BRIEF COMMUNICATION

Cannabinoid receptor 1/2 double-knockout mice develop epilepsy

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Epilepsia, 58(12):e162–e166, 2017
doi: 10.1111/epi.13930

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Summary

The endocannabinoid system has gained attention as an important modulator of activity in the central nervous system. 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-electroencephalographic monitoring. Single-knockout animals did not exhibit seizures. These findings highlight the importance of the endocannabinoid system for maintaining network stability.

KEY WORDS: **Cannabinoid receptors, Epilepsy, Spontaneous seizures.**

Endocannabinoids and their receptors are recognized as important regulators of neuronal activity.[1,2] Attention has focused primarily on cannabinoid 1 (CB1) receptors (encoded by *Cnr1*), which are expressed widely throughout the central nervous system (CNS). The role of cannabinoid 2 (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 brain,[3] 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 epilepsy.[4] Moreover, a majority of studies indicate that increased CB1 receptor activity is anticonvulsant and decreased activity is proconvulsant.[5] On the other hand, $Cnr2^{-/-}$ mice have not been reported to exhibit a seizure phenotype,[6] although neurological deficits—including memory impairment and schizophrenia-like symptoms—have been reported.[7]

CB1 and CB2 receptors are activated by the endogenous cannabinoids anandamide and 2-arachidonoylglycerol. They are primarily coupled to the $G_{i/o}$ family of G-proteins. Both receptors can decrease excitatory synaptic transmission in the CNS.[8] Recent studies have identified novel roles for CB2 receptors in inducing hippocampal pyramidal cell hyperpolarization[3] and suppressing epileptic seizures.[6] 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.

Accepted September 28, 2017; Early View publication November 3, 2017.

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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 National Institutes of Health 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 wild-type (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-electroencephalographic monitoring

Four BL6/CD1-WT, four *Cnr1*$^{-/-}$, four *Cnr2*$^{-/-}$, and five *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mice were implanted with electroencephalographic (EEG) electrodes at approximately 4 months of age (120 ± 8.6, 120.4 ± 3.3, 124, and 125 ± 6.4 days, respectively; p = 0.907, one-way analysis of variance [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 were collected continuously for approximately 1 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 2 weeks after surgery, all mice implanted with EEG electrodes were suspended by the tail with slight agitation for 10 s 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 retested 1 week later. Additionally, cohorts of nonimplanted mice from all groups were tested for behavioral seizures by tail suspension. Testing was done once per week for 3 weeks by tail suspension for 10 s. 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 exact tests for association between two categorical variables (groups and seizure) were conducted using SAS software (version 9.3; SAS Institute, Cary, NC, U.S.A.; RRID: SCR_008567). A probability value of $p < 0.05$ was accepted as significant. Means ± standard error of the mean 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-knockout, or double-knockout animals. Beginning at approximately 2 months of age, however, *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mice were observed to exhibit spontaneous seizurelike 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. Eight of 29 (27.6%) double-knockout mice tested had at least one seizure after handling. Five of these animals were female and three were male (Fig. 1B). No seizures were observed in any of the single-knockout, BL6/CD1-WT, or BL6-WT animals (p = 0.0057 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*$^{-/-}$, two of 21 (9.5%) *Cnr2*$^{-/-}$, and four 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).

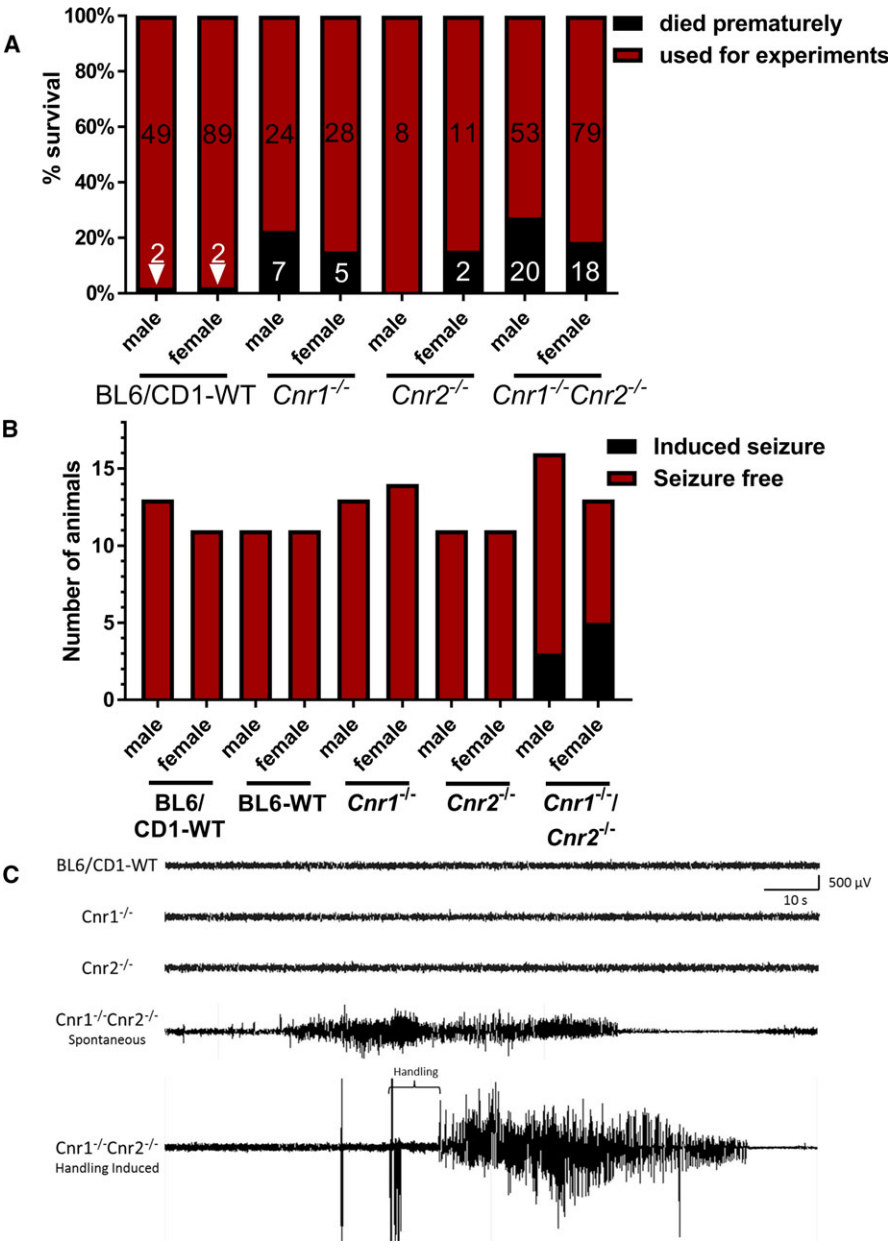

Figure 1.
(**A**) Percentage survival for BL6/CD1–wild-type (WT) and knockout mice. Numerical values in bars are the number of animals for each measure. (**B**) Handling-induced seizures for all experimental groups. (**C**) Sample electroencephalographic recordings from monitored animals showing normal electroencephalograms (top three traces) for controls and spontaneous and handling-induced seizures in double-knockouts.

Because 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, because the incidence of behavioral seizurelike events was similar in males and females. Mice were implanted at approximately 4 months of age and monitored 24/7 by video/EEG for approximately 1 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 of five double-knockout mice (80%) exhibited at least one spontaneous electrographic seizure during the recording period, whereas seizures were absent from BL6/CD1-WT and single-knockout mice. Examples of EEG-recorded baseline traces and seizures are shown in Fig. 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 nonimplanted 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.

Table 1. Summary of 24/7 video-electroencephalography–monitored animals

Group	Age at implantation, days	Time recorded, days	Spontaneous seizures, n	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[a]
Cnr1$^{-/-}$/*Cnr2*$^{-/-}$	125	52	5	Yes[a]
Cnr1$^{-/-}$/*Cnr2*$^{-/-}$	128	49	2	Yes
Cnr1$^{-/-}$/*Cnr2*$^{-/-}$	101	15	11	Yes[b]
Cnr1$^{-/-}$/*Cnr2*$^{-/-}$	137	20	0	No

WT, wild-type.
[a]Died prematurely.
[b]Handling-induced seizure during cage change.

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 s, and died immediately thereafter. The second animal had a milder, class III 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, and 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 thresholds,[4] 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 excitability.[3] Alternatively, functions unique to the CB2 receptor might account for the more severe phenotype observed when both receptors are eliminated. Finally, although *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, because 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 proliferation,[12] and thus the receptor's 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 activation.[13] *Cnr2*$^{-/-}$ mice have increased inflammation in response to stress, whereas transgenic overexpression of *Cnr2* was neuroprotective.[14] Inflammatory changes are hypothesized to contribute to epileptogenesis.[15] 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 anticonvulsant potential.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) National Institute on Drug Abuse (5RO1DA006668-21, S.K.D.), NIH National Institute of Neurological Disorders and Stroke (2R01NS062806, 2R01NS065020, S.D.), and NIH National Institute of Environmental Health Sciences (T32ES007051, S.R.). The authors would like to thank Lili Ding (Cincinnati Children's Hospital Medical Center) for help with statistical analyses and Keri Kaeding for editorial advice on the manuscript.

Disclosure

The authors declare no conflicts of interest. We confirm that we have read the Journal's position on issues involved in ethical

publication and affirm that this report is consistent with those guidelines.

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