MicroRNA-Mediated Downregulation of the Potassium Channel Kv4.2 Contributes to Seizure Onset

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Highlights
- Kv4.2 mRNA is recruited to the miRNA-induced silencing complex after seizure
- Kv4.2 mRNA is a specific target of the microRNA miR-324-5p
- Antagonizing miR-324-5p counteracts seizure-induced reduction of Kv4.2 protein
- Antagonizing miR-324-5p delays seizure onset in wild-type but not in Kcnd2 KO mice

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In Brief
Gross et al. show that the voltage-gated potassium channel Kv4.2 is regulated by microRNA-induced silencing during seizures. Inhibition of the Kv4.2-targeting microRNA miR-324-5p increases Kv4.2 protein levels, counteracts seizure-induced Kv4.2 downregulation, suppresses kainic-acid-evoked seizures and cell death, and delays seizure onset in wild-type, but not in Kcnd2 knockout mice.

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MicroRNA-Mediated Downregulation of the Potassium Channel Kv4.2 Contributes to Seizure Onset

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SUMMARY

Seizures are bursts of excessive synchronized neuronal activity, suggesting that mechanisms controlling brain excitability are compromised. The voltage-gated potassium channel Kv4.2, a major mediator of hyperpolarizing A-type currents in the brain, is a crucial regulator of neuronal excitability. Kv4.2 expression levels are reduced following seizures and in epilepsy, but the underlying mechanisms remain unclear. Here, we report that Kv4.2 mRNA is recruited to the RNA-induced silencing complex shortly after status epilepticus in mice and after kainic acid treatment of hippocampal neurons, coincident with reduction of Kv4.2 protein. We show that the microRNA miR-324-5p inhibits Kv4.2 protein expression and that antagonizing miR-324-5p is neuroprotective and seizure suppressive. MiR-324-5p inhibition also blocks kainic-acid-induced reduction of Kv4.2 protein in vitro and in vivo and delays kainic-acid-induced seizure onset in wild-type but not in Kcnd2 knockout mice. These results reveal an important role for miR-324-5p-mediated silencing of Kv4.2 in seizure onset.

INTRODUCTION

Seizures are episodes of synchronized brain activity, but the underlying molecular mechanisms are not fully understood. The voltage-gated potassium channel Kv4.2 (KCND2; potassium channel, voltage-gated Shal-related subfamily D, member 2) is a major mediator of transient A-type currents in the brain and is important for controlling neuronal excitability (Jerng et al., 2004). Knockout of Kv4.2 or its auxiliary subunits DPP6 (Dipeptidyl-peptidase-like protein 6) and KChIP2 (K channel-interacting protein 2) in mice reduces A-type currents, increases dendritic excitability and enhances susceptibility to provoked seizures (Chen et al., 2006; Barnwell et al., 2009; Sun et al., 2011; Wang et al., 2013). Mutations in KCND2 have been found in humans with epilepsy (Singh et al., 2006; Lee et al., 2014) further suggesting that compromised Kv4.2 function increases the brain’s vulnerability to develop seizures.

Reduced expression and impaired function of Kv4.2 were observed in at least three different rodent models of acquired epilepsy (pilocarpine-induced temporal lobe epilepsy, traumatic brain injury and ischemic insult) (Monaghan et al., 2008; Lei et al., 2012, 2014; Bernard et al., 2004) and in rats acutely following evoked seizure (Francis et al., 1997; Tsaur et al., 1992). Reduction of Kv4.2 expression may thus be a pathological mechanism contributing to seizures and epilepsy; however, the molecular mechanisms regulating Kv4.2 expression during neuronal hyperactivity are unknown, and it is not clear whether downregulation of Kv4.2 expression contributes to seizure onset.

MicroRNAs recognize and bind specific sequences on their target mRNAs via the RNA-induced silencing complex (RISC) followed by RNA degradation or translational suppression (Pasquinelli, 2012). Several recent studies have demonstrated that seizures in rodents cause differential expression of microRNAs, and select microRNAs were shown to be involved in modulating seizure susceptibility and epilepsy-induced neuroinflammation (e.g., Brennan et al., 2016; reviewed in Reschke and Henshall, 2015). The underlying molecular mechanisms and the targeted mRNAs that are regulated by microRNAs during seizures are not well established. In particular, it is not known whether microRNA-mediated silencing of the potassium channel Kv4.2, a crucial regulator of neuronal excitability, plays a role in the reduction of Kv4.2 expression in epileptic mice or after status epilepticus.

This study reveals microRNA-mediated silencing as a mechanism promoting downregulation of Kv4.2 protein expression following status epilepticus. We demonstrate that the microRNA...
miR-324-5p reduces Kv4.2 protein expression and further show that antagonizing miR-324-5p is seizure suppressive, which is mediated partially through Kv4.2. Our work provides insight into how Kv4.2 is downregulated after seizures and highlights the potency of a microRNA targeting an ion channel to regulate the onset of provoked seizures.

RESULTS

Kv4.2 mRNA Is Recruited to the RISC during Neuronal Hyperactivity

To investigate whether microRNA-induced silencing regulates Kv4.2 expression in the brain, we quantified Kv4.2 mRNA in Ago2-specific immunoprecipitates (IPs) from brain tissue and neurons. Ago2 is an essential component of the RNA-induced silencing complex (RISC) that associates with mRNAs prone to microRNA-mediated silencing (Bartel, 2009). Using quantitative real-time PCR, we detected enrichment of Kv4.2 mRNA in Ago2-IPs from mouse hippocampal lysates (Figure 1A) and cultured mouse hippocampal neurons (Figure 1B) compared to IPs with normal mouse immunoglobulin G (IgG) (western blots confirming Ago2 IP shown in Figure S1A). To analyze whether the RISC is involved in the regulation of Kv4.2 expression during seizures and excitotoxicity-inducing neuronal hyperactivity, we quantified the association of Kv4.2 mRNA with Ago2 30 min following onset of kainic-acid-induced status epilepticus in mice as well as 4 hr following neurotoxicity-inducing kainic acid treatment in cultured hippocampal neurons (KAI) (D, 10 μM kainic acid, paired t test, *p = 0.034). (E–H) Seizures or excitotoxicity do not affect total Kv4.2 mRNA levels (E and F) but lead to significantly reduced Kv4.2 protein levels (G and H) (conditions as above, paired t tests, E, p = 0.545; F, p = 0.375; G, *p = 0.006; H, *p = 0.021). Kv4.2 protein was still reduced in the hippocampus 3 hr after onset of status epilepticus (Figure S1B).

MiR-324-5p Is a Specific Suppressor of Kv4.2 Protein Expression

Using online software tools (microRNA.org, TargetScan), we identified miR-324-5p as a putative candidate microRNA targeting Kv4.2 mRNA with a highly complementary seed region found in mouse, rat, and human (Figure 2A). To assess whether miR-324-5p regulates Kv4.2 expression, we generated a luciferase reporter construct containing the rat Kv4.2 3'UTR. Luciferase assays in Neuro2A (N2A) cells showed that miR-324-5p negatively regulates Kv4.2 reporter expression (Figures 2B–2E). Co-expression of the reporter construct with pre-miR-324-5p reduced luciferase activity (Figure 2B), and treatment of the cells with a locked-nucleic acid (LNA)-modified “antagomir” containing the miR-324-5p antisense sequence increased luciferase activity of the reporter (Figure 2C). A reporter construct bearing a double point mutation in the seed region-targeted sequence of the Kv4.2 3'UTR was not affected by overexpression of pre-miR-324-5p (Figure 2D) and had increased basal luciferase activity compared to the wild-type sequence, presumably due to a lack of inhibition by endogenous miR-324-5p in N2A cells (Figure 2E).

MiR-324-5p Regulates Endogenous Kv4.2 Protein Levels

Next, we examined whether miR-324-5p controls endogenous Kv4.2 protein levels in neurons. Previous reports have shown...
that miR-324-5p is expressed in the rodent brain (Kim et al., 2004; Capitano et al., 2016). Using fluorescence in situ hybridization analyses, we confirmed expression of miR-324-5p in the mouse cortex (data not shown) and showed strong expression in both somatic and dendritic areas of the hippocampus, whereas the signal for miR-330, a previously identified somatic microRNA (Kye et al., 2007), was confined to the cell bodies (Figures S2A and S2B). Inhibition of miR-324-5p in cultured cortical and hippocampal neurons using antagonirs significantly increased endogenous Kv4.2 protein levels as quantified by western blotting (Figures 2F and 2G). mRNA levels were not affected (Figure S2C). In vivo, intracerebroventricular (i.c.v.) injection of a miR-324-5p-specific antagonist in mice resulted in significant upregulation of Kv4.2 protein in the cortex and hippocampus after 24 hr (Figures 2H and 2I), but mRNA levels were unchanged (Figures S2D and S2E). Reduction of miR-324-5p by the antagonist was confirmed by qRT-PCR (Figure S2F). Virus-mediated overexpression of pre-miR-324-5p significantly reduced Kv4.2 protein in cortical neurons (Figure 2J) but did not change Kv4.2 mRNA levels (Figure S2G).

Kv4.2 is a transmembrane protein that mediates potassium currents across the plasma membrane. Using surface biotinylation and western blot analyses in cultured cortical neurons (Figure 2K), as well as non-permeabilizing fluorescence immunocytochemistry in hippocampal neurons (Figure 2L), we detected significantly reduced Kv4.2 cell-surface expression following miR-324-5p overexpression. By contrast, GluN1 cell-surface expression was not affected (western blot in Figure 2K, quantification in Figure S2H). Non-permeabilizing fluorescence immunocytochemistry was validated by analyzing Kv4.2 cell-surface levels after kainic acid treatment, which showed a significant reduction (Figure S2I). Previous studies reported that Kv4.2 mRNA translation is regulated by the Fragile X Mental Retardation Protein (FMRP) (Gross et al., 2011; Lee et al., 2011). Using hippocampal Fmr1 knockout (KO) neurons, we showed that FMRP is not necessary for miR-324-5p-mediated regulation of Kv4.2 (Figure S2J).

Antagonizing miR-324-5p Delays Seizure Onset, Reduces EEG Total Power, and Prevents Cell Death after Intra-amygdala Kainic Acid Injection

We next tested whether miR-324-5p is affected by seizures. Total hippocampal miR-324-5p levels were unchanged 30 min after onset of status epilepticus induced by intraperitoneal (i.p.) injection of kainic acid (Figure 3A), but association of miR-324-5p with the RISC component Ago2 was increased (Figure 3B). To test whether inhibition of miR-324-5p affects seizures, we induced status epilepticus by intra-amygdala injection of kainic acid (Jimenez-Mateos et al., 2012) in mice that had received scrambled or miR-324-5p-specific antagonirs by i.c.v. injection 24 hr prior to seizure induction (timeline shown in Figure S3A). Antagonizing miR-324-5p significantly delayed seizure onset (Figure 3C) and also reduced the EEG total power compared to scrambled control (Figures 3D–3F and S3B–S3F). Fluoro-Jade B (FJB) staining 72 hr after status epilepticus showed a neuroprotective effect of anti-miR-324-5p in the hippocampus, resulting in less neuronal death (Figures 3G and S3G–S3I). The neuroprotective effect of miR-324-5p inhibition was confirmed in vitro in cultured hippocampal neurons: kainic acid treatment (10 μM, 4 hr) induced cell death in neurons transfected with control antagonist but not in neurons transfected with miR-324-5p-specific antagonirs (Figures 3H and 3I).

Antagonizing miR-324-5p Prevents Kainic-Acid-Induced Downregulation of Kv4.2 and Delays Seizure Onset in Wild-Type but Not in Kcnd2 KO Mice

We next analyzed whether miR-324-5p-specific antagonirs blocked Kv4.2 reduction following kainic-acid-induced excito-toxicity in vitro or status epilepticus in vivo. Kainic acid treatment significantly reduced Kv4.2 protein expression in cultured hippocampal neurons transfected with control antagonirs (Figure 4A) but not in neurons that were transfected with miR-324-5p-specific antagonirs (Figure 4B). Similarly, in vivo, seizures evoked by i.p. injection of kainic acid reduced hippocampal Kv4.2 expression in mice that had received i.c.v. injections of scrambled antagonirs but not in mice that had received miR-324-5p-specific antagonirs 24 hr prior seizure induction (Figure 4C).

miR-324-5p was experimentally confirmed to silence a few genes and is predicted to target many others (HumanTargetScan 7.1, Agarwal et al., 2015; Table S1). To assess the specific impact of Kv4.2 on the miR-324-5p-mediated seizure-suppressing effects, we tested whether blocking miR-324-5p affects seizure onset in the absence of Kv4.2. We induced status epilepticus by i.p. injection of kainic acid in wild-type and Kcnd2 KO mice (Guo et al., 2005) and assessed seizure onset using cortical EEG recordings. As with the amygdala model of status epilepticus (Figure 3C), antagonizing miR-324-5p prolonged the latency to seizure onset in wild-type mice (Figure 4D). This effect was abolished in Kcnd2 KO mice (Figure 4E). In contrast to a previous study (Barnwell et al., 2009), we did not detect differences in the latency to seizure onset between wild-type and Kcnd2 KO mice (Figures 4D and 4E). This may be caused by different doses of kainic acid and genetic backgrounds used, which were shown to affect the phenotype (Barnwell et al., 2009). However, like Barnwell and colleagues, we observed an increased seizure-induced mortality rate (50% Kcnd2 KO mice versus 22% wild-type mice, scrambled antagonist-injected, Figure S4A). Moreover, EEG total power was higher in Kcnd2 KO mice (Figure S4B). Notably, the miR-324-5p-specific antagonist reduced EEG total power regardless of genotype, although statistical significance was not reached (Figure S4B). This suggests that, in contrast to regulation of latency to seizure onset, other miR-324-5p targets, apart from Kv4.2, might contribute to the effect of miR-324-5p inhibition on EEG total power.

DISCUSSION

Reduced expression and function of the voltage-gated potassium channel Kv4.2 have been associated with epilepsy and seizures, but the molecular mechanisms leading to downregulation of Kv4.2 following seizures are unknown. Here, we present data supporting a model in which microRNA-induced silencing decreases Kv4.2 protein expression during excitotoxic events and seizures. We identify miR-324-5p as a specific regulator of Kv4.2 protein expression and show that miR-324-5p plays a role in regulating seizures and neuronal excitotoxicity: blocking
Figure 2. MiR-324-5p Represses Recombinant and Endogenous Kv4.2 Protein Expression

(A) MiR-324-5p target region on mouse and rat Kv4.2 3'UTRs, seed region is shown in green. Two nucleotides differ in the mouse and rat 3'UTRs (highlighted in yellow) but do not affect miR-324-5p targeting. Luciferase constructs were based on the rat sequence.

(B and C) Neuro2a cells were transfected with Kv4.2 firefly luciferase 3'UTR reporters and pre-microRNA expressing plasmids (B) or antagomirs (C) (paired t tests, B, *p = 0.0015; C, *p = 0.0012).

(D and E) Mutating two nucleotides in the seed region of the Kv4.2 3'UTR (shown on top, mutated nucleotides in red) abolishes the inhibitory effect of miR-324-5p overexpression (D), paired t test, p = 0.005), and increases luciferase activity compared to the wild-type reporter (E), paired t test, *p < 0.0001). Luciferase activity was normalized to co-transfected Renilla firefly; pre-microRNA or antagomir sequences with no known homology served as control (NEG).

(F–L) MiR-324-5p inhibition with a sequence-specific antagomir increases endogenous Kv4.2 protein expression as analyzed by western blots in cultured cortical (F) and hippocampal (G) neurons, and in cortical (H) and hippocampal (I) tissue of mice i.c.v. injected with anti-miR-324-5p or scrambled (SCR) antagomirs 24 hr prior tissue harvest (paired t tests, F, *p = 0.048; G, *p = 0.025; unpaired t tests, H, *p = 0.05; I, *p = 0.025). MiR-324-5p overexpression using pre-microRNA-expressing lentiviral particles reduces Kv4.2 total (J, paired t test, *p = 0.019), and cell-surface expression in cultured cortical neurons (K, paired t test, *p = 0.0007, n indicates separate IPs from six independent experiments from three cultures), as well as cell-surface expression in cultured hippocampal neurons (L, Mann-Whitney U test, *p = 0.0027, n represents individual neurons from three cultures). Cell-surface expression was measured using surface biotinylation and western blot analysis in (K), and using quantitative immunofluorescence under non-permeabilizing conditions in transfected cultured hippocampal neurons in (L). Neurons in (L) were identified by GFP co-expressed by the pre-miRNA plasmid and by morphology. Primary and secondary dendritic segments (15–50 μm, 60–100 μm apart from the cell body, were analyzed. Examples of analyzed dendritic segments are shown in the magnifications on the bottom (white box). Scale bar, 25 μm in the overview images and 10 μm in the inset. Images were deconvolved and maximum intensity projections of ten stacks (15 μm total) are shown. Contrast (legend continued on next page)
miR-324-5p prevents kainic-acid-mediated reduction of Kv4.2 protein, delays kainic-acid-induced seizure onset, decreases EEG total power after kainic acid treatment, and is neuroprotective. MiR-324-5p inhibition does not delay seizure onset in mice that lack Kv4.2, supporting an important role of Kv4.2 in mediating the seizure-delaying effect of miR-324-5p.

Previous studies have detected reduced expression of Kv4.2 mRNA and protein in the hippocampus several hours following seizures (Francis et al., 1997; Tsaur et al., 1992). In this study, we analyzed early events regulating Kv4.2 expression shortly after seizure and demonstrate that Kv4.2 mRNA is recruited to the RISC 30 min after kainic-acid-induced status epilepticus was adjusted equally for both images. Quantification of GluN1 surface expression in biotinylation experiments shown in (K) revealed no significant differences (Figure S2H). MiR-324-5p expression patterns in the mouse brain are shown in Figures S2A and S2B. Kv4.2 mRNA levels were not affected by the miR-324-5p antagomir or by overexpressing pre-miR-324-5p (Figures S2C–S2E and S2G). Cultured neurons were between 11 and 15 days in vitro. N indicated in the figure; error bars represent SEM.

Figure 3. Inhibition of miR-324-5p Reduces Kainic-Acid-Induced Seizure Severity and Excitotoxicity
(A and B) Total miR-324-5p levels are unchanged 30 min following seizure onset induced by i.p. injection of kainic acid (A, paired t test, p = 0.908), but association of miR-324-5p with Ago2 is increased (B, paired t test, *p = 0.030).
(C–F) i.c.v. injections of miR-324-5p antagonirs delayed seizure onset following intra-amygdala kainic acid injection (C, independent t test, *p = 0.048) and reduced total EEG power 40 min post kainic acid (D, independent t test, *p = 0.010) and 60 min post lorazepam (E, independent t test, *p = 0.032). Lorazepam was used to end the seizures. Example EEGs are shown in (F); additional analyses and timeline of the experiment are shown in Figures S3A–S3F.
(G) Antagonizing miR-324-5p reduces seizure-induced cell death in the dorsal hippocampus as shown by Fluoro-Jade B staining (independent t test, *p = 0.025).
(H and I) Propidium iodide staining in living hippocampal neurons (14 days in vitro) shows reduced kainic-acid-induced cell death in neurons transfected with miR-324-5p antagonirs compared to control antagonirs. Neurons were analyzed 4 hr following treatment with 10 μM kainic acid, and DAPI staining was used as a counterstain. Scale bar, 200 μm. Contrasts were adjusted equally for all example images in (H). Shown in (I) is the relative increase in propidium iodide-positive cells normalized to control (Kruskal-Wallis test p = 0.036; Dunn’s post hoc tests *p = 0.036; n.s. p > 0.9999). N indicated in the figure; error bars represent SEM.
Increased microRNA-induced silencing of Kv4.2 contributes to Kv4.2 protein downregulation in the hippocampus (Figure 1G). In line with this hypothesis, inhibition of miR-324-5p, a microRNA that we show suppresses Kv4.2 protein expression in the brain (Figures 2F–2L), counteracts kainic-acid-induced downregulation of Kv4.2 protein (Figures 4A–4C). Two other microRNAs, miR-223-3p and miR-301a-3p, were shown to regulate Kv4.2 in the heart (Panguluri et al., 2013; Liu et al., 2016), but their function in neurons and during seizures is unknown.

We did not detect changes in total miR-324-5p levels shortly after seizure (Figure 3A). A previous study identified miR-324-5p as one of several microRNAs that were downregulated in the hippocampus 24 hr following pilocarpine-induced status epilepticus (Kretschmann et al., 2015), but this result was not reproduced in a similar study using kainic acid to induce seizures (Jimenez-Mateos et al., 2011). Another study showed hippocampal upregulation of miR-324-5p in a rat epilepsy model 60 days following lithium-pilocarpine-induced status epilepticus (Song et al., 2011). In the future, it will be interesting to analyze whether miR-324-5p and Kv4.2 expression are co-regulated at later time points after status epilepticus. We show that miR-324-5p is recruited to the RISC 30 min after seizure (Figure 3B), suggesting early dynamic changes in the composition of miRISC during neuronal hyperexcitation. These findings add to recent discoveries that microRNA association with the RISC is not static, but dynamic changes remodeling RISC can occur in response to synaptic activity (Muddashetty et al., 2011; Jimenez-Mateos et al., 2012).

A prerequisite for our observation that changes in microRNA-induced translational silencing have an immediate impact on Kv4.2 protein expression (within 30 min to 4 hr, Figures 1G, 1H, and 4A–4C) is that the turnover rate of Kv4.2 protein must be fast. A study in COS-1 cells using recombinant Kv4.2 protein has shown that the half-life of Kv4.2 is indeed relatively short, namely 2 hr (Shibata et al., 2003). In cultured neurons, Kv4.2 protein is rapidly degraded within minutes in response to glutamatergic activation (Lee et al., 2011). Thus, under physiological conditions in the brain the turnover rate of Kv4.2 might be even faster than 2 hr. We speculate that the reduction in Kv4.2 evident 30 min after seizure is a combination of increased Kv4.2 degradation and reduced Kv4.2 synthesis. Nonetheless, blocking miR-324-5p-mediated translational suppression of Kv4.2 abolishes kainic-acid-induced Kv4.2 reduction (Figures 4A–4C), implying that increased Kv4.2 protein synthesis can overcome excitotoxically-induced Kv4.2 degradation.

It was previously shown that Kv4.2 mRNA is translationally regulated by the mRNA-binding protein FMRP (Lee et al., 2011; Gross et al., 2011). The exact mechanisms and consequences of loss of FMRP on Kv4.2 expression and function remain controversial, as both decreased and increased levels and function of Kv4.2 have been shown in Fmr1 KO mice (Kalmbach et al., 2015; Gross et al., 2011; Lee et al., 2011; Routh et al., 2013). FMRP interacts with the microRNA pathway (Jin et al., 2004) and cooperates with microRNAs to regulate translation of certain mRNA targets (Muddashetty et al., 2011; Edbauer et al., 2010). Our results suggest that FMRP is not necessary for the gene silencing effects of miR-324-5p on Kv4.2 (Figure S2J), but future studies are needed to investigate the relationship between FMRP- and microRNA-mediated translational control of Kv4.2 and its significance for seizure-induced downregulation of Kv4.2.

Manipulation of miR-324-5p is expected to affect multiple mRNA targets in addition to Kv4.2, since microRNAs usually
regulate numerous mRNAs. Our results in Kcnd2 KO mice show that a miR-324-5p-specific antagomir does not delay seizure onset in the absence of Kv4.2 and thus support a specific role of Kv4.2 in the delay in seizure onset mediated by miR-324-5p inhibition. However, our analyses also suggest that Kv4.2 is dispensable for the effects of miR-324-5p inhibition on EEG total power (Figure S4B), and it is unclear whether the observed neuroprotective effect (Figures 3G–3I) is mediated through Kv4.2. There are a few experimentally confirmed targets of miR-324-5p that might be involved in neuroprotection after miR-324-5p inhibition, and other potassium channels are predicted to be targets that may contribute to seizure suppression (Table S1). In the future, it will be interesting to assess the contribution of individual targets of miR-324-5p to the neuroprotective and seizure-suppressing effects of miR-324-5p inhibition.

EXPERIMENTAL PROCEDURES

Mice and Materials
For a list of mice, antibodies, drugs, and source and sequence of antagomirs, FISH probes, and qRT-PCR primers, see Supplemental Experimental Procedures.

Ago2 Co-immunoprecipitation
Ago2 co-IPs were done using a mouse monoclonal Ago 2 antibody and Protein-G-coupled agarose. For details, see Supplemental Experimental Procedures.

RNA Isolation and qRT-PCR
RNA was extracted using Trizol (Life Technologies), followed by cDNA preparation and qRT-PCR analysis using SYBR green or TaqMan assays. For details, see Supplemental Experimental Procedures.

 Luciferase Assays
Luciferase assays were carried out using the Dual-Glo Luciferase Assay System (Promega) and a Luminometer (Veritas, Promega). For details, see Supplemental Experimental Procedures.

Fluorescence In Situ Hybridization
Fluorescence in situ hybridization on mouse brain slices was performed as described in Muddashetty et al. (2011). For details, see Supplemental Experimental Procedures.

Intracerebroventricular Injection of MicroRNAs
For i.c.v. injections of miR-324-5p-specific or scrambled antagomirs, mice received a 2-μl infusion of 0.5 nmol of either scrambled or miR-324-5p-specific antagomirs in artificial cerebrospinal fluid (aCSF). For experiments shown in Figures 3C–3G, a cannula was implanted for kainic acid injection at the time of antagonist injection. Mice were euthanized to quantify miR-324-5p or Kv4.2 protein and mRNA after 24 hr. Alternatively, status epilepticus was induced after 24 hr by i.p. or intra-amygdala kainic acid injection.

Seizure Models
For experiments shown in Figures 1C, 1E, 1G, 3A, 3B, 4C–4E, and S4, seizures were induced in male C57BL/6 mice or male Kcnd2 KO mice in C57BL/6 background (Figures 4E and S4) by i.p. injection of kainic acid (15 mg/kg). Control mice received PBS injections. For experiments in Figures 1C, 1E, 1G, 3A, and 3B, mice were between postnatal days 24 and 28 and were euthanized 30 min after behavioral seizure onset. In Figures 4C–4E, mice were between 6 and 9 weeks old (20–23 g). In Figure 4C, mice were euthanized 90 min after injection of kainic acid. Behavioral seizure onset was defined as a full tonic-clonic seizure. In Figures 4D and 4E, seizure onset was identified by cortical EEG recordings using a wireless EEG system (TA11ETAF10) from Data Sciences International. A seizure was defined as sudden onset of high amplitude activity (>2× background) and a duration greater than 10 s. Seizures were terminated by subcutaneous injection of diazepam 90 min after seizure onset (10 mg/kg). Experiments shown in Figures 3C–3F and S3 were done as described (Jimenez-Mateos et al., 2012). Also, see Supplemental Experimental Procedures.

Surface Biotinylation
Cultured Cortical neurons at 12 days in vitro were transcifted with lentiviral particles expressing pre-miR-324-5p or control sequence. After 5–6 days, surface biotinylation was performed as in Gross et al. (2011). For details, see Supplemental Experimental Procedures.

Fluorescence Immunocytochemistry
For fluorescence immunocytochemistry under non-permeabilizing conditions, neurons between days 10 and 12 in vitro were transfected with plasmids expressing GFP and pre-miR-324-5p or a control sequence using Lipofectamine 2000 (Invitrogen). After 24 hr, neurons were stained for Kv4.2 without detergent. Cells were imaged using a Nikon Eclipse Ti inverted microscope. Hippocampal pyramidal neurons were identified according to their morphology. Images were deconvolved using AutoQuant X2 (Media Cybernetics) or NIS Elements (Nikon), and fluorescence intensities in primary and secondary dendritic segments (15–50 μm in length, 15-μm stack), 60–100 μm apart from the cell body, were quantified using ImageJ (NIH). For details (including image processing), see Supplemental Experimental Procedures.

Propidium Iodide Staining
Propidium iodide staining was performed as described in Gauthier et al. (2012). For details, see Supplemental Experimental Procedures.

Fluoro–Jade B Staining
Fluoro–Jade B staining (EMD Millipore) was performed and analyzed as described previously (Moran et al., 2013). Mice were sacrificed 72 hr after seizure induction. Postfixed sections were incubated in 0.006% potassium permanganate solution, followed by 0.001% FJB solution and imaged using a Nikon 2000s epifluorescence microscope and a Hamamatsu Orca 285 camera.

Statistical Analyses
Data were analyzed using GraphPad Prism6. Data were tested for normality using Shapiro-Wilk tests, and appropriate parametric or non-parametric tests were used as indicated in each figure legend. Significance level was set to p < 0.05. For two-group/two-factor analyses, two-way ANOVA with Tukey’s post hoc assays was performed, except for Figure 3I, where a Kruskal-Wallis test followed by Dunn’s post hoc analyses was performed, as the data were not normally distributed. Data are expressed as means with SEM. Repetitions of experiments are indicated in the legends.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.074.

AUTHOR CONTRIBUTIONS

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REFERENCES


