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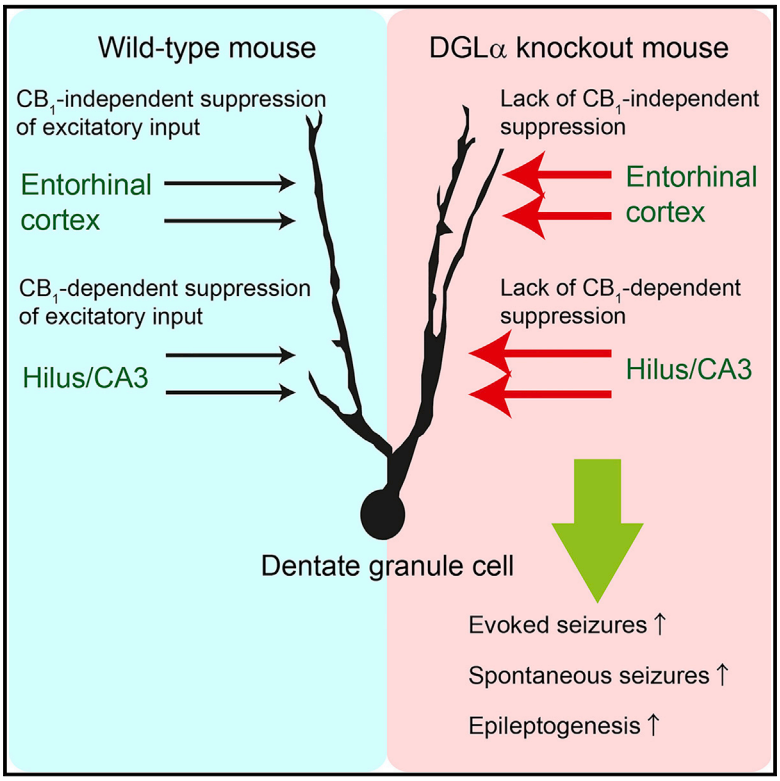
Crucial Roles of the Endocannabinoid 2-Arachidonoylglycerol in the Suppression of Epileptic Seizures

Graphical Abstract



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In Brief

Endocannabinoid signaling suppresses epileptic seizures, but the precise mechanism of this action is undetermined. Sugaya et al. demonstrate that the endocannabinoid 2-arachidonoylglycerol can suppress seizures and epileptogenesis by reducing excitatory synaptic inputs in the dentate gyrus through CB_1 and presumably CB_2 cannabinoid receptors.

Highlights

- 2-AG antagonizes seizure generation through CB_1 and presumably CB_2 receptors
- 2-AG suppresses excitatory synaptic inputs in the dentate gyrus during seizures
- Kindling epileptogenesis is markedly enhanced in mice lacking 2-AG signaling
- Augmentation of 2-AG signaling suppresses the occurrence of spontaneous seizures

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Crucial Roles of the Endocannabinoid 2-Arachidonoylglycerol in the Suppression of Epileptic Seizures

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SUMMARY

Endocannabinoid signaling is considered to suppress excessive excitability of neural circuits and to protect the brain from seizures. However, the precise mechanisms of this effect are poorly understood. Here, we report that 2-arachidonoylglycerol (2-AG), one of the two major endocannabinoids, is crucial for suppressing seizures. We found that kainate-induced seizures in mice lacking the 2-AG synthesizing enzyme, diacylglycerol lipase α, were much more severe compared with those in cannabinoid CB_1 receptor knockout mice and were comparable to those in mice lacking both CB_1- and CB_2-receptor-mediated signaling. In the dentate gyrus, 2-AG suppressed excitatory input around the inner and middle molecular layers through CB_1 and presumably CB_2 receptors, respectively. This 2-AG-mediated suppression contributed to decreased granule cell excitability and the dampening of seizures. Furthermore, lack of 2-AG signaling enhanced kindling epileptogenesis and spontaneous seizures after kainate-induced status epilepticus. These results highlight critical roles of 2-AG signaling in the suppression of epileptic seizures.

INTRODUCTION

Epilepsy is a common and disabling neurological disorder with multiple etiologies that affects about 0.8% of the world's population (Moshé et al., 2015). Some forms of epilepsy, such as mesial temporal lobe epilepsy, are highly resistant to medication (Stephen et al., 2001); therefore, there is a continuous need for the development of new anti-epileptic drugs. Enhancement of GABAergic inhibition and suppression of glutamatergic excitation, voltage-dependent Na^+ channels, or voltage-gated Ca^{2+} channels are the major modes of action of currently used anti-epileptic drugs (Rogawski and Löscher, 2004). However, the endocannabinoid system has emerged as a novel target for anti-epileptic drugs (Porter and Jacobson, 2013).

Endocannabinoids are produced and released from postsynaptic neurons in activity-dependent manners, act retrogradely on cannabinoid CB_1 receptors in presynaptic terminals, and suppress neurotransmitter release (Kano et al., 2009; Ohno-Shosaku and Kano, 2014). CB_1 receptors and the molecules for endocannabinoid synthesis are present throughout the nervous system; therefore, endocannabinoid-mediated retrograde suppression is considered to be a ubiquitous and important form of activity-dependent synaptic modulation. In line with this notion, endocannabinoid signaling has been shown to play crucial roles in a variety of brain functions including learning and memory (Kano et al., 2009), anxiety (Lafenêtre et al., 2007), addiction (Sidhpura and Parsons, 2011), pain modulation (Nadal et al., 2013), feeding behavior (Silvestri and Di Marzo, 2013), and neuroprotection (Sánchez and García-Merino, 2012). Above all, control of excessive excitability and prevention of epileptic seizures appear to be important functions of the endocannabinoid system (Katona and Freund, 2008; Soltesz et al., 2015). Indeed, many previous studies demonstrate that CB_1-receptor-mediated endocannabinoid signaling has suppressive effects on seizures (Marsicano et al., 2003; Monory et al., 2006). However, it remains to be determined which of the two major endocannabinoids, 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995) or anandamide (Devane et al., 1992), mediates the suppression of seizures. It is also not known how endocannabinoid signaling suppresses seizures. Inhibition of glutamate release is a likely mechanism, but there are no in vivo physiological studies that directly support this notion. Another important but unsolved issue is whether endocannabinoids also affect the development of epileptic focus, also termed epileptogenesis. Suppression, but not enhancement, of CB_1-receptor-mediated endocannabinoid signaling was shown to significantly increase the seizure threshold in a febrile seizure model (Chen et al., 2007). However, a recent study reported that blockade of 2-AG hydrolysis by JZL184 retarded the development of amygdala kindling epileptogenesis (von Rüden et al., 2015a).

In the present study, we tackled the issues whether and how 2-AG signaling prevents seizures and epileptogenesis. Our

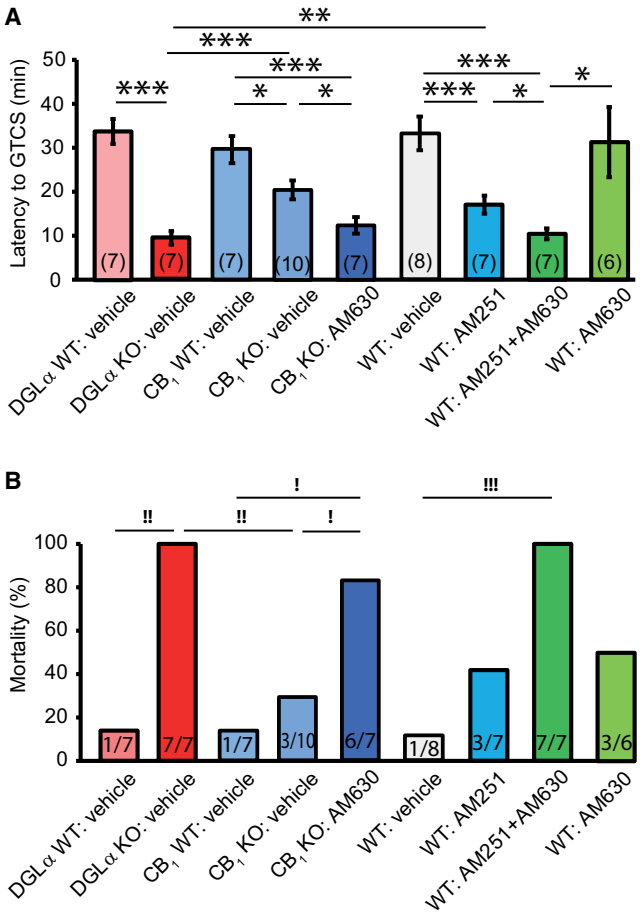

Figure 1. Kainate-Induced Acute Seizures in Several Mouse Models with Deleted/Blocked 2-Arachidonoylglycerol Signaling

(A) Latency to generalized tonic-clonic seizure (GTCS) after intraperitoneal injection of kainate (KA, 30 mg/kg, i.p.). Sixty minutes before the KA injection, wild-type (WT) mice, diacylglycerol lipase α knockout (DGLα KO) mice, CB_1 knockout (CB_1 KO) mice, and their WT littermates had undergone one of the following drug treatments: the CB_1 antagonist AM251 (20 mg/kg, i.p.) alone, the CB_2 antagonist AM630 (2 mg/kg, i.p) alone, combined AM251 and AM630, or vehicle. The number inside each column represents the number of mice that developed seizures. Error bars attached to individual columns represent ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by Welch's t test.

(B) Mortality during 20 min after KA injection. The number inside each column shows ([the number of mice that died] / [the number of mice that developed seizures]). !p < 0.05, !!p < 0.01, !!!p < 0.001, by Fisher's exact test.

results highlight the importance of 2-AG signaling in the dentate gyrus for the suppression of seizures and epileptogenesis and suggest that enhancement of 2-AG signaling may be a promising strategy for the development of new anti-epileptic drugs.

RESULTS

2-AG Signaling Is Crucial for the Suppression of Seizure Generation

We first asked whether 2-AG has suppressive effects on seizure generation in the non-epileptic mouse brain. We used mice lacking diacylglycerol lipase (DGL) α (Tanimura et al., 2010), one of the two 2-AG synthesizing enzymes, and CB_1 knockout (KO) mice (Figure S1). We injected DGLα KO mice and wild-type (WT) littermates with kainate (KA) (30 mg/kg, i.p.) and estimated the severity of seizures. Latency from the KA injection to the onset of generalized tonic-clonic seizure (GTCS) was significantly shorter in DGLα KO mice than in WT littermates (t test, t = 8.26, p < 0.0001; Figure 1A). All DGLα KO mice died within 20 min after the initiation of seizures, whereas only 14.3% of WT littermates died (Fisher's exact test, p = 0.00466; Figure 1B). CB_1 KO mice also showed significantly shorter latency to seizure onset after KA injection compared with their WT littermates (t test, t = 2.54, p = 0.0225; Figure 1A), but both genotypes exhibited similar mortality rates (30% for CB_1 KO mice versus 14.3% for WT littermates, Fisher's exact test, p = 0.603; Figure 1B). Notably, the latency was shorter (t test, t = 3.78, p = 0.00182; Figure 1A) and the mortality was higher (100% versus 30%, Fisher's exact test, p = 0.00982; Figure 1B) in DGLα KO than in CB_1 KO mice. WT mice treated with a CB_1 antagonist, AM251 (20 mg/kg, i.p.), also showed significantly shorter latency compared with vehicle-treated WT mice (t test, t = 3.51, p = 0.00383; Figure 1A). The latency was significantly longer (t test, t = 3.02, p = 0.0108; Figure 1A) and the mortality rate lower (42.9%, Fisher's exact test, p = 0.0699; Figure 1B) in AM251-treated mice than in DGLα KO mice. These results suggest that the suppressive effect of 2-AG on KA-induced seizures involves not only CB_1-mediated processes but also other mechanisms independent of CB_1.

While CB_1 is widely expressed in the CNS, the other cannabinoid receptor, CB_2, is mainly expressed in the immune system (Kano et al., 2009). However, accumulating evidence suggests that CB_2 receptors are functional in the CNS (Li and Kim, 2016; Stempel et al., 2016). A recent study reported the antiepileptic effect of CB_2 antagonist, AM630, when co-administered with cannabinoid receptor agonist, WIN 55,212-2 (Rizzo et al., 2014). However, the antagonist alone did not affect seizures evoked in the non-epileptic brain (Rizzo et al., 2014). Because 2-AG is known to activate CB_2 receptors (Van Sickle et al., 2005), we examined the possibility that CB_2 receptors contribute to the suppression of seizures in our experimental conditions. We treated CB_1 KO mice with a CB_2 antagonist (AM630, 2 mg/kg, i.p.) and injected these mice with KA. The latency to seizure onset was significantly shorter (t test, t = 2.59, p = 0.0206; Figure 1A) and the mortality rate was significantly higher in CB_1 KO mice treated with AM630 than in CB_1 KO mice treated with vehicle (85.7% versus 30%, Fisher's exact test, p = 0.0498; Figure 1B). Similarly, the latency to seizure onset of WT mice treated with AM251+AM630 was significantly shorter than that of WT mice treated with AM251 alone (t test, t = 2.75, p = 0.0177; Figure 1A). All of the AM251+AM630-treated WT mice died within 20 min (mortality: 100%, Fisher's exact test, p = 0.00140 versus WT mice; Figure 1B). Importantly, however, WT mice treated with AM630 alone had a latency and mortality rate that were comparable to those of vehicle-treated WT mice (t test, t = 0.223, p = 0.831 for latency, 50%, Fisher's exact test, p = 0.245 for mortality; Figures 1A and 1B). These results suggest that 2-AG suppresses generation of seizures primarily through CB_1 and secondarily through CB_2 receptors.

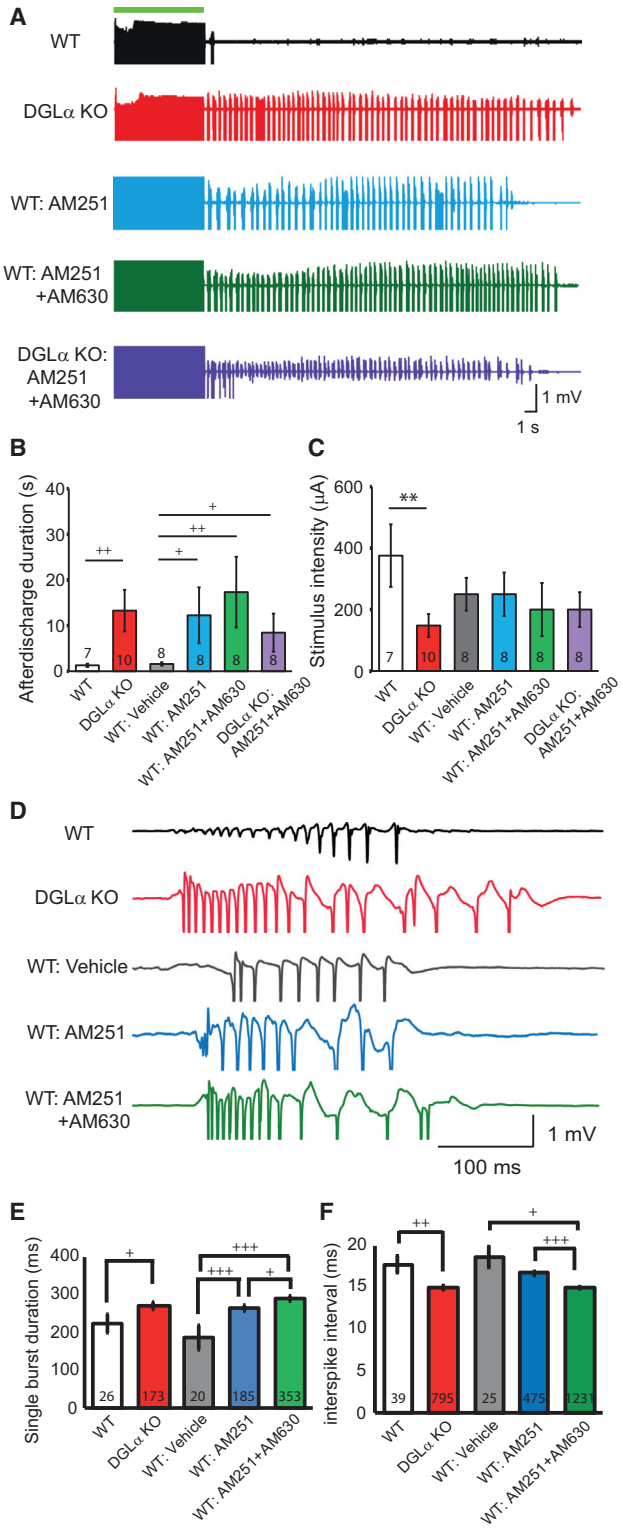

Figure 2. Suppression of Epileptic Afterdischarge in the Dentate Hilus by 2-Arachidonoylglycerol Signaling through CB_1 and Presumably CB_2 Receptors

(A) Example traces of afterdischarges recorded in the dentate hilus of WT mice, DGLα KO mice, WT mice treated with AM251 (WT: AM251), WT mice treated with AM251 and AM630 (WT: AM251+AM630) and DGLα KO mice treated with AM251 and AM630 (DGLα KO: AM251+AM630). The green bar above the recordings indicates the period during which the perforant path was stimulated.

(B and C) Summary data for the duration of afterdischarge (B) and for the minimal stimulus intensity required to induce afterdischarges (C). The number inside each column represents the number of mice examined.

(D) Example traces of single burst discharge observed during afterdischarges in the dentate hilus of WT or DGLα KO mice that underwent one of the indicated treatments.

(E and F) Summary data for the duration of single burst discharge (E) and for the interspike interval (F). The number inside each column represents the number of burst discharges (E) and the number of interspike intervals (F).

Error bars attached to individual columns represent ± SEM +$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$ by Mann-Whitney U test. **$p < 0.01$ by Welch's t test.

In line with these results, recent reports show the localization of CB_2 receptors in the hippocampal CA1, CA3 and the dentate gyrus (Li and Kim, 2015; Stempel et al., 2016). Using real-time PCR, we quantified the level of mRNA for CB_2 receptors in the hippocampus to be 0.00109% ± 0.000487% of that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, n = 10), which is in the same order of magnitude as that described in the report of Li and Kim (2015). Because the severity of KA-induced seizures was very similar between genetic deletion and pharmacological blockade of CB_1 receptors, we hereafter used AM251 treatment to investigate the role of CB_1-receptor-mediated processes in seizure suppression.

2-AG Suppresses Dentate Afterdischarges through CB_1 and Presumably CB_2 Receptors

CB_1-mediated protection against KA-induced seizures depends on CB_1 receptors around the dentate gyrus (Monory et al., 2006). This suggests that CB_1-receptor-mediated signaling may antagonize epileptic discharge in the dentate gyrus. To examine this possibility, we recorded local field potentials (LFPs) in the dentate gyrus of anesthetized mice and induced afterdischarges by perforant path stimulation. Burst stimuli induced significantly longer afterdischarges in DGLα KO mice compared with WT mice (Mann-Whitney U test, U = 1, $p < 0.01$; Figures 2A and 2B). Minimal stimulus intensity required to induce afterdischarges was significantly lower in DGLα KO mice compared with WT littermates (t test, t = 2.39, p = 0.0306; Figure 2C). Prior treatment of WT mice with AM251 or AM251+AM630 significantly increased the duration of afterdischarges (Mann-Whitney U test, U = 10, $p < 0.05$, and U = 6, $p < 0.01$, respectively; Figures 2A and 2B). In contrast to the duration, AM251 or AM251+AM630 treatment did not affect the minimal stimulus intensity (Figure 2C). There was no significant difference in these two parameters between AM251 and AM251+AM630 treatment (Figures 2A–2C). Importantly, treatment with AM251+AM630 had no additive effect in DGLα KO mice (Figures 2A–2C; DGLα KO versus DGLα KO: AM251+AM630). Furthermore, the duration of afterdischarges and the minimal stimulus intensity were comparable among DGLα KO mice, WT mice treated with AM251 alone, and WT mice treated with AM251+AM630 (Figures 2A–2C). These results suggest that the effect of AM251 and/or AM630 is occluded in DGLα KO mice, and that 2-AG decreases the

duration of afterdischarges induced by perforant path stimulation mainly through CB_1 receptors.

In WT mice, a burst of epileptic discharge occurred only once in most cases (Figure 2A). In contrast, bursts of epileptic discharge often repeated in DGLα KO mice (Figure 2A). The duration of single epileptic burst discharge was significantly longer in DGLα KO mice than in WT littermates (Mann-Whitney U test, $Z = 2.13$, $p = 0.0329$; Figures 2D and 2E). The duration in AM251-treated WT mice was also significantly longer than that in vehicle-treated mice (Mann-Whitney U test, $Z = 3.31$, $p = 0.000922$; Figures 2D and 2E). Furthermore, the duration in WT mice treated with AM251+AM630 was significantly longer than that in WT mice treated with AM251 alone (Mann-Whitney U test, $Z = 2.61$, $p = 0.0448$; Figures 2D and 2E). To further assess the extent of hyperexcitability in the dentate gyrus, we measured the interspike interval (ISI) during epileptic burst discharges. The ISI was significantly shorter in DGLα KO mice than in WT littermates (Mann-Whitney U test, $Z = 2.85$, $p = 0.00441$; Figures 2D and 2F). Treatment with AM251+AM630 induced a significantly shorter ISI in WT mice than with AM251 treatment alone (Mann-Whitney U test, $Z = 5.09$, $p < 0.00001$; Figures 2D and 2F). However, treatment with AM251 alone caused no significant effect on the ISI when compared with vehicle treatment (Mann-Whitney U test, $Z = 1.41$, $p = 0.159$; Figures 2D and 2F). Taken together, these results suggest that 2-AG suppresses perforant path stimulation-induced epileptic burst discharges in the dentate gyrus through both CB_1 and CB_2 receptors.

Optogenetic Inhibition of Dentate Output Suppresses Afterdischarges

We next checked whether neural activity in the dentate gyrus was necessary for sustaining seizure activities in vivo. It is possible that afterdischarges recorded in the dentate gyrus are driven by other brain regions such as the CA1 (Bragin et al., 1997). To suppress dentate neural activity optogenetically, we injected adenoassociated viral (AAV) vectors for the expression of Archaerhodopsin T (ArchT) under control of the CaMKII promoter (AAV1-CaMKII-ArchT/GFP) bilaterally into the dentate gyrus of DGLα KO mice (Figure S2A) or into the right dentate gyrus of WT mice (Figure S2E). Four weeks after the injection, we successfully suppressed the population spike in the dorsal dentate gyrus by optogenetics (Figures S2B and S2F). The optogenetic suppression of the bilateral dorsal dentate gyrus during afterdischarges significantly decreased the durations of afterdischarges in DGLα KO mice (Mann-Whitney U test, $Z = 2.87$, $p = 0.00413$; Figures S2C and S2D). Similarly, optogenetic suppression in AM251-treated WT mice significantly decreased the number of burst discharges (Mann-Whitney U test, $Z = 2.54$, $p = 0.0110$; Figures S2G and S2H). These results suggest that excessive neural activity in the dentate gyrus is necessary for long-lasting afterdischarges evoked by perforant path stimulation.

Increased Synaptic Input during Afterdischarges in the Dentate Gyrus of DGLα KO Mice

Long-lasting afterdischarges in mice lacking 2-AG signaling could be ascribed to strong depolarization and heightened activity of granule cells by burst stimulation to the perforant path. To test this possibility, we made in vivo whole-cell recordings from granule cells and measured their membrane potentials in AM251+AM630-treated WT mice (Figure S3A). During burst stimulation of the perforant path, granule cells became gradually depolarized and their membrane potentials reached plateau levels (Figure S3B). After burst stimulation, membrane potentials gradually returned to the pre-stimulation level (Figure S3C). There was no significant correlation between the minimal stimulus intensity required to induce afterdischarges and the membrane potential at the end of burst stimulation (Pearson correlation coefficient, $r = 0.357$, $p = 0.175$) nor between the membrane potential at the end of burst stimulation and the number of burst synaptic input (Pearson correlation coefficient, $r = 0.257$, $p = 0.336$; Figure S3D). However, we found that the amplitude of the first excitatory postsynaptic potential (termed "epileptic synaptic input"; Figure S3C) positively correlated with the number of burst synaptic input (Pearson correlation coefficient, $r = 0.717$, $p = 0.00179$; Figure S3E). These results suggest that the strong excitatory synaptic input to granule cells during burst discharges is a cause of long-lasting afterdischarges in DGLα KO mice.

To examine whether synaptic inputs to dentate granule cells are increased in DGLα KO mice, we recorded LFPs of the dentate gyrus using a 16 channel silicon probe and calculated current source density (CSD). The CSD analysis enables us to separately evaluate the synaptic input in each layer of the dentate gyrus. We found that sink currents in the inner molecular layer (IML) of DGLα KO mice were significantly larger than those of WT littermates during burst discharges (Mann-Whitney U test, $Z = 3.88$, $p = 0.000104$; Figures 3A and 3B). WT mice treated with AM251 alone or with AM251+AM630 also had significantly larger sink currents in the IML than vehicle-treated mice (Mann-Whitney U test, $Z = 3.18$, $p = 0.00146$ for AM251-treated mice, $Z = 2.76$, $p = 0.00576$ for AM251+AM630-treated mice; Figure 3B). The strength of excitatory synaptic input in the IML was comparable among the three groups (Figure 3B), suggesting that CB_1-mediated mechanisms are essential for the suppression of the IML input during afterdischarges. Because CB_1 receptors are strongly expressed at mossy cell terminals in the IML (Uchigashima et al., 2011), changes in excitatory synaptic inputs from mossy cells to granule cells are presumably the major cause of increased excitatory synaptic input in the IML.

In the MML, sink currents of DGLα KO mice were significantly larger than those of WT littermates (Mann-Whitney U test, $Z = 2.22$, $p = 0.0263$; Figures 3A and 3C). Likewise, sink currents in the MML of AM251+AM630-treated WT mice were significantly larger than those of vehicle-treated control mice (Mann-Whitney U test, $Z = 1.96$, $p = 0.0308$; Figure 3C). Unexpectedly, MML sink currents of AM251-treated mice were comparable to those of vehicle-treated control mice and were significantly smaller than those of AM251+AM630-treated WT mice (Mann-Whitney U test, $Z = 2.25$, $p = 0.0242$; Figure 3C). These results suggest that 2-AG suppresses excitatory synaptic currents in the MML presumably through CB_2 receptors. Since excitatory synapses in the MML originate from the perforant path, these results suggest that excitatory synaptic inputs from the entorhinal cortex are regulated by 2-AG through CB_2-dependent mechanisms during seizures.

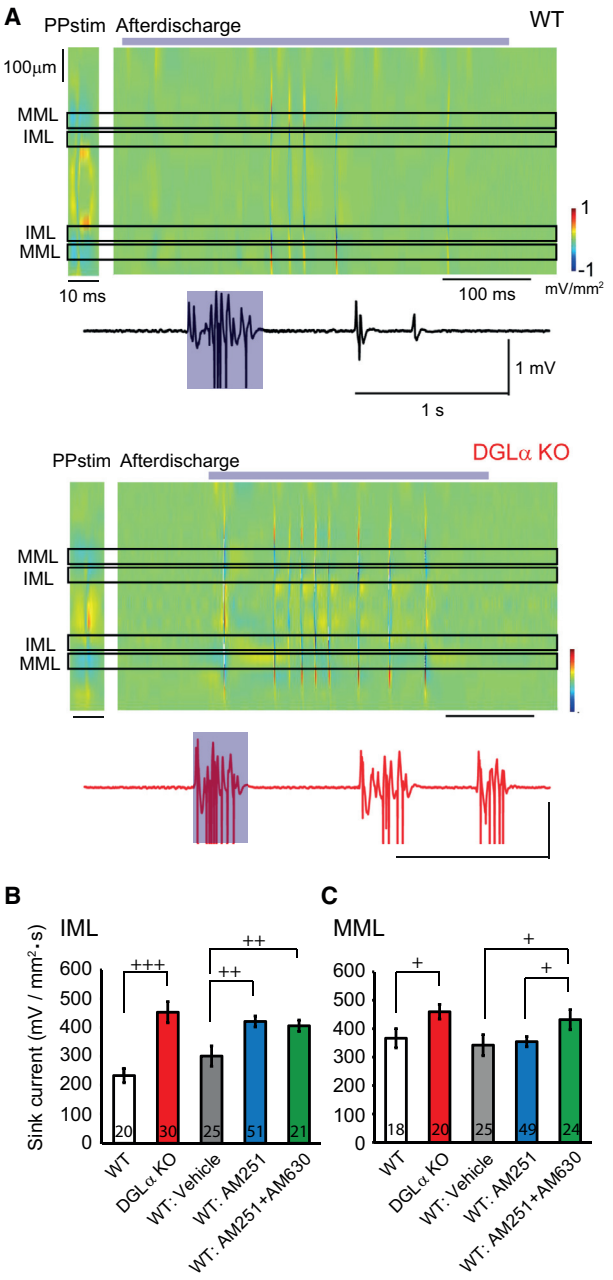

Figure 3. Current Source Density Analysis of Local Field Potentials in the Dentate Gyrus Induced by Perforant Path Stimulation

(A) Color maps showing current source density (CSD) in the dentate gyrus after single perforant path stimulation (PP stim) and during afterdischarges in WT (upper panel) and DGLα KO mice (lower panel). The areas of the CSD map that correspond to the middle molecular layer (MML) and the inner molecular layer (IML) are surrounded with black lines. Current sink (blue) after perforant path stimulation indicates inward current at the MML. Sections of the local field potential (LFP) traces shaded by gray rectangles correspond to the period indicated with gray bars over the CSD maps during afterdischarges.

(B and C) Magnitudes of sink currents during single burst discharge in the IML (B) and in the MML (C). The number inside each column represents the number of burst discharges.

Error bars represent means ± SEM +p < 0.05, ++p < 0.01, +++p < 0.001 by Mann-Whitney U test.

Optogenetic Activation of Mossy Cell Terminals Promotes Afterdischarges

To confirm the promoting effect of mossy cells on dentate afterdischarges, we selectively stimulated mossy cell axons during afterdischarges by optogenetics. Mossy cells are known to project to the contralateral IML. Therefore, injection of AAV containing channelrhodopsin/wide receiver (Wang et al., 2009) (AAV2-CAGGS-ChRWR/Venus) into the right dentate gyrus resulted in the expression of ChRWR in nerve terminals in the IML of the left dentate gyrus (Figure 4A). We confirmed that optogenetic stimulation of ChRWR-expressing cells on the right side induced current sink around the IML of the left dentate gyrus (Figure 4B). Then, in WT mice, we optogenetically stimulated mossy cell axons in the left dentate gyrus for 10 s after electrical stimulation of the perforant path. We found a significant increase in the number of burst discharges by optogenetic stimulation (one-way repeated-measures ANOVA, $p = 0.0362$; Figures 4C and 4D), suggesting that the activation of mossy cells can promote seizures. Taken together, these results suggest that dentate granule cells in DGLα KO mice are hyperexcited by the increased excitatory synaptic input from mossy cells and the entorhinal cortex, leading to long-lasting seizures by excess activation of mossy cells through recurrent collaterals.

2-AG Suppresses Epileptogenesis in the Perforant Path Kindling Model

Next, we examined whether 2-AG signaling influences epileptogenesis in mice using the perforant path kindling model (Maru and Goddard, 1987). Stimulation and recording electrodes were chronically implanted, and afterdischarges were induced in the dentate gyrus. We found that DGLα KO mice exhibited a significantly longer first afterdischarge in the dentate gyrus than WT littermates (Mann-Whitney U test, $U = 27$, $p < 0.05$; Figures 5A and 5B). The stimulus intensity required to induce the first afterdischarge was significantly lower in DGLα KO mice than in WT littermates (t test, $t = 2.20$, $p = 0.0435$; Figure 5C). As kindling proceeded, the evoked seizures became more severe (Figure 5D). The numbers of kindling stimuli required to develop the first class V seizures (Racine, 1972) (t test, $t = 2.66$, $p = 0.0206$; Figure 5E), and three consecutive class V seizures (t test, $t = 2.61$, $p = 0.0230$; Figure 5E) were both significantly smaller in DGLα KO mice than in WT littermates. Twenty-five percent (two of eight) of DGLα KO mice developed spontaneous seizures, whereas no WT littermates did (Figure 5F). Waveforms of burst discharge during spontaneous seizures in awake DGLα KO mice were similar to those observed during evoked afterdischarges under anesthesia (Figure 5G), suggesting that identical neural circuits were involved.

We also investigated the effects of CB_1 and CB_2 receptor antagonists on dentate afterdischarges and kindling epileptogenesis. Administration of either AM251 or AM630 alone did not affect the duration of the first afterdischarges or the minimal stimulus intensity (Figures 6A and 6C). However, co-administration of AM251 and AM630 to WT mice significantly increased the duration of the first afterdischarge compared with that after vehicle treatment (Mann-Whitney U test, $Z = 2.49$, $p = 0.0129$; Figure 6A). Because the duration of afterdischarges varied greatly at the initial stage of kindling, we measured the mean duration

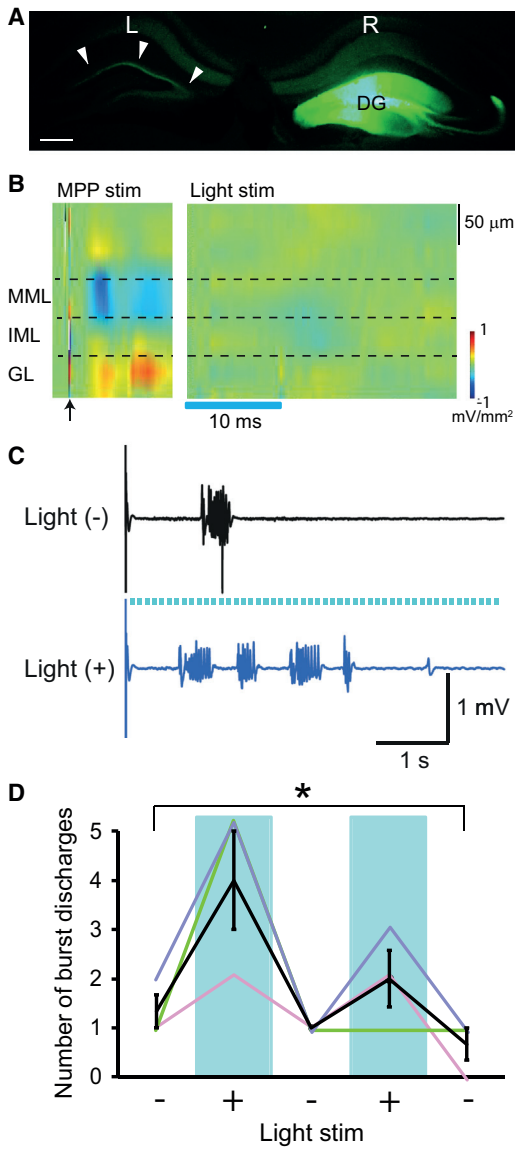

Figure 4. Optogenetic Activation of Mossy Cell Axons Enhances Epileptic Discharges in the Dentate Gyrus In Vivo

(A) Expression of channelrhodopsin/wide receiver (ChRWR)-Venus in the right (R) dentate gyrus (DG) and the left (L) dentate IML (arrow heads) 4 weeks after injection of the AAV carrying cDNAs for ChRWR and Venus. Scale bar, 500 μm.

(B) CSD map of the left dentate gyrus after stimulation of the left medial perforant path (MPP stim, left panel) and after optogenetic stimulation of the right dentate gyrus (right panel). The upward arrow and the blue bar, respectively, indicate the timing of left perforant path stimulation and the period of blue light stimulation to the right dentate gyrus. MML, IML, and granule cell layer (GL) are separated by dotted lines.

(C) Example traces of afterdischarges induced by perforant path stimulation recorded in the left dentate hilus with (blue) or without (black) blue light stimulation. Burst stimulation was applied to the left perforant path and 50 ms light stimulation at 10 Hz (blue dotted line) was applied to the left IML.

(D) The average number of burst discharges in five successive afterdischarges with (second and fourth trials) or without (first, third, and fifth trials) light stimulation. Colored lines represent data from individual mice, and the black line shows the mean with error bars representing ± SEM. *$p < 0.05$ by one-way analysis of variance.

of the five initial afterdischarges. Daily administration of AM251+AM630 to WT mice significantly increased the mean duration of the initial five afterdischarges compared with that produced by vehicle treatment (Mann-Whitney U test, $Z = 3.70$, $p = 0.000219$; Figure 6B). Treatment with AM630 but not with AM251 significantly increased the mean duration of the initial five afterdischarges (Mann-Whitney U test, $Z = 2.20$, $p = 0.0276$; Figure 6B). The number of kindling stimuli required to induce three consecutive class V seizures was significantly smaller in mice treated with AM251+AM630 compared with vehicle-treated mice (Mann-Whitney U test, $Z = 1.96$, $p = 0.0497$; Figure 6D). However, treatment with either AM251 or AM630 alone had no effect on the number of kindling stimuli (Figure 6D). Taken together, these results suggest that 2-AG suppresses epileptogenesis in the hippocampus through CB_1 and CB_2-mediated mechanisms.

Augmentation of 2-AG Levels Suppresses Seizure Generation, Epileptogenesis, and Spontaneous Seizures

Finally, we investigated whether enhancement of 2-AG signaling can ameliorate seizure generation, epileptogenesis, or the occurrence of spontaneous seizures. To elevate 2-AG levels in brain tissues, we used JZL184, a specific inhibitor of the major 2-AG hydrolyzing enzyme, monoacylglycerol lipase. We found that administration of JZL184 (40 mg/kg, i.p.) to WT mice significantly decreased the occurrence of GTCS during 60 min after KA injection (46.7% for vehicle versus 0% for JZL184, Fisher's exact test, $p = 0.0225$), clearly indicating that augmentation of 2-AG levels suppresses seizure generation.

We then examined the effect of JZL184 treatment on epileptogenesis in the perforant path kindling model. We found that JZL184 treatment in WT mice significantly increased the stimulus intensity required to induce the first afterdischarges and decreased the first afterdischarge duration compared with vehicle treatment (stimulus intensity: Mann-Whitney U test, $Z = 2.58$, $p = 0.00992$; afterdischarge duration: Mann-Whitney U test, $Z = 2.31$, $p = 0.0210$; Figures 6A and 6C). The treatment also decreased the mean duration of the initial five afterdischarges (Mann-Whitney U test, $Z = 2.57$, $p = 0.0101$; Figure 6B). The number of kindling stimuli required to achieve three consecutive class V seizures was significantly larger in JZL184-treated WT mice than in vehicle-treated WT mice (Mann-Whitney U test, $Z = 2.44$, $p = 0.0146$; Figure 6D). These results indicate that augmentation of 2-AG levels suppresses epileptogenesis.

We then asked whether JZL184 treatment can suppress spontaneous seizures by using a KA-induced chronic seizure model (Bouilleret et al., 1999). KA was injected into the right dentate gyrus of 8-week-old mice. Two weeks later, daily injection of JZL184 (4 mg/kg, i.p.), AM251 (20 mg/kg, i.p.), or vehicle was started and continued for 10 days. From the second day of drug treatment, the number of spontaneous seizures that occurred during 6 hr per day were counted for 9 consecutive days (Figure 7A). We found that JZL184 treatment significantly decreased the number of spontaneous seizures compared with vehicle treatment (t test, $t = 2.11$, $p = 0.0491$; Figures 7B and 7C). In contrast to JZL184, AM251 treatment significantly increased the number of spontaneous seizures (t test, $t = 2.91$,

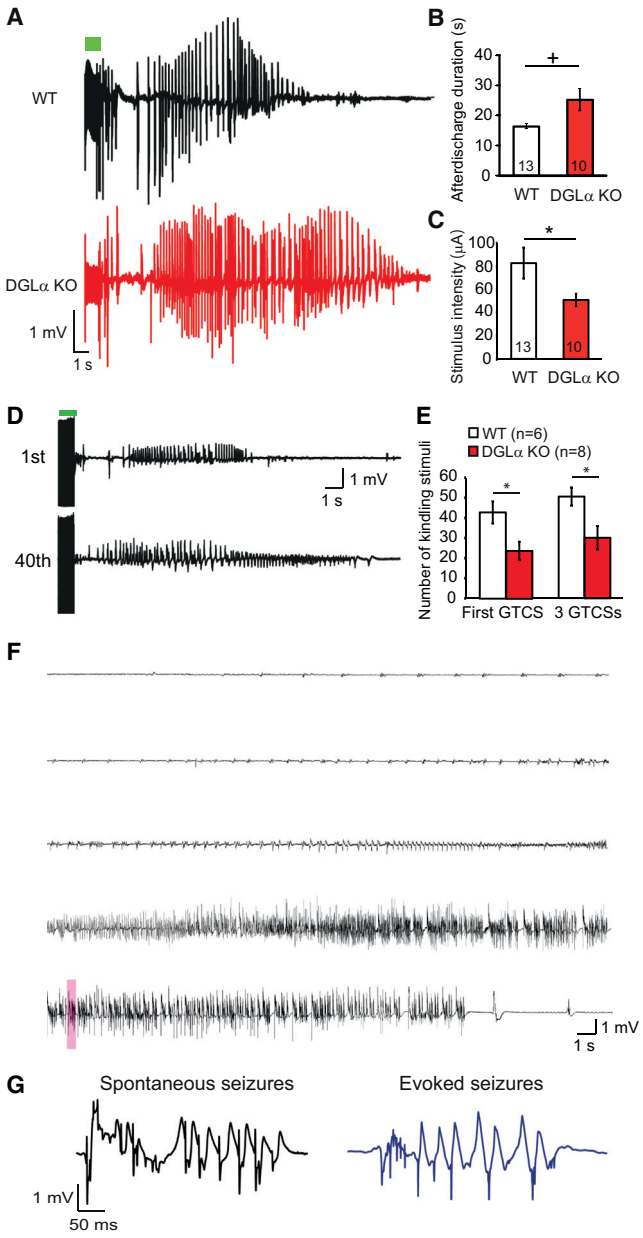

Figure 5. Enhanced Epileptogenesis in DGLα KO Mice in the Perforant Path Kindling Model

(A) Example traces of the first afterdischarges recorded in the dentate hilus of freely moving DGLα KO mice (red) and WT littermates (black). The green bar indicates the period of perforant path stimulation.

(B and C) Summary for the duration of the first afterdischarge (B) and the stimulus intensity required to induce the first afterdischarges (C) in WT and DGLα KO mice. The number inside each column represents the number of mice examined.

(D) Example traces of afterdischarges in the dentate gyrus of a WT mouse in the first and the 40th kindling trials by perforant path stimulation indicated with the green bar.

(E) Summary for the number of kindling stimuli required to induce the first GTCS and three consecutive GTCSs (three GTCSs) in WT and DGLα KO mice.

Error bars attached to individual columns represent ± SEM (B, C, and E). +$p < 0.05$ by Mann-Whitney U test (B). *$p < 0.05$ by Welch's t test (C and E).

p = 0.0179; Figures 7B and 7C) and the mortality rate (3/8 for AM251-treated mice versus 0/13 for vehicle-treated mice, Fisher's exact test, p = 0.0117). Our immunohistochemical analysis showed that in the dentate gyrus, the expression of CB_1 receptors was significantly decreased (Mann-Whitney U test, Z = 3.87, p = 0.000109; Figures S4A and S4B) and that of DGLα tended to become weaker (Mann-Whitney U test, Z = 1.85, p = 0.0647; Figures S4A and S4C) 4 weeks after the KA injection. We also found a significant decrease in the expression of CB_1 receptors (Mann-Whitney U test, Z = 2.42, p = 0.0153; Figures S4A and S4B) and of DGLα (Mann-Whitney U test, Z = 2.60, p = 0.00937; Figures S4A and S4C) in the hippocampal CA1 area. Thus, attenuation of 2-AG signaling appears to underlie KA-induced chronic seizure. Taken together, our results suggest that augmentation of 2-AG signaling could be an effective therapy for certain forms of epilepsy.

DISCUSSION

2-AG mediates retrograde signals for endocannabinoid-dependent short-term synaptic suppression and long-term depression (Tanimura et al., 2010). Of two enzymes that can synthesize 2-AG, DGLα and DGLβ, DGLα but not DGLβ is responsible for the production of 2-AG in the brain. 2-AG functions as a synaptic retrograde messenger (Gao et al., 2010; Tanimura et al., 2010) and is therefore considered to prevent seizure in physiological conditions by regulating neural circuit activity through retrograde synaptic suppression. Indeed, genetic deletion of phospholipase Cβ1, the enzyme upstream of DGLα and crucial for 2-AG synthesis (Hashimotodani et al., 2005), resulted in increased seizure susceptibility in mice (Kim et al., 1997). Furthermore, in patients with severe mesial temporal lobe epilepsy, decreased 2-AG signaling in the hippocampus was suggested (Ludányi et al., 2008). Although the level of 2-AG in the brain was reported not to increase during KA-induced seizure (Marsicano et al., 2003), in our study, all of the DGLα KO mice died soon after KA injection, suggesting that 2-AG signaling is crucial for suppressing KA-induced seizure. We also demonstrated that DGLα KO mice exhibited enhanced kindling epileptogenesis in the dentate gyrus. Thus, these lines of evidence strongly support 2-AG signaling to be essential for the suppression of hyperexcitability of dentate gyrus neural circuits and the prevention of epileptogenesis.

A recent study showed that inhibition of 2-AG catabolism and elevation of 2-AG levels in postsynaptic neurons caused an enhancement of $GABA_A$ receptor signaling in a cannabinoid-receptor-independent manner, and that this $GABA_A$ receptor enhancement had a suppressive effect on pentylenetetrazol (PTZ)-induced seizures (Naydenov et al., 2014). In contrast, we demonstrated in the present study that there was no additive effect of the blockade of CB_1 and CB_2 receptors in DGLα-deleted mice in perforant path stimulation-induced seizures, suggesting

(F) Example traces showing spontaneous seizures recorded in the dentate gyrus of freely moving DGLα KO mice. A burst discharge indicated by the pink rectangle is enlarged in (G).

(G) Representative waveform of a single burst discharge observed during spontaneous seizures in a freely moving DGLα KO mouse (left) and that of evoked seizures in an anesthetized DGLα KO mouse (right).

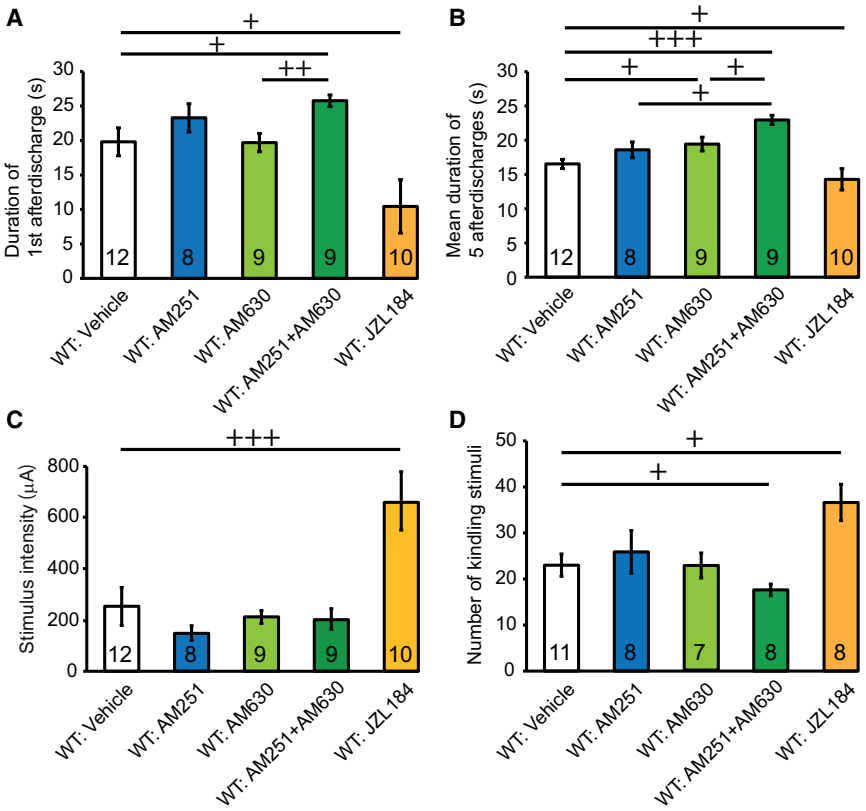

Figure 6. Seizure Generation and Kindling Epileptogenesis in the Dentate Gyrus of Freely Moving WT Mice Treated with AM251, AM630, AM251+AM630, or JZL184

(A–C) Summary bar graphs for the duration of the first afterdischarge (A), the mean duration of the initial five afterdischarges (B), and the stimulus intensity required to induce the first afterdischarge (C) in WT mice treated with vehicle, AM251, AM630, AM251+AM630, or JZL184.

(D) Summary bar graph for the number of kindling stimuli required to induce three consecutive GTCS in WT mice treated with vehicle, AM251, AM630, AM251+AM630, or JZL184. The number inside each column represents the number of mice examined.

Error bars attached to individual columns represent ± SEM +p < 0.05, ++p < 0.01, +++p < 0.001, by Mann-Whitney U test.

that 2-AG acted exclusively on CB_1 and CB_2 receptors. Previous studies also suggest that seizures induced by electrical stimulation or by KA injection are regulated strongly by CB_1-dependent mechanisms (Marsicano et al., 2003; Monory et al., 2006; von Rüden et al., 2015b). One possible explanation for this apparent discrepancy may be the different stimuli used to induce seizures. PTZ is thought to suppress $GABA_A$ receptor function (Squires et al., 1984) and therefore 2-AG-induced $GABA_A$ receptor augmentation may potently antagonize the effect of PTZ. In contrast, KA injection and perforant path stimulation are thought to enhance excitatory neural circuit activity, and therefore 2-AG-mediated suppression of excitatory transmission through CB_1 receptors seems to be crucial.

In the present study, WT mice with acute blockade of CB_1 and CB_2 receptors showed a similar epileptic phenotype to that of DGLα KO mice. In addition, acute treatment with JZL184 had a strong suppressive effect on seizure generation and epileptogenesis in WT mice, suggesting the importance of acute effects of 2-AG on cannabinoid receptors. However, deletion of CB_1 receptors decreases adult neurogenesis in the hippocampus (Jin et al., 2004). Furthermore, lack of CB_1 receptor signaling in GABAergic neurons results in increased numbers of inhibitory synapses in pyramidal neurons (Berghuis et al., 2007), and the dendritic spines are decreased in the hippocampus of CB_2 KO mice (Li and Kim, 2016). These changes could potentially affect the excitability of neural circuits in the hippocampus. Therefore, we cannot exclude the possibility that compensatory mechanisms may affect the epileptic phenotypes of DGLα KO mice.

In addition, previous reports suggest a decrease of neuronal excitability by acute activation of CB_2 receptors located on postsynaptic neurons (den Boon et al., 2012; Stempel et al., 2016). Thus, mechanisms other than retrograde suppression of synaptic transmission might contribute to the roles of 2-AG in the suppression of epileptic seizures.

In the dentate gyrus, mossy cells form both mono-synaptic excitatory and di-synaptic inhibitory feedback connections onto granule cells. Specific ablation of mossy cells by diphtheria toxin increases the excitability of granule cells (Jinde et al., 2012), suggesting that mossy cells exert net inhibition on dentate granule cells. In contrast, selective aspiration of mossy cells in acute slices decreased field excitatory postsynaptic potential amplitude in the dentate gyrus (Ratzliff et al., 2004), suggesting net excitation exerted by mossy cells on granule cells. Our present results with CSD analysis in the dentate gyrus and optogenetic excitation of mossy cell terminals in vivo support the latter notion. The apparent controversy about the net effect of mossy cells on the excitability of granule cells might be partly attributable to high levels of DGLα expression in granule cells and mossy cells with a virtual absence of DGLα in inhibitory interneurons in the dentate gyrus (Uchigashima et al., 2011). Mossy cell terminals on granule cells but not those on inhibitory interneurons are considered to be suppressed by 2-AG released from granule cells. Therefore, it may depend on the overall neural circuit activity and the extent of 2-AG signaling whether mossy cells exert net excitation or inhibition on granule cells.

In the dentate gyrus, inhibitory terminals projecting onto granule cells also express CB_1 receptors (Uchigashima et al., 2011). Because activation of CB_1 receptors decreases the GABAergic current in dentate granule cells (Isokawa and Alger, 2005), CB_1 receptor signaling at inhibitory synapses is potentially pro-epileptic. In accordance with this notion, conditional deletion of CB_1 receptors from inhibitory neurons increased the

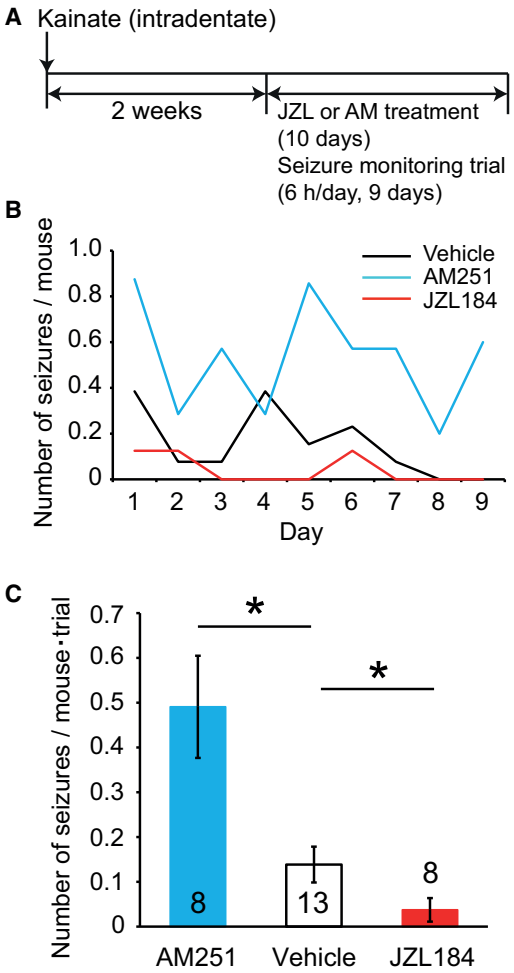

Figure 7. 2-Arachidonoylglycerol Signaling Suppresses Spontaneous Epileptic Seizures in the KA-Induced Chronic Seizure Model

(A) Basic experimental scheme. KA was injected to the right dentate gyrus of 8-week-old WT mice. Two weeks after KA injection, mice were treated with JZL184 (4 mg/kg, i.p.) or AM251 (20 mg/kg, i.p.) once a day for 10 days. Monitoring of seizures (6 hr/day) was started on day 2 of drug treatment and continued for 9 days.

(B and C) Mean number of spontaneous seizures per mouse during the daily monitoring period plotted for 9 consecutive days (B) and ensemble average of the daily scores (C). The number inside each column in (C) represents the number of mice examined. Error bars attached to individual columns represent ± SEM. *$p < 0.05$ by Welch's t test.

afterdischarge duration by amygdala stimulation (von Rüden et al., 2015b) or did not affect KA-induced seizures (Monory et al., 2006). Therefore, the suppressive effect of endocannabinoid signaling on epileptic activity is indicated to be largely mediated by CB_1 receptor signaling at excitatory synapses.

As for the effect of cannabinoid signaling on epileptogenesis, conflicting results have been reported (Chen et al., 2007; Ma et al., 2014). Blockade of 2-AG synthesis or CB_1 receptors immediately after the initial insult prevents epileptogenesis in status epilepticus (Ma et al., 2014) or the hyperthermia-induced seizure model (Chen et al., 2007). In these models, initial spontaneous seizures are so strong that animals often die. Such severe insults are thought to accompany intense activation of endocannabinoid signaling and may result in the downregulation of CB_1 receptors in the excitatory terminals (Falenski et al., 2009). The blockade of CB_1 receptors during the initial insult may prevent their downregulation and preserve their function, which may eventually prevent epileptogenesis. In the present study, DGLα KO mice exhibited significantly faster kindling development compared with WT littermates. Furthermore, pharmacological blockade of CB_1 and CB_2 receptors also promoted kindling epileptogenesis. These results clearly show that the lack of 2-AG signaling throughout the process of kindling promotes epileptogenesis. We also found that after epileptogenesis, inhibition of 2-AG hydrolysis effectively suppressed, whereas blockade of the CB_1 receptor increased, the occurrence of spontaneous seizures. These results strongly suggest that 2-AG signaling is essential for the suppression of seizures in epileptic brains and that enhancement of 2-AG signaling may be a promising way to treat epilepsy.

EXPERIMENTAL PROCEDURES

Full details of experimental procedures are described in the Supplemental Information.

Animals

Two- to 4-month-old male and female C57BL/6N mice, DGLα KO mice (Tanimura et al., 2010), CB_1 KO mice (Figure S1), and their WT littermates were used. Animals were housed in a plastic cage in a room illuminated with a 12-hr-light/12-hr-dark cycle and fed with standard mouse chow and water ad libitum. All procedures involving animal care were approved by the University of Tokyo Animal Care and Use Committee.

KA-Induced Seizures

Mice were treated with drugs (AM251, AM630, and JZL184) or vehicle 60 min before the injection of KA (30 mg/kg dissolved in saline, i.p., Tocris). After the injection of KA, latency to GTCS was determined in each animal by an experimenter blind to the drug treatment and genotype.

Induction of Afterdischarges in the Dentate Gyrus of Anesthetized Mice

In WT mice, drugs (AM251 or AM251+AM630) or vehicle was administered after setting the electrodes. Afterdischarges were induced by a 10-s stimulus train composed of 500-μs square pulses at 20 Hz. The stimulus intensity was initially set at 50 μA. The stimulus train was applied at 1-min intervals with stimulus strength increasing by steps of 50 μA until afterdischarges were detected in the LFPs.

Whole-Cell Recordings from Dentate Granule Cells In Vivo

The method for whole-cell recording from dentate granule cells in vivo was the same as that described previously (Sugaya et al., 2013). After recording resting membrane potential, burst stimuli were applied to the perforant path and evoked dentate afterdischarges as described above.

Optogenetic Inhibition of Granule Cells

AAV1-CaMKII-ArchT-GFP (Vectorcore) was injected into the bilateral dentate gyrus of 2- to 4-month-old DGLα KO mice or into the right dentate gyrus of 8-week-old C57BL/6N mice. Four weeks after the injection, afterdischarges were induced more than five times, alternately without and with optogenetic inhibition.

Optogenetic Activation of Mossy Cell Axon Terminals

AAV2-CAGGS-ChRWR/Venus was injected into the right dentate gyrus of 8-week-old C57BL/6N mice. Four weeks after the injection, dentate afterdischarges were induced five times by perforant path stimulation as described

above. On the second and fourth trials of afterdischarge induction, optogenetic stimulation (50-ms pulse stimuli at 10 Hz) of mossy cell axons was performed 50 ms after the end of burst stimulation of the perforant path.

Perforant Path Kindling Protocol

One week after the implantation of electrodes, afterdischarges were induced twice daily with an interval of more than 6 hr while the mice were exploring freely in their cages. Drugs (JZL184, AM251, AM630, and AM251+AM630) or vehicle were injected 3 hr before the first kindling stimulus of the day. Behavioral seizures were rated according to Racine's seizure rating scale (Racine, 1972). Animals were judged to be fully kindled when three consecutive seizures of class V or greater were observed.

Monitoring Spontaneous Seizures after KA-Induced Status Epilepticus

KA (0.2 μg in 100 nl saline, 50 nl/min) was injected into the right dentate gyrus of 8-week-old C57BL/6N mice under isoflurane anesthesia (1.5%). Fourteen days after the KA injection, JZL184, AM251, or vehicle was injected once a day for 10 consecutive days. From day 2, the occurrence of spontaneous seizures was monitored for 6 hr per day for 9 consecutive days. For the immunohistochemical analysis of DGLα and CB_1 receptors, a different set of mice injected with KA was used.

Statistics

All data were expressed as mean ± SEM, and statistical information of each test is indicated. Datasets with normal distributions were analyzed with unpaired two-tailed Welch's t test or one-way ANOVA. Datasets with non-Gaussian distributions were analyzed with the Mann-Whitney U test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.083.

AUTHOR CONTRIBUTIONS

Y.S. and M.K. conceived and designed the experiments; Y.S. performed the experiments; Y.S. analyzed data; M.Y., M.U., M.W., and K.S. contributed characterizing animals. K.K. made AAV. Y.S., M.Y., and M.K. wrote the paper.

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