Silencing microRNA-134 produces neuroprotective and prolonged seizure-suppressive effects

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Temporal lobe epilepsy is a common, chronic neurological disorder characterized by recurrent spontaneous seizures. MicroRNAs (miRNAs) are small, non-coding RNAs that regulate post-transcriptional expression of protein-coding mRNAs, which may have key roles in the pathogenesis of neurological disorders. In experimental models of prolonged, injurious seizures (status epilepticus) and in human epilepsy, we found upregulation of miR-134, a brain-specific, activity-regulated miRNA that has been implicated in the control of dendritic spine morphology. Silencing of miR-134 expression in vivo using antagonirs reduced hippocampal CA3 pyramidal neuron dendrite spine density by 21% and rendered mice refractory to seizures and hippocampal injury caused by status epilepticus. Depletion of miR-134 after status epilepticus in mice reduced the later occurrence of spontaneous seizures by over 90% and mitigated the attendant pathological features of temporal lobe epilepsy. Thus, silencing miR-134 exerts prolonged seizure-suppressant and neuroprotective actions; determining whether these are anticonvulsant effects or are truly antiepileptogenic effects requires additional experimentation.

Epilepsy is a serious, chronic neurological disorder characterized by recurrent spontaneous seizures that affects about 50 million people worldwide. Antiepileptic drugs typically control seizures in two-thirds of patients but probably do not alter the underlying pathophysiology1. The development of symptomatic epilepsy is thought to involve altered expression of ion channels, synaptic remodeling, inflammation, gliosis and neuronal death, among other factors2–5. However, few antiepileptogenic interventions targeting these processes have shown sufficient efficacy in vivo1, and our understanding of the cell and molecular mechanisms of epileptogenesis is incomplete.

Evidence is emerging that miRNAs may be crucial to the pathogenesis of several neurological disorders6–7, including epilepsy8–9. MiRNAs are a family of small (~22 nt), endogenously expressed noncoding RNAs that regulate mRNA translation by imperfect base-pairing interactions within the 3′ untranslated region10,11. Depending on the degree of sequence complementarity, miRNA binding, which occurs with the help of Argonaute proteins within the RNA-induced silencing complex (RISC), results in either cleavage of the target mRNA or a reduction in its translational efficiency10,11.

MiR-134 is a brain-specific, activity-regulated miRNA that has been implicated in the control of neuronal microstructure12,13. Pyramidal cells are the most common neurons in the neocortex and hippocampus2. They are the major source of intrinsic excitatory cortical synapses, and their dendritic spines are the main postsynaptic target of excitatory synapses, with their spine size acting as an index of synaptic strength14–16. Spine remodeling occurs during learning and memory formation, as well as in the setting of neuropsychiatric disorders and pathological brain activity17–21. Spine collapse is mediated in part by the N-methyl-D-aspartate (NMDA)-receptor–dependent and calcium-dependent depolymerization of actin by cofilin19,22,23. LIM kinase-1 (Limk1) phosphorylates cofilin and inactivates the ability of cofilin to depolymerize actin, and loss of Limk1 results in abnormal spine morphology24. In hippocampal neurons, miR-134 targets Limk1 mRNA, thereby preventing Limk1 protein translation13. Overexpression of miR-134 in vitro has been reported to reduce spine volume13, whereas overexpression of miR-134 in vivo reduces total dendritic length25 and abrogates long-term potentiation26. Mice haploinsufficient for the miRNA biogenesis component DiGeorge syndrome critical region gene 8 do not produce several mature miRNAs, including miR-134, and have lower hippocampal spine density compared to wild-type mice27. Spine loss may have divergent consequences according to context28, promoting excitability29 or uncoupling NMDA-receptor–driven currents in neurons and preventing excitotoxicity30.

Here we investigated the role of miR-134 in epilepsy and explored the in vivo effect of inhibiting miR-134. We report that miR-134 is upregulated

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in experimental and human epilepsy and show that silencing miR-134 generates a seizure-refractory state and attenuates epileptic seizures and the pathophysiological features of temporal lobe epilepsy (TLE).

RESULTS

MiR-134 is regulated by status epilepticus and in epilepsy

We first investigated whether pathologic brain activity in vivo affects miR-134 levels. We triggered prolonged seizures (status epilepticus) in C57BL/6 mice by intra-amygdala microinjection of the glutamate receptor agonist kainic acid31,32. The resultant seizures caused neuronal damage mainly within the hippocampal CA3 subfield (Fig. 1a).

To identify the cell populations expressing miR-134 in vivo, we performed in situ hybridization on tissue sections from C57BL/6 control mice labeled with a probe specific for mature miR-134. We detected a strong signal in the soma of hippocampal pyramidal neurons and hilar interneurons, as well as in neurons in the neocortex and amygdala (Fig. 1b and Supplementary Fig. 1).

Real-time quantitative PCR (qPCR) analysis showed that status epilepticus resulted in an increase in mature miR-134 levels in the ipsilateral CA3 ($P = 0.016$) and CA1 ($P = 0.035$) subfields (Fig. 1c). The levels of mature miR-134 were not changed in the undamaged contralateral CA3 subfield (Supplementary Fig. 2a). Nonharmful, nonconvulsive seizures induced by a low dose of systemic kainic acid, a model of epileptic preconditioning33 in which status epilepticus does not develop, did not alter miR-134 levels compared to vehicle controls in the CA3 ($P = 0.89$) or CA1 subfields ($P = 0.56$) ($n = 6$ mice per group; data not shown).

To determine whether miR-134 was functional, we measured its levels within the RISC, where targeting of miRNAs to mRNA occurs34, in control mice and in mice after status epilepticus. We eluted Argonaute-2 from the CA3 subfield35,36 and extracted miRNA. We detected a low level of miR-134 in the RISC in the controls, whereas the levels of Argonaute-2–bound miR-134 were higher ($P = 0.035$) in mice in which we induced status epilepticus (Fig. 1d). Protein concentrations of the miR-134 target, Limk1 (ref. 13), were lower ($P = 0.001$) in mice after status epilepticus than in the controls (Fig. 1e).

We next investigated whether miR-134 levels and the expression of Limk1 were altered in experimental epilepsy. Recurrent spontaneous

Figure 1. MiR-134 upregulation after status epilepticus and in epilepsy. (a) Photomicrographs show neuronal death (FJB) in the CA3 stratum pyramidale (s.p., within dashed lines in the top left image) 24 h after status epilepticus induced by intra-amygdala kainic acid compared to control (Cont) mouse. Arrows in the image on the top right point to damaged neurons. Below is a depiction of electrographic seizure frequency and amplitude during status epilepticus. Scale bar, 200 µm (b) In situ hybridization showing miR-134 in the soma of CA3 pyramidal neurons. Scale bar, 200 µm. ‘Sense’ indicates the sense-labeled control section as a control for probe specificity. (c) Real-time qPCR measurement of miR-134 (normalized to RNU19) for the CA3 and CA1 24 h after status epilepticus (SE). $n = 5$ mice per group. $^{*} P < 0.05$ compared to Cont subfield by $t$ test. (d) Argonaute-2 (Ago2)-immunoprecipitated (IP) miR-134 from control mice and mice 24 h after status epilepticus. $n = 3$ mice per group. $^{*} P < 0.05$ compared to Cont by $t$ test. (e) Limk1 western blot and densitometry. Actin was used as the loading control. $n = 5$ mice per group. $^{**} P < 0.05$ compared to Cont by $t$ test. (f) Photomicrographs showing loss of CA3 neurons (NeuN, arrows) and astrogliosis (gli fibrillary acidic protein, GFAP) in epileptic mice 14 d after status epilepticus (top) and a telemetry-recorded spontaneous seizure (bottom; denoted by arrows). Scale bar, 200 µm. (g) MiR-134 levels in the CA3 and CA1 subfields 1 and 3 weeks after status epilepticus. $n = 5$ mice per group. $^{*} P < 0.05$ compared to time-matched Cont by $t$ test. (h) Western blot showing Limk1 concentrations 1 and 3 weeks after status epilepticus and densitometry. $n = 4$ mice per group. $^{*} P < 0.05$ compared to Cont by analysis of variance (ANOVA) followed by Bonferroni’s $post hoc$ test. (i) MiR-134 levels in surgically resected temporal lobe samples from individuals with TLE compared to autopsy controls (Cont). $n = 3$ samples per group. $^{*} P < 0.05$ compared to Cont by $t$ test. Western blot (above) shows LIMK1 concentrations ($n = 1$ sample per lane). All data are mean ± s.e.m.
seizures emerge 3–4 d after status epilepticus in the model we used\(^1,\)\(^3,\)\(^3\), and within 3 weeks, the mice show pathologic hallmarks of TLE, including neuron loss and astrogliosis (Fig. 1f). MiR-134 levels were elevated in the CA3 subfield 3 weeks after status epilepticus (P = 0.049) and 1 (P = 0.003) and 3 (P = 0.008) weeks after status epilepticus in the CA1 subfield compared to controls (Fig. 1g). Limk1 protein concentrations followed an opposite trend and were lower (P = 0.028) in epileptic mice than control mice (Fig. 1h). The concentrations of cAMP responsive element binding protein 1 (Crep1), another validated miR-134 target\(^2,\)\(^6\), were also lower in epileptic mice than control mice (Supplementary Fig. 2b).

We next analyzed surgically obtained temporal lobe material from individuals with pharmacoresistent TLE. We detected higher levels (P = 0.029) of mature miR-134 in the TLE specimens compared to autopsy control samples from people who died of causes unrelated to neurological disease (Fig. 1i). This difference was not an artifact of postmortem delay (Supplementary Fig. 2c–e). Protein concentrations of LIMK1 were lower (P = 0.039, t test) in individuals with TLE compared to the autopsy controls (Fig. 1j).

**In vivo depletion of miR-134 using antagomirs**

To explore the function of miR-134 in vivo, we injected mice with locked nucleic acid (LNA) 3'-cholesterol-conjugated oligonucleotides (‘antagomirs’)\(^3,\)\(^6–\)\(^9\), We injected antagomirs targeting miR-134 (Ant-134) (Supplementary Fig. 3a) or a non-targeting scrambled sequence (Scr) into the mouse ventricle (intracerebroventricularly, i.c.v.) (Supplementary Fig. 3b) and measured the miRNA levels 1, 4, 8 and 12 h later and then after 1, 3, 5 and 7 d and after 1 and 2 months. Knockdown of miR-134 was first evident 12 h after injection of 0.12 nmol Ant-134 (Supplementary Fig. 4a–d), and by 24 h after injection, the levels of miR-134 in the hippocampus of these mice were reduced by over 95% (P = 0.005) (Fig. 2a). This is similar to effects reported for antagomirs in other tissues\(^3,\)\(^7\). Hippocampal levels of an unrelated miRNA, miR-19a, were not changed by injection of Ant-134 (Fig. 2b). Increasing the amount of Ant-134 injected to 1 nmol seemed to produce off-target knockdown of miRNAs (Supplementary Fig. 4e,f). The miR-134 levels began to recover by 7 d after Ant-134 injection, although they remained lower than the miR-134 levels in the mice injected with Scr at 1 month after injection (P = 0.034), which is consistent with other reports\(^7,\)\(^3,\) and were no longer different from the levels in mice injected with Scr (P = 0.469) by 2 months after injection (Fig. 2c).

Brains from mice injected with the antagomirs had grossly normal anatomy (data not shown). We found no evidence of hippocampal neuronal death when we stained sections from the mice injected with antagomirs for Fluoro-Jade B (FJB), DNA fragmentation (terminal deoxynucleotidyl dUTP nick end labeling; TUNEL)\(^43\) and the neuronal marker NeuN (Fig. 2d,e and data not shown).

To determine whether the reduction in the levels of miR-134 had any gross effects on mouse behavior, we performed ethological tests\(^41\). We injected mice with either Scr or Ant-134 and assessed them 24 h later. We found no differences in measures of animal exploratory activity including ambulatory counts, distance traveled or vertical counts between the two groups (Fig. 2f), suggesting that silencing of miR-134 does not alter normal exploratory activities.

**MiR-134 antagomirs reduce pyramidal neuron spine density**

Because in vitro and in vivo evidence supports a role for miR-134 in controlling dendritic spine morphology\(^1,\)\(^3,\)\(^5,\)\(^2,\)\(^7\), we examined whether antagomirs caused changes to dendritic spines in vivo. We micro-injected Lucifer yellow into individual CA3 pyramidal neurons in hippocampal slices from control mice 24 h after injection of 0.12 nmol Ant-134 or Scr and imaged them using confocal microscopy, as previously described\(^42,\)\(^43\) (Fig. 3a,b).

We analyzed 218 neurons in the Scr-injected mice (n = 7) and 181 neurons in the Ant-134–injected mice (n = 7). The structures of the basal dendritic trees were grossly normal in both groups, as were the distributions of spines (Fig. 3c–e). Dendrites from the Scr-injected mice had an average of 68 nodes (ramifications) compared to 72 nodes in the Ant-134–injected mice. The number of ramification points per μm was also similar in the two groups (0.0127 nodes per μm in the Scr-injected mice compared to 0.0131 nodes per μm in the Ant-134–injected mice). We then analyzed spine density, assessing a total length of 5,343.7 μm of dendrites in the Scr-injected mice (7,455 spines) and a similar length (5,477.5 μm) of dendrites in the Ant-134–injected mice (6,196 spines). Spine density in the Scr-injected mice was within the expected range\(^42\) (Fig. 3d–f). Notably, spine density was 21% lower (P = 0.037) in Ant-134–injected mice than in the Scr-injected mice (Fig. 3d–f). Thus, injecting miR-134 antagomirs in vivo results in a reduction in spine density.

![Figure 2](https://example.com/fig2.png)

**Figure 2** Antagomir-mediated silencing of miR-134 in mouse hippocampus. (a,b) Real-time qPCR measurement of miR-134 (a) and miR-19a (b) in mouse hippocampus 24 h after i.c.v. injection of Ant-134 or Scr. n = 3 mice per group. **P < 0.001 compared to artificial cerebrospinal fluid (aCSF) and ##P < 0.001 compared to Scr by ANOVA followed by Bonferroni’s post hoc test. (c) MiR-134 levels in hippocampus after injection of Ant-134 or Scr at 1, 3, 5, 7, 10 and 21 days after injection. n = 3 or 4 mice per group. *P < 0.05, **P < 0.01 compared to Scr by ANOVA followed by Bonferroni’s post hoc test. NS, not significant. (d) NeuN counts at two different levels of the dorsal hippocampus in mice 24 h after injection with either Scr or Ant-134. n = 4 mice per group. (e) NeuN staining of the CA3 subfield 24 h after injection of Scr or Ant-134. Scale bar, 200 μm. (f) Behavioral analysis of mice 24 h after injection with Scr or Ant-134. The graph shows indices of exploratory activity: total ambulatory counts; distance traveled (cm); and vertical counts. All data are mean ± s.e.m.
Silencing miR-134 reduces status epilepticus and neuronal death

To test the idea that antagomirs might influence pathologic brain activity in vivo, we compared seizures evoked by intra-amygdala microinjection of kainic acid in mice 24 h after injection with either Scr or Ant-134 (Fig. 4 and Supplementary Fig. 3c). There was no difference in basal electroencephalography (EEG) measures between the two groups of mice (Supplementary Fig. 5a). Scr-injected mice experienced typical status epilepticus, comprising episodes of high-amplitude, high-frequency discharges (HAHFDs) [Fig. 4a–c; compare Fig. 4c to Fig. 1a]. An EEG analysis revealed that the duration of the HAHFDs (P = 0.0051), which are associated with damage-causing pathologic activity [45], and total EEG power (P = 0.033) were lower in Ant-134–injected mice compared to Scr-injected mice without kainic-acid–induced status epilepticus (Scr + C) and after status epilepticus in mice given Scr (Scr + KA) or Ant-134 (Ant + KA). The graph shows Limk1 protein concentrations normalized to actin. n = 4 mice per group. **P < 0.01 compared to Scr + KA compared to Scr + C; $P < 0.05$ for Scr + KA compared to Ant + KA, by ANOVA with Bonferroni’s post hoc test. All data are mean ± s.e.m.

Figure 4 Antagomir silencing of miR-134 reduces seizure severity during status epilepticus. (a) Graphs show HAHFDs and total EEG power during status epilepticus in mice 24 h after injection with Scr or Ant-134. n = 4–8 mice per group. **P < 0.05, ***P < 0.01 compared to Scr by t test. (b,c) Total EEG power (b) and the frequency and amplitude parameters (c) during status epilepticus in representative Scr-injected and Ant-134–injected mice covering the period between kainic acid injection and lorazepam administration. (d) MiR-134 levels in Scr- and Ant-134–injected mice 24 h after status epilepticus. n = 4 mice per group. **P < 0.01 compared to Scr by t test. (e) Limk1 protein concentrations in Scr-injected mice without kainic-acid–induced status epilepticus (Scr + C) and after status epilepticus in mice given Scr (Scr + KA) or Ant-134 (Ant + KA). The graph shows Limk1 concentrations normalized to actin. n = 4 mice per group. **P < 0.01 for Scr + KA compared to Scr + C; $P < 0.05$ for Scr + KA compared to Ant + KA, by ANOVA with Bonferroni’s post hoc test. All data are mean ± s.e.m.
reduction in FJB and TUNEL staining and less of a loss of NeuN staining after status epilepticus (Fig. 5a–f).

To specifically link the neuroprotective effects of antagonomirs to miR-134 and Limk1, we treated cultures of primary hippocampal neurons from C57BL/6 mice with kainic acid to model excitotoxic injury. Treatment of hippocampal neurons with kainic acid increased miR-134 levels (P = 0.019) (Fig. 5g). Next, neurons were co-transfected with shRNAs that targeted Limk1 (Fig. 5h), and either Scr or Ant-134, and then treated 48 h later with kainic acid. Ant-134 prevented kainic-acid–induced neurotoxicity in hippocampal neurons co-transfected with a non-targeting shRNA, and this protective effect was blocked in neurons cotransfected with shRNA targeting Limk1 (Fig. 5i,j).

Silencing miR-134 reduces spontaneous recurrent seizures

Based on our findings so far, we hypothesized that silencing miR-134 might also affect epilepsy. To avoid a confounding influence of shortened status epilepticus duration or neuroprotection on epileptogenesis, we injected antagomirs 1 h after triggering status epilepticus with kainic acid. An analysis of EEG measures confirmed that when we injected antagonism 1 h after kainic acid injection, there was no difference in duration of HAHFDs during status epilepticus between the Ant-134–injected and Scr-injected groups (Fig. 6a). In addition, CA3 damage assessed 24 h after triggering status epilepticus was similar between the Ant-134–injected and Scr-injected mice (Fig. 6b,c).

We then equipped groups of mice with EEG telemetry units and undertook continuous EEG recording for 2 weeks after status epilepticus. In agreement with the normal course of epilepsy in this model, mice injected with Scr after status epilepticus experienced the first spontaneous seizures on the third day, and all mice were epileptic by the fourth day after status epilepticus (Fig. 6d, and Supplementary Fig. 6c). The median epileptic seizure count during 14 d of monitoring of Scr-injected mice was 25 (range 8–79), with 200 epileptic seizures recorded in total for all the Scr-injected mice over this time period (Fig. 6d and Supplementary Fig. 6c). In contrast, only 60% of mice injected with Ant-134 had had a spontaneous seizure by the eleventh day after status epilepticus (Fig. 6d and Supplementary Fig. 6c); one mouse had only a single seizure on day 14 and another had no seizures in the 14 d after status epilepticus (Fig. 6d). Ant-134–injected mice had a median epileptic seizure count of 2 during the 14-d recording period (range 0–7), with just 16 epileptic seizures recorded in total for these mice during the 2 week recording period (P = 0.0001, compared to the Scr-injected group by two-way ANOVA) (Fig. 6e). The total amount of time spent in seizures also differed between the two groups, with Scr-injected mice spending more time in seizures than Ant-134–injected mice (P < 0.001 by two-way ANOVA; Supplementary Fig. 6c). The durations of the individual epileptic seizures were similar between the groups (Supplementary Fig. 6d).
Silencing miR-134 alters pathologic hallmarks of TLE

Progressive neuron loss, gliosis and rearrangement of mossy fibers are common pathologic hallmarks of TLE\textsuperscript{37,48}. We therefore examined whether Ant-134 altered the underlying pathology in the telemetry-equipped mice. Hippocampal CA3 neuron counts in the Ant-134–injected mice were lower than in the Scr-injected mice when the antagomirs were administered 1 h after inducing status epilepticus. n = 4 or 5 mice per group. Groups were not different when compared by t test. (f) Hippocampal NeuN (arrows indicate areas of reduced NeuN staining) and GFAP (astrogliosis) staining in Scr-injected and Ant-134–injected mice after 2 weeks of epilepsy monitoring. Scale bar, 200 µm. (g) NPY scores in mice 14 d after status epilepticus (left) and representative NPY-stained sections from Scr-injected and Ant-134–injected mice (right). **P < 0.01 compared to Scr by t test. Scale bar, 1 mm. (h) Graphs show the number of generalized tonic-clonic seizures (GTCS) each day for individual mice during two periods of 5 d of continuous video monitoring after status epilepticus for Scr-injected (left) and Ant-134–injected (right) mice. Scr1–Scr6, the six mice injected with Scr; Ant1–Ant5, the five mice injected with Ant-134. (i) Total GTCS counts averaged from the two monitoring periods. n = 5 or 6 mice per group. **P < 0.01 compared to Scr by t test. All data are mean ± s.e.m.

We first measured miR-134 levels in Ant-134–injected mice at the end of 14 d of telemetry recordings, which established that the miR-134 levels were ~55% of those in the Scr-injected mice (Supplementary Fig. 7a). Reducing miR-134 levels to ~55% of Scr in additional mice by injecting Ant-134 did not produce an anticonvulsant effect against kainic-acid–induced status epilepticus (Supplementary Fig. 7b,c). Likewise, mice subjected to kainic-acid–induced status epilepticus 14 d after i.c.v. injection with Ant-134, when levels of miR-134 are ~70% of those in Scr-injected mice (Supplementary Fig. 7d), were as sensitive to kainic-acid–induced status epilepticus as Scr-injected mice (Supplementary Fig. 7e–h), and mice were not protected against status epilepticus–induced hippocampal damage (Supplementary Fig. 7l).

Finally, longer-term video monitoring revealed that mice injected with Ant-134 after status epilepticus had fewer generalized tonic-clonic seizures and more seizure-free days at up to 2 months after...
injection compared to Scr-injected mice, although the seizure rates did increase toward the rate of Scr-injected mice in three out of the 5 Ant-134–injected mice (Fig. 6h,i and Supplementary Fig. 8).

**DISCUSSION**

Our study shows that silencing miR-134 in mice using antagomirs suppresses evoked seizures, the occurrence of spontaneous seizures and the associated pathologic hallmarks of epilepsy. These are the first in vivo data, to our knowledge, to show that inhibition of a single mature miRNA can alter pathologic electrical activity in the brain, and they offer a new therapeutic target for the treatment of epilepsy.

An association between seizures and changes in miRNA expression has been suggested by recent profiling work5,9,36,50 and by the phenotype of mice lacking Dicer31, although our experiments are the first, to our knowledge, to link upregulation of miR-134 to evoked, harmful seizures and chronic epilepsy. Brief, nonharmful generalized seizures were insufficient to alter miR-134 expression. This suggests that in vivo regulation of miR-134 is not only a response to increased neuronal activity19 but is also coupled to epileptic or pathogenic brain activity. Crucially, our miRNA silencing experiments support a role for miR-134 in facilitating pathologic neuronal activity in vivo because status epilepticus was potently suppressed in mice in which miR-134 was depleted by antagomirs. Notably, the seizure-suppressing effect of the antagomirs was nearly comparable to that of benzodiazepines45,46,52. Injecting antagomirs before status epilepticus also protected against seizure-induced hippocampal damage. This probably arises because of the resulting shortened seizure duration46,52, but antagomirs also inhibited direct kainic acid toxicity in vitro. Our in vitro experiments also implicated Limk1 in the mechanism of protection, but rescuing Creb1 may also contribute53, and our data are consistent with the neuroprotective effect of sirtn1, which negatively regulates miR-134 expression in brain20.

Despite progress in understanding the pathogenesis of epilepsy, few studies targeting epileptogenic processes have shown sufficient efficacy at reducing the occurrence, course or severity of the disease1,54. A second major finding here was that silencing miR-134 after status epilepticus resulted in a substantial reduction in the number of epileptic seizures in mice. Epilepsy developed normally in Scr-injected mice31, whereas spontaneous seizures seldom occurred in the Ant-134–injected mice. Thus, the effect of Ant-134 injection after status epilepticus was superior to that of neuroprotection by other means applied at the time of status epilepticus40,55 and was comparable to or exceeded the performance of other experimental antiepileptic treatments1. Did the antagomirs interrupt epileptogenesis or simply prevent epileptic seizures from occurring as a result of a prolonged anticonvulsant effect, or did they work in a manner similar to antiepileptic drugs? We detected spontaneous seizures, albeit infrequently, in the mice treated with antagomirs, and the increase in seizure rates over extended monitoring in these mice supports the interpretation of a prolonged anticonvulsant or antiepileptic effect. Nevertheless, seizure rates in these mice never recovered to baseline, and seizure frequency 2 months after status epilepticus, a time when antagonim suppression of miR-134 was no longer occurring, was over 70% lower than baseline, so an antiepileptic effect is possible. Additional experiments examining seizure frequency at time points later than 2 months after antagonist administration will be required to distinguish between these possibilities.

The mechanism by which miR-134 antagomirs suppress seizures is unknown, but our study offers the possibility that the effect is through changes to dendritic spines. The finding that Ant-134 reduced hippocampal CA3 dendritic spine density in vivo was notable and contrasts with reports that spine size, not density, is controlled by miR-134 (ref. 13). Thus, a different phenotype results from miR-134 inhibition in vivo, which may be explained by the scale of miRNA suppression achieved, the distribution of the antagonim (somal compared to dendritic), the targets affected or the potency of LNA-modified antagomirs over 2′-O-methyl oligonucleotides39. Coincidently, a similar reduction in spine density was reported in mice lacking an miRNA biogenesis component in which the hippocampal levels of mature miR-134 were also reduced27. A key target of miR-134 is Limk1, which regulates dendritic spine dynamics13. Limk1 protein concentrations followed an opposite pattern as miR-134 expression, and miR-134 silencing prevented seizure-induced downregulation of Limk1, although other explanations for a decrease in spines are possible45,55. Could the reduction in spines account for the suppression of seizures? Dendritic spines are targets of excitatory axons in the brain14,15. Although spines have been suggested to operate as barriers against potentially harmful afferent input38, our data are consistent with evidence showing that spine loss reduces excitatory responses17,27 and transient spine reduction uncouples excitotoxic NMDA-mediated signaling28. The antagomirs seem to curtail pathologic activity in the brain without impairing tonic neuronal communication; an impairment would have been detected by the ethogram, and this result is consistent with reports that a ~20% reduction in the number of hippocampal pyramidal neuron spines does not alter basal neurotransmission58. Spine-localized synaptic signaling is also implicated in the pathogenesis of TLE3, but whether a similar mechanism may account for the seizure-suppressive effects of miR-134 silencing is unknown. Loss of Limk1, which was countered with antagomirs, can result in increased hippocampal excitability24. However, other miR-134 targets with effects on excitability and seizure suppression are known, so we cannot exclude the involvement of other targets in the effects we observed.

Although the therapeutic application of miR-134 antagomirs for status epilepticus is probably barred by a need for pretreatment, the longevity of the suppression after a single injection, consistent with antagonist silencing data from mouse and primate experiments57,39, suggests applications in refractory epilepsy or in disease modification in the wake of epilepsy-precipitating injuries. Alternate routes for antagomir delivery, such as an intranasal route, which has been considered for antiepileptic drug delivery59, may avoid the blood-brain barrier exclusion of antagonim in vivo57 and may facilitate translation to the clinic for the treatment of epilepsy.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
E.M.J.-M. performed expression analyses, tissue culture, spine imaging, histology and epilepsy monitoring. T.E., K.T., G.M. and T.S. performed mouse modeling and telemetry. P.M.-S. and J.D. performed spine injections and data analysis. R.C.M. and S.P. performed expression studies. C.O. and J.L.W. conducted and analyzed the behavioral studies. N.D., D.F.O. and M.A.F. organized the human studies. R.M.C. performed statistical analyses. R.L.S. contributed to study design and analysis. D.C.H. and E.M.J.-M. conceived of the study, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Seizure models. All mouse experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (REC #205) under license from the Department of Health, Dublin, Ireland. Adult (20–22 g) male C57BL/6 mice were purchased from Harlan. Food and water was available to the mice ad libitum. Induction of status epilepticus was performed as described previously31. Mice were anesthetized with isoflurane and placed in a mouse-adapted stereotaxic frame. After making a midline scalp incision, the bregma was located, and three partial craniectomies were performed for the placement of skull-mounted recording screws (Bilaney Consultants). A fourth craniectomy was drilled for the placement of a guide cannula (coordinates from the bregma: anterior-posterior (AP) = –0.94 mm, lateral (L) = –2.85 mm) based on a stereotaxic atlas60. The cannula and electrode assembly was fixed in place, and the mouse was placed in an open Perspex box, which allowed free movement. The EEG was recorded using a Grass Comet digital EEG. After the baseline EEG was established, the mouse was lightly restrained while an injection cannula was lowered to 3.75 mm below the brain surface for injection of kainic acid (Sigma-Aldrich) or vehicle (PBS pH adjusted to 7.4) into the basolateral amygdala nucleus. After 40 min, all mice received lorazepam (Ativan, 6 mg per kg body weight, intraperitoneally (i.p.)). Mice were recorded for up to 1 h thereafter before being disconnected and placed in a warmed recovery chamber. Nonharmful seizures were induced by a single injection (i.p.) of kainic acid (15 mg per kg body weight), as previously described36. Nonharmful seizures were induced by a single injection (i.p.) of kainic acid (15 mg per kg body weight), as previously described36. Nonharmful seizures were induced by a single injection (i.p.) of kainic acid (15 mg per kg body weight), as previously described36.

EEG analysis during status epilepticus. EEG was analyzed using TWin software, and the duration of the HAHFDs, also termed type 4 seizures45, was calculated between the time of kainic acid injection and the time of lorazepam administration by an observer blinded to the treatment group. Additional frequency and amplitude analyses of EEG data were performed by uploading the data to an automated program for EEG analysis (LabChart Pro v7 software, ADInstruments Ltd).

Intracerebroventricular injections. For i.c.v. injections, additional mice were affixed with a cannula ipsilateral to the side of kainic acid injection. The coordinates from the bregma were: AP = −0.3 mm, L = −1.0 mm, ventral (V) = −2.0 mm. Mice received a 1 μl infusion of either Scr or Ant-132 LNA-modified and 3'-cholesterol-modified oligonucleotides (Exiqon) in aCSF (Harvard Apparatus). Mice were either euthanized or underwent status epilepticus. For the experiments involving intranasal administration of antagomir, 0.12 nmol of Scr or Ant-134 in a 5 μl volume was administrated into each nostril of the mice. Mice were euthanized at various time points after antagonim administration (1, 4, 8, 12 or 24 h, 5, 7, 14 or 28 or 2 months), after kainic acid or vehicle administration (24 h) or once mice began to show spontaneous seizures at 1, 2, 4 or 8 weeks after intra-amygdala injections.

Analysis of spontaneous seizures using EEG telemetry. Epilepsy monitoring by implanted EEG telemetry units was performed as previously described31,35. EEG data were acquired with EEG transmitters (Model: F20-EET, Data Systems International) configured to record a two-channel EEG that was skull-affixed over the dorsal hippocampus and the temporal cortex when the mice were under anesthesia at the time of surgery for intra-amygdala injection. Transmitter units were placed in a subcutaneous pocket along the dorsal flank. Continuous EEG data were collected for 14 consecutive days after status epilepticus. EEG data were reviewed and manually scored by an observer unaware of the experimental treatment, with epileptic seizures being defined as high-frequency (>5 Hz), high-amplitude (>2× baseline) polyspike discharges of ≥5 s duration. Analysis of spontaneous seizures by continuous video monitoring. Mice were subjected to intra-amygdala kainic acid–induced status epilepticus and then injected 1 h later with Scr or Ant-134 i.c.v., as described above. Mice were allowed to recover and were then housed in pairs (the mice were distinguished by ear clips) in clear Perspex cages (dimensions: 32 cm (long) × 17 cm (wide) × 14 cm (deep)). Webcam-style cameras connected to laptop computers were placed 40 cm from the cages in a room equipped with safe lights for nighttime recordings. Images were captured using VirtualDub 1.9.11 (SourceForge.net) with a sampling rate of 10 frames per second and a data transfer rate of 140 kb s–1. Videos from 5 d of continuous monitoring during weeks 4–6 and weeks 7–8 after status epilepticus were reviewed by an observer unaware of the experimental treatment. Seizures were counted using a modified six-point Racine scale for mice. The scores were determined as follows: 2, forelimb and or tail extension, rigid posture, 3, repetitive movements, head bobbing; 4, rearing and falling; 5, continuous rearing and falling; or 6, severe tonic-clonic seizures. Clinical events scoring below 2 such as sudden freezing and immobility were not included. The numbers of seizures per day were analyzed using Poisson regression with robust standard errors to express the experimental effect as an incidence rate ratio. The average Racine score and the number of seizure-free days were compared using the Wilcoxon Mann-Whitney test.

Mouse tissue samples. Mice were killed by pentobarbital overdose and perfused with ice-cold saline to remove the intravascular blood components. The brains to be used for molecular and biochemical work were microdissected over wet ice, with the hippocampus being further subdivided to obtain the separate CA3-enriched portion, as previously described55. For histology, mice were either perfused fixed with paraformaldehyde (4%) or their brains were fresh frozen in 2-methylbutane (at −30 °C).

Human samples. This study was reviewed and approved by the Beaumont Hospital Ethics (Medical Research) Committee (approval REC 05/18), and informed consent was obtained from each subject. Subjects (n = 3) were determined to have medically intractable TLE and had been referred by an epileptologist (N.D.) for surgical resection of the temporal lobe (D.F.O.) after neurological assessment, video EEG recordings and neuroimaging (magnetic resonance imaging). All patients were taking antiepileptic drugs before surgery. Each temporal lobe neocortex sample was inspected by a pathologist (M.A.F.) and deemed to be absent of any obvious neuron loss or lesion. Subject 1 was a 27-year-old male who underwent right posterior temporal lobectomy. Subject 2 was a 53-year-old male who underwent right anterior temporal lobectomy. Subject 3 was a 45-year-old male who underwent temporal lobectomy. Temporal neocortex samples were flash frozen in liquid nitrogen and stored at −70 °C until use. Each sample was divided and used for either miRNA measurement or protein analysis. Human control samples were also fresh frozen, were donated by people who died of causes not related to known neurological disease and were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, Maryland, USA. Samples were all from people of European ancestry (mean age 26.3 years, range 25–28 years). The causes of death for these individuals were (for subjects C1 and C2) multiple injuries from a road traffic accident and (for subject C3) cardiac tamponade. The average autopsy delay after death was 6.7 h (range 7–12 h).

Simulated postmortem delay. To simulate the postmortem delay in the human subjects, mice were killed, and the cortices from the temporal association region were extracted either immediately (0 h) or were removed after a delay of 6 h or 12 h (to simulate the postmortem interval). Samples were then processed for miRNA measurement.

Histopathology. Coronal sections from two levels of the dorsal (septal) hippocampus were analyzed (rostral, AP = −1.46 mm; medial, AP = −1.70 mm). For detection of neurodegeneration, sections were stained using FJB55 (Millipore). Irreversible DNA damage was detected using a fluorescein-based TUNEL kit (Promega). For immunohistochemistry, sections were postfixed, permeabilized, blocked in 5% goat serum and then incubated overnight with antibodies to NeuN (1:400, Clone A60, Millipore), GFAP (1:400, G9269) or NPY (1:1,000, N9528) (both from Sigma-Aldrich). Sections were washed and incubated with secondary antibodies raised in goat conjugated with Alexa Fluor 488 (for NeuN) or Alexa Fluor 568 (for GFAP and NPY) (BioSciences Ltd). Sections were mounted with medium containing DAPI (Vector Laboratories Ltd). Sections were examined using a Hamamatsu Orca 285 camera attached to a Nikon 2006s epifluorescence microscope (Micron Optical). Counts were the average of those from two adjacent sections of the ipsilateral CA3 subfield scored by an observer blinded to the experimental treatment.

Synaptic reorganization was assessed by an observer blinded to the experimental treatment in NPY-stained sections. Immunostaining was rated from...
0 to 5 using a scale adapted from methods of mossy fiber sprouting assessment. The staining was scored as follows: 0, no NPY between the tips and crest of the dentate gyrus; 1, sparse staining in the supragranular region in a patchy distribution between the tips and crest of the dentate gyrus; 2, more abundant NPY in the supragranular region in a continuous distribution between the tips and crest of the dentate gyrus; 3, prominent NPY in the supragranular region in a continuous pattern between the tips and crest, with occasional patches of confluent staining between the tips and crest of the dentate gyrus; 4, prominent NPY in the supragranular region that forms a confluent dense laminar band between the tips and crest; or 5, confluent dense laminar band of NPY in the supragranular region that extends into the inner molecular layer.

**miRNA expression.** Total RNA was extracted using the miRNeasy kit (QIAGEN), and 250 ng was reverse transcribed using stem-loop Multiplex primer pools (Applied Biosystems). We used reverse-transcriptase–specific primers for the mouse miRNAs miR-19a and miR-134 (Applied Biosystems), and qPCR was carried out on a 7900HT Fast Realtime System (Applied Biosystems) using TaqMan miRNA assays (Applied Biosystems). RNU19 or U6B was used for normalization. A relative fold change in expression of the target gene transcript was determined using the comparative cycle threshold method (2^{-ΔΔCT}).

**Western blotting.** Protein was extracted from the whole hippocampus or individual microdissected CA3 subfields, subjected to SDS-PAGE, transferred to nitrocellulose membranes and incubated with the primary antibodies to the following: Limk1 (1:1,000, 3842) or CREB (1:1,000, 86B10) (both from Cell Signaling Technology), β-actin (1:2,000, AC40, Sigma-Aldrich), α-tubulin (1:2,000, sc-8035) or GFP (1:500, sc-9996) (both from Santa Cruz Biotechnology). Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch), and bands were visualized using Supersignal West Pico Chemiluminescence Substrates (Pierce). Images were captured using a Fuji-Film LAS-300, and densitometry was performed using AlphaEaseFC4.0 gel-scanning integrated optical density software (Alpha Innotech).

**Argonaute-2 immunoprecipitation.** Pools of three individual CA3 subfields for each condition were homogenized in immunoprecipitation buffer (300 mM NaCl, 5 mM MgCl₂, 0.1% NP-40 and 50 mM Tris HCl pH 7.5) and centrifuged, and then 400 µl of the lysate was incubated overnight at 4 °C with 5 µg of antibodies to Argonaute-2 (1:50, C34C6, Cell Signaling Technology). Protein A agarose beads (Santa Cruz Biotechnology) were added, mixed and incubated for 1 h at 4 °C and then centrifuged, and the supernatant was removed. The pellet was washed and processed for miRNA extraction, stem-loop reverse transcription and real-time qPCR (Applied Biosystems).

**In situ hybridization.** Mice (n = 3) were perfused with 4% paraformaldehyde, and 12-µm tissue sections were mounted on SuperFrost-Plus slides (VWR). Using RNase-free solutions, slides were washed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% IGEPA, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and 50 mM Tris, pH 8.0), followed by treatment with 0.25% acetic anhydride in 0.1 M triethanolamine, rinsed with 0.1% Tween-20 in PBS and treated with 5 µg ml⁻¹ proteinase K. Next, slides were rinsed in hybridization buffer (1× saline solution, 50% formamide and 1× Denhardt's solution) for 1 h at 56 °C (melting temperature (Tm) = 20 °C, Tm provided by Exiqon). The probe to detect miR-134 was the reverse complement to the mature miRNA and was 5'-digoxigenin-labeled, 5′-O,4′-C methylene bicyclocyclolysine monomer–containing oligonucleotide (LNA-modified). Sections were also labeled using a sense control. Probes were incubated at a dilution of 1:200 in hybridization buffer overnight at 56 °C in a humidified chamber. The next day, sections were washed in FAM buffer (2× saline sodium citrate (SSC) buffer, 50% formamide and 0.1% Tween 20) for 1 h at 60 °C. Sections were then rinsed in B1 buffer (150 mM NaCl, 100 mM maleic acid and 0.4% IGEPA, pH 7.5) for 1 h at room temperature and B2 buffer (2% blocking reagent and 10% goat serum in B1 buffer) for 30 min. Polyclonal antibodies to digoxigenin (1:1,000, 11093274910, Roche) were incubated in B2 buffer overnight at 4 °C. The next day, sections were washed in B1 buffer and incubated in B3 buffer (100 mM NaCl, 50 mM MgCl₂, 0.025% Tween 20 and 100 mM Trizma, pH 9.5) for 30 min. Then, 200 µl of color substrate solution (nitroblue tetrazolium in 2.5-mano-4-chloro-indolyl-phosphatase stock solution (Roche) diluted 1:50 in B3 buffer) was added to each slide until the signal appeared. Slides were then rinsed, mounted with medium and covered with a cover slip.

**Antagomirs.** Scr or Ant-134 (Exiqon, LNA-modified and 3′-cholesterol-modified oligonucleotides) in aCSP (Harvard Apparatus) was injected into the lateral ventricles of the mice.

**Behavior analysis.** Exploratory activity was assessed in Ant-134–injected and Scr-injected mice in the open field. Each mouse was placed individually in the center of a open-field apparatus (ENV-510; 27.9 cm × 27.9 cm; Med Associates). Total ambulatory and vertical counts—counts defined as consecutive break of three infrared beams when interbreak interval < 500 ms—and distance traveled (cm) were recorded. Data were collected over a 60-min period.

**Spine density analysis.** Coronal mouse brain sections (150 µm) were cut on a vibratome and prelabeled with DAPI. Pyramidal cells from the CA3 subfield were individually injected with Lucifer yellow (8% in 0.1 M Tris buffer, pH 7.4). Lucifer yellow was applied to each injected cell by continuous current until the individual dendrites of each cell could be traced to an abrupt end at their distal tips that fluoresced brightly. Sections were then processed with a rabbit polyclonal antibody to Lucifer yellow produced at the Cajal Institute (1:400,000 in 2% BSA (A3425, Sigma-Aldrich), 1% Triton X-100 and 5% sucrose in phosphate buffer) followed by streptavidin coupled to Alexa Fluor 488 (1:1,000, Molecular Probes). The sections were washed and mounted with ProLong Gold Antifade Reagent (Invitrogen). For each pyramidal neuron (4–7 neurons from each mouse, 7 mice per group, 83 neurons total), one randomly selected dendrite was scanned from the soma to the tip. Images of dendrites from Scr-injected and Ant-134–injected mice (n = 40 for each group) were acquired with a Zeiss (LSM 710) confocal laser scanning microscope, and the fluorescence of Alexa Fluor 488 was recorded. Image stacks were obtained that consisted of 10–100 image planes (voxel size: 0.057 µm × 0.057 µm × 0.14 µm; area: 58.36 µm × 58.36 µm) with a 63× oil-immersion lens (AN, 1.40; refraction index 1.45) using a calculated optimal zoom factor of 2.3. For each stack, the laser intensity and detector sensitivity were set so that the fluorescence signal from the dendritic spines occupied the full dynamic range of the detector. Therefore, some pixels were saturated in the dendritic shaft, but no pixels were saturated in the dendritic spines. After the acquisition, stacks were opened with Imaris 7.1 (Bitplane AG). The images were coded and were not broken until the quantitative analysis had been completed. After acquisition, the stacks were processed with a three-dimensional blind deconvolution algorithm (AutoDeblur; Autoquant, Media Cybernetics) for ten iterations to reduce the out-of-focus light. Image stacks were imported to the confocal module of Neuronucila Explorer (MicroBrightfield, Inc.) and dendritic spine density was determined by tracing the image of the acquired dendrites in three dimensions. Spines were marked during tracing, and all protrusions were considered to be spines. After tracing, the reconstructed data were exported to Neuronucila Explorer (MicroBrightfield Inc.) for quantitative analysis. The spine density was calculated for each dendrite by dividing its length by the number of spines, and the spine density was also analyzed as a function of its distance from the soma (using a Sholl analysis) by dividing the length of the dendritic segment by the number of spines in this distance for every 10 µm of distance from the soma.

**Cell culture.** SH-SY5Y cells were grown in 10% FCS, 2 mM glutamine and 100 U ml⁻¹ penicillin and streptomycin in DMEM/Ham’s F12 medium. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. Hippocampal neurons were prepared from embryos at gestational day 18. The harvested embryos were transferred to Hank’s balanced salt solution without calcium and magnesium medium (Invitrogen), the hippocampi were isolated, and the surrounding meninges were removed. The tissue was then incubated with papain (Worthington Biochemical Corp.) for 30 min, and the cells were gently dissociated, counted and then seeded in dishes coated with 1 mg ml⁻¹ poly(t-lysine) and 20 µg ml⁻¹ laminin in horse serum andneurobasal medium (10% horse serum, 2 mM glutamine, 2 mM pyruvate and 100 U ml⁻¹ penicillin and streptomycin in neurobasal medium). After 3 h, the medium was removed.
and N2/B27/neurobasal medium (N2 (Invitrogen), B27 (Invitrogen), 2 mM glutamine, 2 mM pyruvate and 100 U ml^{-1} penicillin and streptomycin in neurobasal medium) was added. Half of the N2/B27/neurobasal medium was replaced every 2 d.

Two days after transfection, the hippocampal neurons were exposed to 0.3 µM kainic acid for 6 h, and then neuronal damage was assessed. Neurons were live stained with Hoechst 33258 (1 µg ml^{-1}) and propidium iodide (5 µM) in the medium for 30 min. Nuclear morphology was assessed with an epifluorescence microscope under the 20× objective. For each condition, images of the nuclei were captured in six different areas for each individual experiment. GFP+ and propidium iodide-positive cells showing condensed and/or fragmented nuclei were scored as dead and were expressed as a percentage of the total GFP+ cell population.

Plasmids and transfection. SH-SY5Y cells were transfected using lipofectamine 2000 (Invitrogen). Briefly, DNA was incubated with the lipofectamine reagent for 20 min at a ratio of 1:2 (v/v). Then, the DNA and lipofectamine mix was added to the cells for 4 h. The medium was removed, the cells were washed, and new medium was added. Two days later, cells were harvested, and the samples were prepared for western blotting. Hippocampal neurons were transfected after 6 d in culture with 3 µg of the expression plasmid pGFP-V-RS-scramble or pGFP-V-RS-shLimk (Origene) and 0.01 nmol of either Scr or Ant-134 (Exiqon) using the calcium-phosphate technique. The following sequences were used: control shRNA: 5′-GCACTACCAGAGCTAACTCAGATACTACT-3′; shLimk1: 5′-AAGGACAAGAGGCTCAACTTCATCACTGA-3′; and shLimk2: 5′-CTGCACGCAAGCTCTCCAAGTGTTGTG-3′.

Data analyses. All data are presented as means ± s.e.m. Two-group comparisons were made using unpaired Student’s t test, and multi-group comparisons were made using one- or two-way ANOVA followed by the appropriate post hoc testing (GraphPad Instat). Significance was accepted at P < 0.05.

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