SUPPLEMENTARY INFORMATION

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

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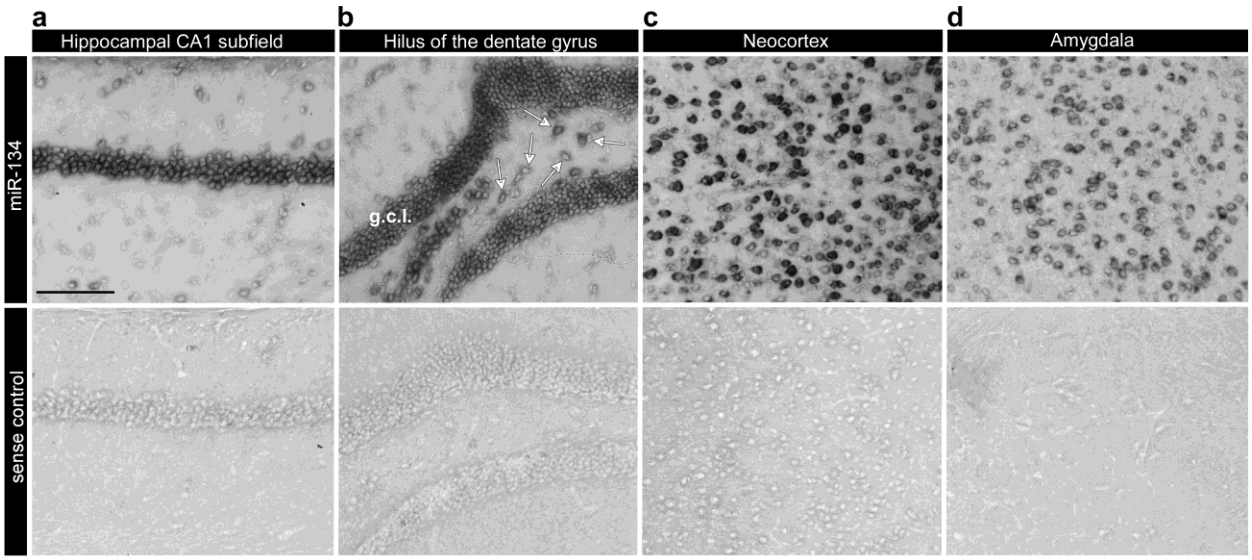
Supplementary Figure 1

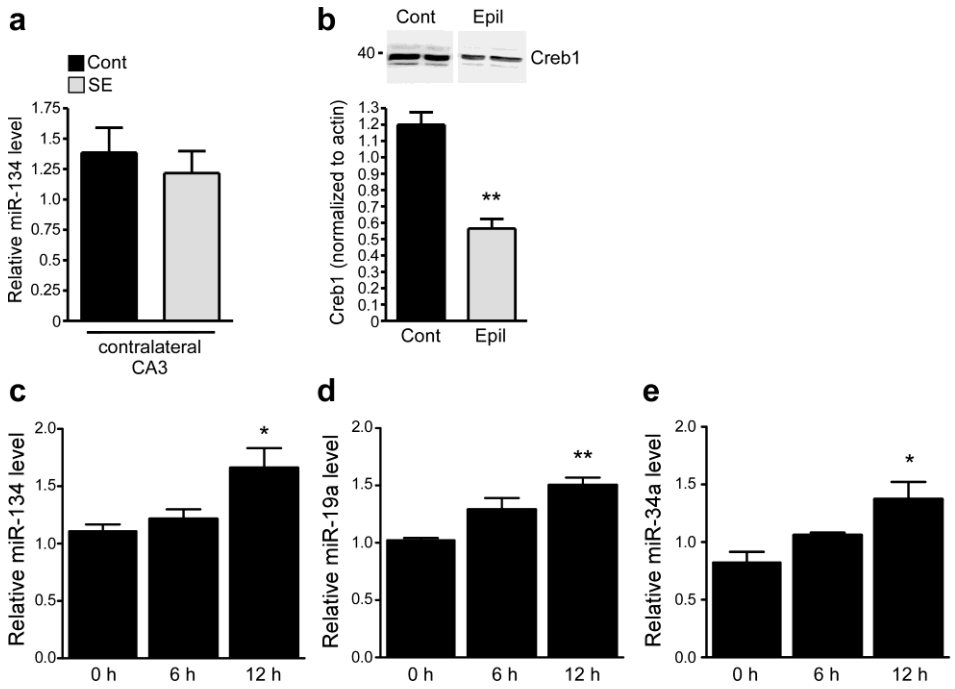
Cellular localization of miR-134 in adult mouse brain by *in situ* hybridization



Supplementary Figure 1: *Cellular localization of miR-134 in adult mouse brain by in situ hybridization.* Representative photomicrographs showing (top) miR-134 hybridization signal and (below) sense control, in normal adult mouse brain within (**a**) CA1 subfield of the hippocampus, (**b**) hilus and granule neurons of the dentate gyrus, (**c**) neocortex (temporal association cortex) and (**d**) amygdala. Arrows in *b* identify probable hilar interneurons. Scale bar in **a**, 180 µm. g.c.l. granule cell layer. Images taken using Leika DM 4000B, 20 x objective.

Supplementary Figure 2

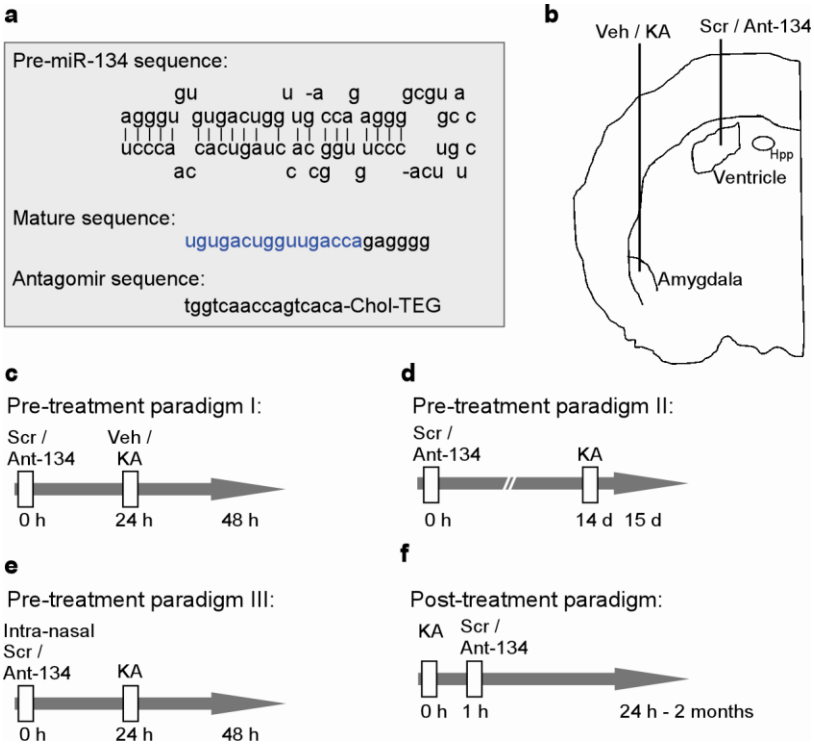
miR-134 levels in contralateral hippocampus after SE, protein levels of a second miR-134 target in experimental epilepsy, and simulated autopsy delay effects on miRNAs



Supplementary Figure 2: *miR-134 levels in contralateral hippocampus after SE, protein levels of a second miR-134 target in experimental epilepsy, and simulated autopsy delay effects on miRNAs.* (**a**) RT-qPCR measurement of miR-134 levels (normalized to RNU6b) in the contralateral CA3 subfield of mice 24 h after SE ($P = 0.566$, $n = 3$ per group). (**b**) Representative western blots ($n = 1$ per lane) and densitometry for Creb1, another target of miR-134, in the hippocampus of epileptic (Epil) mice (3 weeks post-SE) compared to time-matched vehicle controls (Cont) ($P = 0.001$, $^{**}P < 0.01$, $n = 4$ per group). (**c–e**) Simulated autopsy delay experiment. Mice were killed and neocortex removed either immediately (0 h) or after a delay of 6 h (corresponding to the average autopsy delay in human control samples) or 12 h (the longest autopsy delay in our samples). Graphs show expression normalized to RNU19 for (**c**) miR-134; 0 h vs. 6 h, $P = 0.541$; 0 h vs. 12 h, $P = 0.03$.$^{*}P < 0.05$ compared to 0 h, $n = 3$ per group; (**d**) miR-19a; 0 h vs. 6 h, $P = 0.07$; 0 h vs. 12 h, $P = 0.008$.$^{**}P < 0.01$ compared to 0 h, $n = 3$ per group; (**e**) miR-34a; 0 h vs. 6 h, $P = 0.158$; 0 h vs. 12 h, $P = 0.02$. $^{*}P < 0.05$ compared to 0 h, $n = 3$ per group. Thus, the average autopsy delay in human studies (6.7 h) is unlikely to have affected miR-134 levels in human studies. Although a 12 h simulated post-mortem interval changed miRNA levels, the effect was to increase miR-134 levels. This would lead to, at worst, an underestimation of the higher levels of miR-134 in TLE patients. a.u. arbitrary units.

Supplementary Figure 3

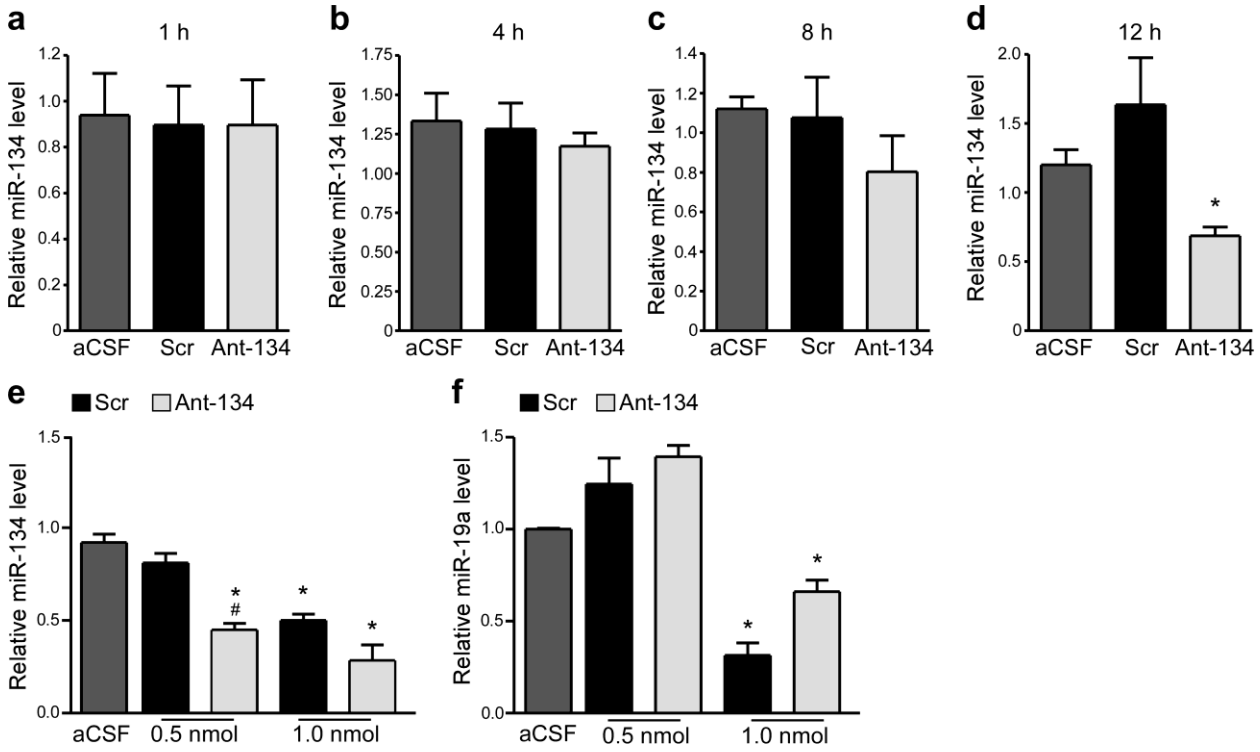
Antagomir sequence and injection routes and paradigms for studies involving KA and antagomirs



Supplementary Figure 3: *Antagomir sequence and injection routes and paradigms for studies involving KA and antagomirs.* (**a**) Precursor (pre-miR-134) sequence of miR-134, sequence of the mature form of miR-134 and sequence of the antagomir targeting miR-134. Region in blue corresponds to the region of miR-134 that the antagomir binds. (**b**) Vehicle (Veh) or KA was microinjected into the right amygdala of mice to trigger SE. Scr or Ant-134 was injected i.c.v. hpp, hippocampus. (**c–f**) Dosing regimens. (**c, d**) For i.c.v. pre-treatment paradigms, mice received Scr or Ant-134 followed either 24 h or 14 days later by vehicle or KA, and animals were killed 24 h later. (**e**) In the intra-nasal pre-treatment regime, mice received 0.12 nmol Scr or Ant-134 in 5 µl into each nostril. Twenty-four hours later, hippocampus was either extracted for miR-134 measurement or KA was given to trigger SE. (**f**) In the post-treatment paradigm, KA was injected to trigger SE and then either Scr or Ant-134 injected 1 h later. Mice were then sacrificed from 24 h to up to two months later.

Supplementary Figure 4

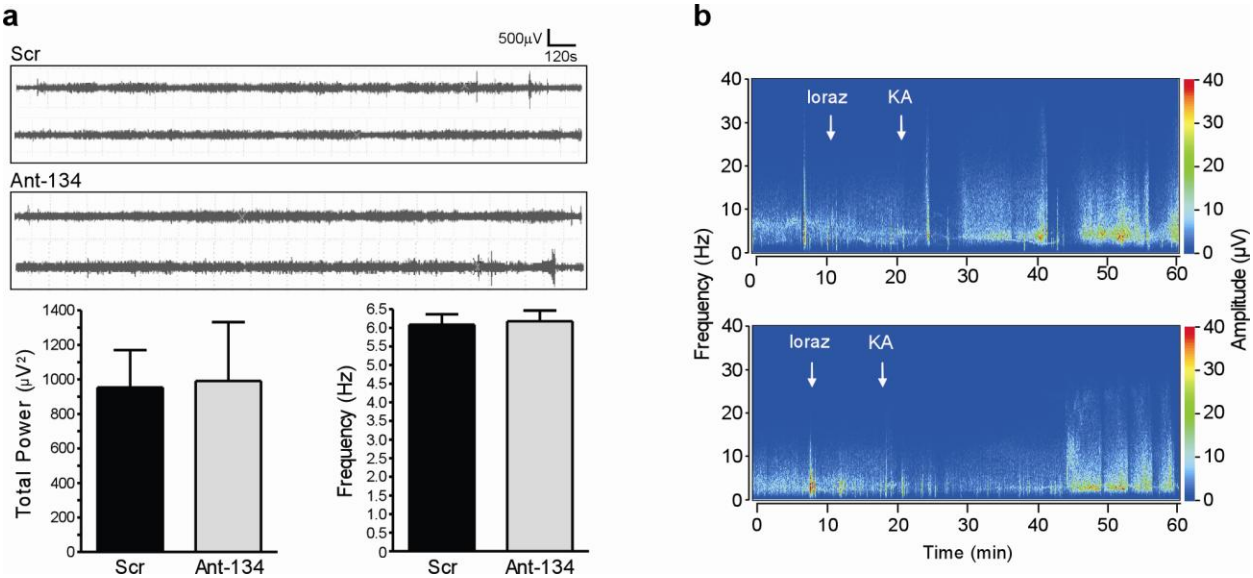
Antagomir time course and dose-response effects on miR-134 knockdown



Supplementary Figure 4: *Antagomir time course and dose-response effects on miR-134 knockdown.* (**a–d**) miR-134 levels, normalized to RNU19, over time following i.c.v. injection of aCSF, Scr or Ant-134. Graphs show miR-134 level in hippocampus (**a**) 1 h (P = 0.981), (**b**) 4 h (P = 0.745) and (**c**) 8 h (P = 0.383) post-injection. By 12 h post-injection (**d**), miR-134 levels were significantly reduced by Ant-134 (aCSF vs. Scr, P = 0.288; aCSF vs. Ant, P = 0.015; Scr vs. Ant, P = 0.049). *P < 0.05, n = 3 per group. (**e**) miR-134 levels 24 h after injection of 0.5 nmol and 1.0 nmol of either Scr or Ant-134 (n = 3 per group). aCSF vs. Scr (0.5 nmol), P = 0.263; aCSF vs. Ant-134 (0.5 nmol), P = 0.01; Scr vs. Ant-134 (0.5 nmol), P = 0.02; aCSF vs. Scr (1 nmol), P = 0.01; aCSF vs. Ant-134 (1 nmol), P = 0.006; Scr vs. Ant-134, P = 0.102 (ANOVA, *P < 0.05 compared to aCSF, #p < 0.05 compared to Scr). (**f**) miR-19a levels 24 h after injection of 0.5 nmol and 1.0 nmol of Scr or Ant-134 (n = 3 per group). aCSF vs. Scr (0.5 nmol), P = 0.182; aCSF vs. Ant-134 (0.5 nmol), P = 0.065; Scr vs. Ant-134 (0.5 nmol), P = 0.222; aCSF vs. Scr (1 nmol), P = 0.001; aCSF vs. Ant-134 (1 nmol), P = 0.011; Scr. vs. Ant-134, P = 0.22 (ANOVA, *P < 0.05 compared to aCSF).

Supplementary Figure 5

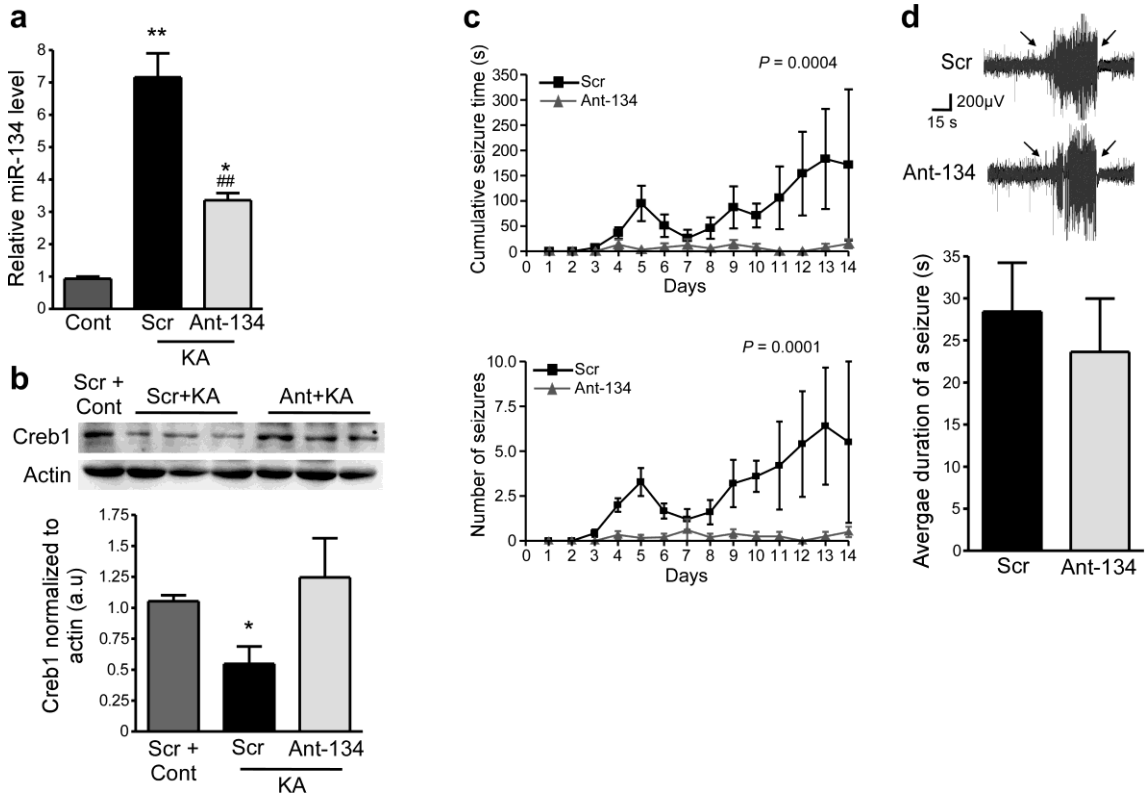
Baseline EEG in Scr and Ant-134 mice and effect of lorazepam pre-treatment on SE



Supplementary Figure 5: *Baseline EEG in Scr and Ant-134 mice and effect of lorazepam pre-treatment on SE*. (**a**) Representative EEG traces (top) from two individual mice injected i.c.v. 24 h previously with Scr, and (below) traces from two individual Ant-134 mice. Graphs below show total EEG power (P = 0.929) and frequency (P = 0.830) during 40 min recordings were not different between the groups. (**b**) As a guide to the seizure-suppressing effect of Ant-134, additional mice (n = 3) were pre-treated with lorazepam (loraz; 6 mg/kg, i.p.) 10 min before intra-amygdala KA injection. Figure shows representative frequency-amplitude EEG heat-maps from two animals given lorazepam followed by KA with recordings continued for 40 min. The extent of seizure suppression was qualitatively similar to that seen with pre-treatment with Ant-134 (see **Fig. 4c**).

Supplementary Figure 6

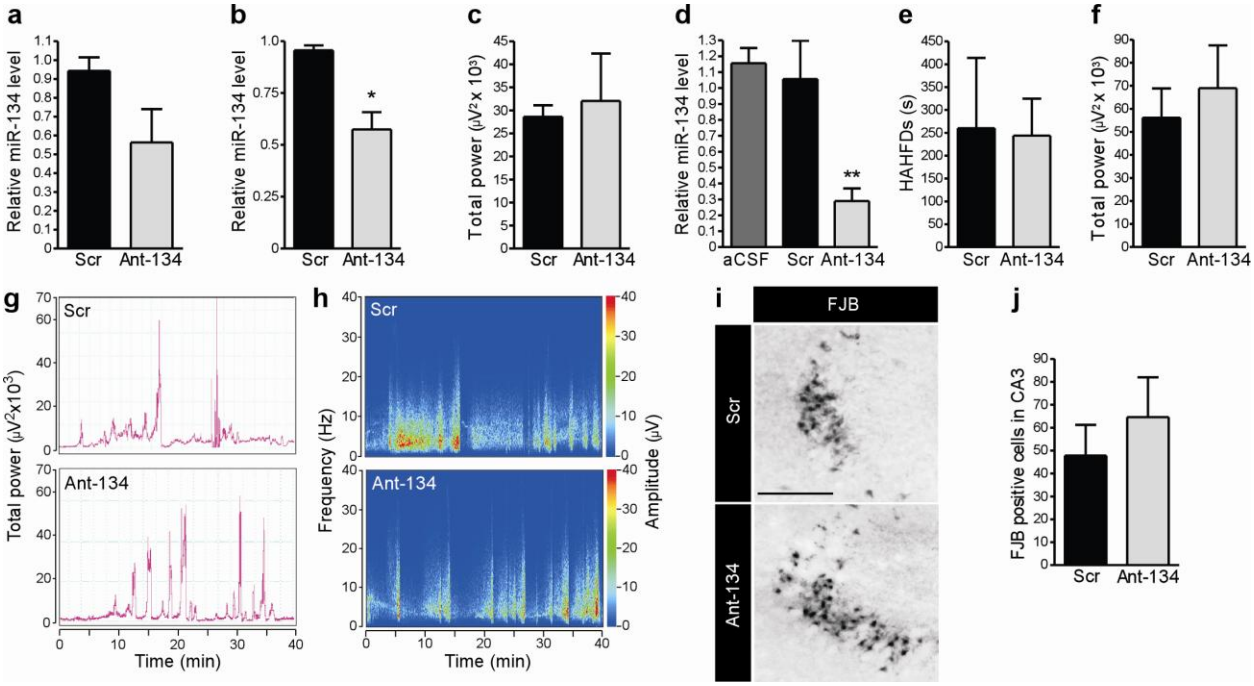
miR-134 and Creb1 levels in antagomir mice after intra-amygdala KA-induced seizures and effects of Ant-134 on epileptic seizures



Supplementary Figure 6: *miR-134 and Creb1 levels in antagomir mice after intra-amygdala KA-induced seizures and effects of Ant-134 on epileptic seizures*. (**a**) miR-134 levels in CA3 at 24 h after SE in Ant-134-injected mice compared to Scr. Cont vs. Scr + KA, $P = 0.005$; Cont vs. Ant-134 + KA, $P = 0.02$; Scr + KA vs. Ant-134 + KA, $P = 0.002$. *$P < 0.05$, ##$P < 0.01$. (**b**) Western blot (top, $n = 1$ per lane) and densitometry (below) show levels of Creb1 after SE and the effects of antagomir. Creb1 levels were reduced after KA-induced seizures in Scr, whereas levels were similar to control in animals given Ant-134 24 h before KA injection. Graph; Scr + Cont vs. Scr + KA, $P = 0.03$; Scr vs. Ant-134 after KA, $P = 0.07$; Scr + Cont vs. Ant-134 + KA, $P = 0.664$). *$P < 0.05$, $n = 3$ in Scr + Cont group, $n = 6$ each for Scr/Ant-134 + KA. (**c**) Graphs showing cumulative time in epileptic seizures per day and mean number of epileptic seizures per day for Scr and Ant-134 animals recorded during the two weeks telemetry study. Two-way ANOVA confirmed groups were statistically different. (**d**) EEG traces of typical spontaneous seizures captured using EEG telemetry. Graph below shows the duration of an individual seizure, when they occurred, was not different between groups ($P = 0.610$, $n = 5$ per group).

Supplementary Figure 7

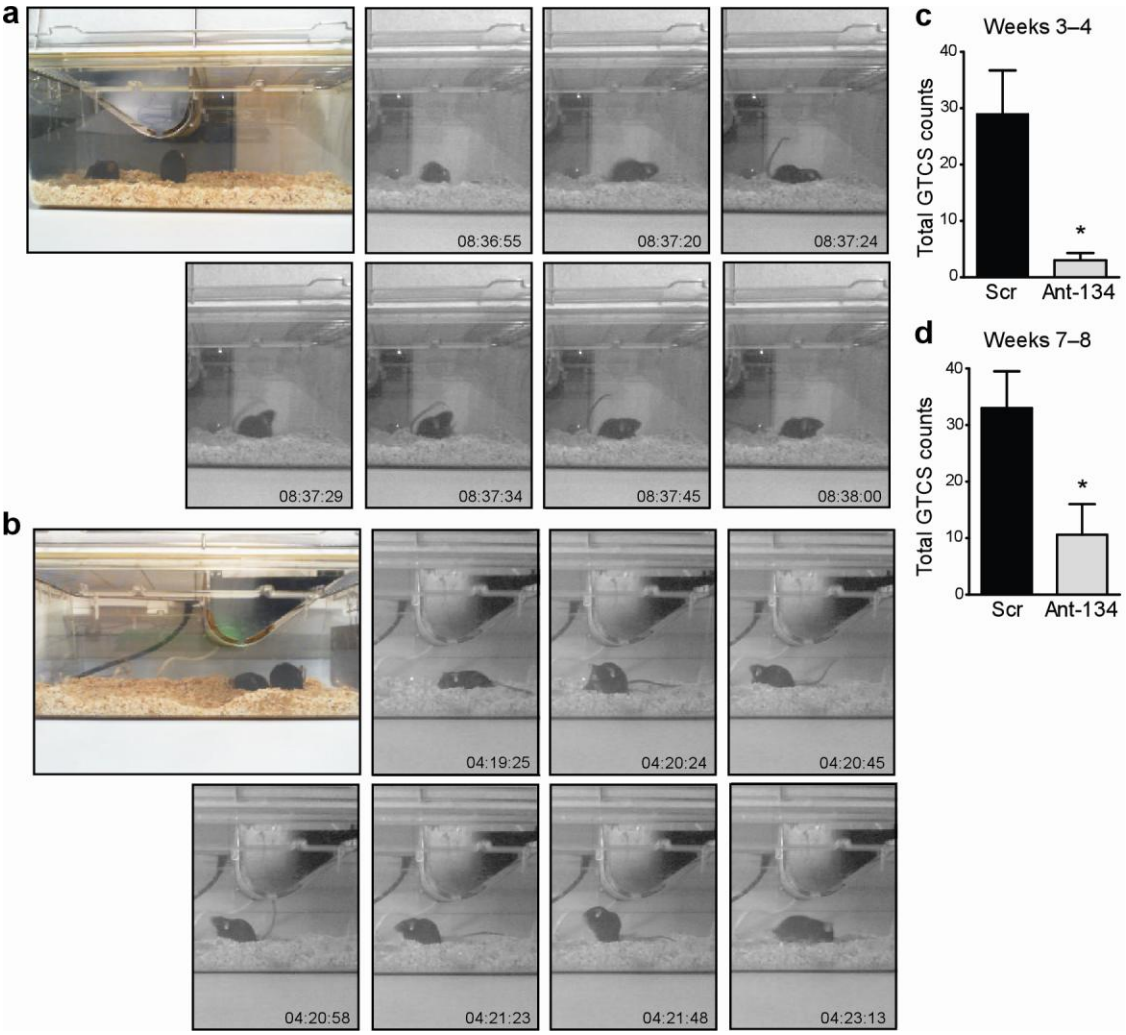
Experiments on the issue of prolonged anticonvulsive versus anti-epileptogenic effects of antagomirs



Supplementary Figure 7: *Experiments on the issue of prolonged anticonvulsive versus anti-epileptogenic effects of antagomirs.* (**a**) miR-134 levels in hippocampus of telemetry mice, 14 days after SE. Levels of miR-134 in Ant-134 animals were ~55 % of levels in Scr mice (P = 0.07, n = 5 per group). (**b**) miR-134 levels 24 h after intranasal Ant-134 (0.12 nmol per nostril). The reduction in miR-134 levels matched that detected in Ant-134-treated mice at the end of epilepsy monitoring (~55 % of Scr level; P = 0.02). *P < 0.05, n = 4 per group. (**c**) Total EEG power during SE in mice was not different between groups treated 24 h earlier with intranasal administration of Scr or Ant-134 (P = 0.755, n = 4 per group). Thus, lowering miR-134 levels to ~55 % of Scr does not have an anticonvulsant effect in this model. (**d**) Levels of miR-134 in hippocampus 14 days after i.c.v. injection of aCSF, Scr or Ant-134 (0.12 nmol) (P = 0.005).**P < 0.01, n = 3 per group. (**e, f**) Graphs show (**e**) HAHFDs, and (**f**) total EEG power, during SE in mice injected i.c.v. with Scr or Ant-134 14 days previously (HAHFDs; P = 0.924; Total EEG power: P = 0.538; n = 5 per group). As with the intranasal route, this reduction in miR-134 levels was insufficient to have an anticonvulsant effect. Representations of (**g**) total EEG power and (**h**) frequency and amplitude parameters during SE from animals given Scr- or Ant-134 14 days before SE. Recorded period covers time between KA injection and anticonvulsant administration. (**i, j**) FJB staining and counts in CA3 between Scr and Ant-134 mice when i.c.v. injections were performed 14 days prior to SE (P = 0.467, n = 4 per group). Scale bar, 170 µm.

Supplementary Figure 8

Long-term effects of antagomirs; continuous video monitoring of spontaneous seizures at one and two months post-SE



Supplementary Figure 8: *Long-term effects of antagomirs; continuous video monitoring of spontaneous seizures at one and two months post-SE.* (**a, b**) Image panels showing representative generalized tonic-clonic seizures (GTCS) (Racine scale 3) in (**a**) Scr and (**b**) Ant-134 animals captured during week 3–4 monitoring post SE. Both recordings were during the night under safe-light conditions and time stamp in lower right of each panel indicates hours:minutes:seconds into recording. Color panel at start is a full-cage view immediately prior to lights out. (**c**) Summary graph showing the average total GTCS counts for each group during the 5 days monitoring between weeks 3–4 (**P* = 0.02, *n* = 5–6 per group). (**d**) Graph showing the average total GTCS counts for each group during the 5 days monitoring between weeks 7–8 (**P* = 0.029, *n* = 5–6 per group).

Supplementary Figure 8 continued:

Full description of results from weeks 3–4 monitoring: Video-monitoring of Scr animals over 5 days during weeks 3–4 detected a total of 174 seizures (range 10 – 53 seizures), an average of 5.8 seizures per day. All Scr mice had at least one GTCS per day. In contrast, video-monitoring of Ant-134 animals during the same period detected a total of just 15 seizures (range 0 – 7), an average of 0.6 per day. Thus, Ant-134 mice had 91% fewer GTCS than Scr animals during video monitoring at the end of the first month post-SE.

Statistical analysis of results from weeks 3–4 monitoring: The seizure rate was lower in the Ant-134 group, with an incidence rate ratio of 0.16 (95% CI 0.06 to 0.41, $P < 0.001$). The number of seizure-free days was significantly higher in the Ant-134 group (Wilcoxon Mann-Whitney test: $z = -2.6$, $P = 0.009$). The average Racine score was slightly higher in Ant-134 compared to Scr animals (4.2 vs. 3.1, respectively; Wilcoxon Mann-Whitney test, $P = 0.044$).

Full description of results from weeks 7–8 monitoring: Video-monitoring of Scr animals during the 5 days between weeks 7–8 detected a total of 198 seizures (range 13 – 55), an average of 6.6 seizures per day. All Scr animals had at least one GTCS per day with the exception of one mouse (Scr 5) that had a single seizure-free day (final day of the second monitoring period). In contrast, video-monitoring of Ant-134 animals during the same period detected 53 seizures (range 0 – 26), an average of 2.1 per day. Four out of five mice in the group had seizure-free days. Thus, Ant-134 mice had 73 % fewer GTCS than Scr animals during video monitoring of the second month post-SE.

Statistical analysis of results from weeks 7–8 monitoring: The seizure rate was significantly lower in the Ant-134 group, with an incidence rate ratio of 0.32 (95% CI, 0.12 to 0.87, $P = 0.026$). The number of seizure-free days was significantly higher in Ant-134 animals (Wilcoxon Mann-Whitney test: $P = 0.012$). There was no difference between Scr and Ant-134 groups for Racine scale scores ($P = 0.286$).

A within-group comparison of the total seizure counts at weeks 3–4 versus weeks 7–8 showed that seizure rates did not significantly change for scrambled animals ($P = 0.702$; $n = 6$ per group) or for Ant-134 mice ($P = 0.24$, $n = 5$ per group) (unpaired t test, Welch corrected).