WT, BL6-WT, *Cnr1*^{-/-}, *Cnr2*^{-/-} and *Cnr1*^{-/-}/*Cnr2*^{-/-} mice were tested for handling induced seizures. 8 of 29 (27.6%) double-knockout mice tested had at least one seizure after handling. 5 of these animals were female and 3 were male (Fig. 1B). No seizures were observed in either of the single knockout, BL6/CD1-WT, or BL6-WT animals ($p=.0057$ for double knockout vs. all other groups; one way-ANOVA). There was no significant difference between males and females in any of the groups ($p=0.406$). Thirty-eight of 170 *Cnr1*^{-/-}/*Cnr2*^{-/-} mice (22.3%) died prematurely

before experiments could be performed. By comparison, 12 of 64 (18.8%) $Cnr1^{-/-}$, 2 of 21 (9.5%) $Cnr2^{-/-}$ and 4 of 142 (2.8%) BL6/CD1-WT mice died during this same time period (Fig. 1A). There was no difference between males and females in premature mortality ($p=0.914$).

Since a variety of conditions can produce abnormalities reminiscent of seizures – such as movement disorders – a subset of male BL6/CD1-WT, $Cnr1^{-/-}$, $Cnr2^{-/-}$, and double-knockout mice were implanted with cortical electrodes to confirm the presence of electrographic seizures. Only males were used for video-EEG studies, since the incidence of behavioral seizure-like events was similar in males and females. Mice were implanted at approximately four months of age and monitored 24/7 by video/EEG for approximately one month (BL6/CD1-WT, $n=4$, 28.3 ± 8.2 days; $Cnr1^{-/-}$, $n=4$, 25.2 ± 7.4 days; $Cnr2^{-/-}$, $n=3$, 27 ± 3 days; $Cnr1^{-/-}/Cnr2^{-/-}$, $n=5$, 31.6 ± 7.8 ; $p=0.930$, one-way ANOVA). One of the original four $Cnr2^{-/-}$ animals was euthanized following surgical complications. Four out of five double-knockout mice (80%) exhibited at least one spontaneous electrographic seizure during the recording period, while seizures were absent from BL6/CD1-WT and single knockout mice. Examples of EEG-recorded baseline traces and seizures are shown in figure 1C. Electrographic seizures were associated with class III, freezing/staring episodes in one double-knockout animal. The remaining three animals exhibited class V, tonic/clonic seizures with rearing and falling; consistent with observations in non-implanted animals. Finally, three of four double knockout animals tested experienced behavioral seizures during tail suspension. EEG recording confirmed that these behavioral events were associated with electrographic seizures (Fig. 1). Compiled seizure details are provided in table 1.

Two of five $Cnr1^{-/-}/Cnr2^{-/-}$ mice died before the end of the study. One of these animals had a severe tonic/clonic seizure, lasting 76 seconds, and died immediately thereafter. The second animal had a milder, class three seizure, became lethargic, and died later in the day. The remaining animals survived the entire study period, but varied in morbidity. The one seizure-free double-knockout mouse was behaviorally indistinguishable from the BL6/CD1-WT mice, while the remaining two double-knockout mice exhibited lethargy after seizure events but appeared otherwise healthy.

Discussion

Here, we demonstrate that $Cnr1^{-/-}/Cnr2^{-/-}$ mice develop epilepsy; a qualitatively more severe phenotype than has been reported for either $Cnr1$ or $Cnr2$ single-knockouts. $Cnr1^{-/-}$ knockout mice have been reported to exhibit reduced seizure thresholds⁴, but spontaneous seizures have not been described following single deletion of either gene. EEG and behavioral measures of seizure activity in the present study confirm these prior findings for single knockout mice. The absence of seizures in single knockouts could reflect compensatory functions of the remaining receptor, as both receptors are expressed in brain and regulate neuronal excitability³. Alternatively, functions unique to the CB2 receptor might account for the more severe phenotype observed when both receptors are eliminated. Finally, while $Cnr1^{-/-}$ mice had similar mortality to double knockouts, they did not show spontaneous seizure activity, suggesting that early mortality is driven by factors other than seizures. Other groups have reported increased mortality in $Cnr1^{-/-}$ mice^{10,11} but the cause

remains unclear. Cardiovascular or other neurological problems besides seizures could lead to the observed increase in mortality.

Several mechanisms may lead to epilepsy in mice with combined loss of both cannabinoid receptors. Most notably, since both receptors are now implicated in regulating neuronal activity in the CNS, it follows that loss of both receptors might produce a more severe deficit than single gene deletion. A second possibility is that loss of both receptors produces developmental changes in the brain that contribute to epileptogenesis. CB2 receptor activation has been shown to promote neuronal proliferation¹², and thus the receptors absence might impair neuronal development. Brains from double-knockout animals appeared grossly normal in the present study (data not shown), but more in-depth anatomical studies will be needed to determine whether subtle deficits are present. A third possibility is that genetic background could play a role. Double knockout animals were on a mixed C57BL/6J; CD1 background. Phenotypes can vary by background strain, so future studies will be needed to assess the potential role of interacting genetic factors. A final possibility is that loss of *Cnr2* contributes to epileptogenesis by enhancing inflammation, as CB2 receptors are widely expressed throughout the immune system, and suppress peripheral immune function upon activation¹³. Indeed, *Cnr2*^{-/-} mice have increased inflammation in response to stress, whereas transgenic overexpression of *Cnr2* was neuroprotective¹⁴. Inflammatory changes are hypothesized to contribute to epileptogenesis¹⁵. Enhanced inflammation, combined with central loss of CB1 receptors, might be sufficient in combination to initiate epileptogenesis.

The present findings add to a growing body of literature implicating CB1 and CB2 receptors as important regulators of neuronal activity, and as potential targets for the treatment of epilepsy. The exact mechanisms by which combined loss of both cannabinoid receptors produces epilepsy remains to be determined. The simple observation that this loss does cause epilepsy, however, underscores the importance of these receptors for regulating brain excitability, and strengthens the rationale for examining various agonists for anti-convulsant potential.

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Figure 1.

A: Percent survival for BL6/CD1-WT and knockout mice. Numerical values in bars are the number of animals for each measure. **B:** Handling-induced seizures for all experimental groups. **C:** Sample EEG recordings from monitored animals showing normal EEGs (top three traces) for controls and spontaneous and handling-induced seizures in double knockouts.

Table 1

Summary of 24/7 video-EEG Monitored Animals

Group	Age at Implantation (Days)	Time Recorded (Days)	#Spontaneous Seizures	Handling Induced Seizure?
BL6/CD1-WT	138	7	0	No
BL6/CD1-WT	111	47	0	No
BL6/CD1-WT	131	28	0	No
BL6/CD1-WT	101	31	0	No
Cnr1 ^{-/-}	110	49	0	No
Cnr1 ^{-/-}	130	9	0	No
Cnr1 ^{-/-}	122	12	0	No
Cnr1 ^{-/-}	122	34	0	No
Cnr2 ^{-/-}	124	21	0	No
Cnr2 ^{-/-}	124	30	0	No
Cnr2 ^{-/-}	124	30	0	No
Cnr1 ^{-/-} /Cnr2 ^{-/-}	134	22	2	not tested **
Cnr1 ^{-/-} /Cnr2 ^{-/-}	125	52	5	Yes **
Cnr1 ^{-/-} /Cnr2 ^{-/-}	128	49	2	Yes
Cnr1 ^{-/-} /Cnr2 ^{-/-}	101	15	11	Yes *
Cnr1 ^{-/-} /Cnr2 ^{-/-}	137	20	0	No

* handling induced seizure during cage change

** died prematurely