

Supplementary Methods

Electrical post-status epilepticus model (angular bundle stimulation)

Rats (Envigo, Horst, the Netherlands, weighing 300-350 g at the start of the experiment) were housed individually in a controlled environment ($21\pm 1^\circ\text{C}$, $60\pm 15\%$ humidity, 12 h light/dark cycle with lights on between 8:00-20:00; with water and standard laboratory food available ad libitum).

Surgery was carried out to implant electrodes for EEG recordings. Rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine (74 mg/kg; Alfasan, Woerden, the Netherlands) and xylazine (11 mg/kg; Bayer AG, Leverkusen, Germany) and placed in a stereotactic frame. In order to record hippocampal EEG, a pair of bipolar polyimide insulated stainless-steel electrodes (California Fine Wire Company, Grover Beach, CA, USA; CFW 100-192; 70 μm wire diameter, tips 800 μm apart) was implanted into the dentate gyrus (3.9 mm posterior and 1.7 mm lateral to bregma) under electrophysiological control, as previously described.¹ A pair of stimulation electrodes was implanted in the angular bundle (7.2 mm posterior and 4.5 mm lateral to bregma). A stainless-steel screw reference electrode was implanted contralaterally on the skull over the right motor cortex. Three stainless steel screws were inserted into the skull (above the left motor cortex, right somatosensory cortex, and right visual cortex) and served as anchors for dental acrylic. The socket contacts of all electrodes were placed in a multi-channel electrode pedestal that was attached to the skull with dental acrylic (Simplex Rapid Power and Liquid; Kemdent, Swindon, UK). After 2 weeks of recovery, rats were placed in individual cages (40x40x80 cm) and connected to the recording and stimulation set-up.

For EEG recording, the signals from the headstage were fed through commutators and a custom designed filter and amplification unit (BR-20D Breakout Box, NPI electronic GmbH,

Tamm, Germany). Signals were then sampled by a computer-controlled digitized card (NI USB-6255, National Instruments Netherlands, Woerden, the Netherlands) that also controlled the stimulation patterns in a synchronized way. EEG signals were amplified (10x within the headstage), band-pass filtered (0.1–1000 Hz), and then digitized at 2 kHz (16 bit; 30.5 μ V/bit) using an in-house data acquisition software running under MATLAB (MathWorks, Natick, MA, USA). Stimulation was performed by the same NI USB-6255 multifunction I/O device, which was able to deliver biphasic, bipolar voltage stimulation pulses (max 20 V) at microsecond resolution to the selected stimulation channels of the headstage. The above-mentioned data acquisition software was used to evoke field potentials, to deliver the electrical stimulus, and to analyze EEG signals.

To induce SE, rats underwent tetanic stimulation (50 Hz) of the angular bundle in the form of a succession of trains of pulses every 13 seconds. Each train was of 10 s duration and consisted of biphasic pulses (pulse duration 0.5 ms, maximal amplitude 20 V, \sim 700 μ A). Stimulation was stopped when rats displayed sustained forelimb clonus and salivation for several minutes, which usually occurred within 1 hour. Immediately after termination of the stimulation, periodic epileptiform discharges (PEDs) occurred at a frequency of 1-2 Hz, characteristic for SE. Rats had frequent seizures as observed by both their behavior and EEG. To stop SE and yield \sim 50% of animals with spontaneous recurrent seizures (SRSs), rats were injected with diazepam (20 mg/kg i.p.), 2.5 hours after SE onset, as described previously.² Age-matched control animals had electrodes implanted but did not receive electrical stimulation.

The EEG was recorded continuously (24/7) starting 1 week before electrical stimulation (baseline) and until 9 days after stimulation. Animals were reconnected to the EEG set-up 6 weeks after SE and the EEG was recorded continuously for another 2 weeks.

Finally, animals were reconnected 14 weeks after SE, and the EEG was recorded for yet another 2 weeks. Next, animals were sacrificed. All EEG recordings were visually screened, and seizures were confirmed by trained observers. An EEG seizure was defined as a high-amplitude rhythmic discharge that clearly represented an atypical EEG pattern (i.e., repetitive spikes, spike-and-wave discharges, poly-spike-and-wave, or slow-waves; frequency, and amplitude modulation) lasting >10 s.³ Rats were defined as having epilepsy if at least 1 unprovoked EEG seizure was detected.

These procedures were approved by the Animal Ethics Committee of the University of Amsterdam, according to Dutch law.

Electrical post-status epilepticus model (amygdala stimulation)

Rats (Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, weighing 290-350 g at the start of the experiment) were housed individually in a controlled environment (24±1°C, 60±10% humidity, 12 h light/dark cycle with lights on between 7:00-19:00, water and standard laboratory food available ad libitum).

Surgery was carried out to implant the electrodes for EEG recordings. Rats were anesthetized using isoflurane anesthesia (2–2.5% in 100% oxygen) that was preceded by an injection of butorphanol (Butomidor, Richter Pharma AG, Wells, Austria; 0.5 mg/kg, i.p.). Stimulating and recording bipolar electrodes (Plastics One, Roanoke, VA, USA; catalog no. E363-3-2WT-SPC; 125 µm wire diameter) were implanted in the left lateral nucleus of the amygdala 3.6 mm posterior and 5.0 mm lateral to bregma, 6.5 mm ventral to the surface of the brain. A stainless-steel screw electrode (Plastics One, Roanoke, VA, USA) was implanted contralaterally in the skull over the right motor cortex (3.0 mm anterior and 2.0 mm lateral to bregma) as a surface EEG recording electrode. Two stainless-steel screw electrodes were

placed bilaterally over the cerebellum (10.0 mm posterior and 2.0 mm lateral to bregma) as ground and reference electrodes. These screws, together with another one inserted into the skull above the left motor cortex, also served as anchors for dental acrylic. The socket contacts of all electrodes were placed in a multi-channel electrode pedestal (Plastics One, Roanoke, VA, USA; catalog no. MS363) that was attached to the skull with dental acrylic.

Rats were housed in pairs. After 2 weeks of recovery, they were electrically stimulated via the intra-amygdala electrode to evoke SE, as previously described.^{4,5} Stimulation consisted of a 100-ms train of 1-ms biphasic square-wave pulses (400 μ A peak to peak) that were delivered at 60 Hz every 0.5 s for 30 min. If the animal did not exhibit SE, then the stimulation was continued for an additional 10 min. Immediately after termination of the stimulation, PEDs occurred at a frequency of 1-2 Hz, characteristic for SE. To stop SE and yield ~50% of animals with recurrent spontaneous seizures, rats were injected with diazepam (20 mg/kg i.p.), 1.5-2 hours after SE onset, as described previously.² If the first dose of diazepam did not suppress SE, then the animal received subsequent doses of 5 mg/kg diazepam. Age-matched control animals had electrodes implanted but did not receive electrical stimulation.

Rats were monitored by vEEG (Comet EEG, Grass Technologies, West Warwick, RI, USA) using a Panasonic WV-CP480 camera from the time of stimulation until week 5 of the study (first vEEG recording) and from week 9 to week 12 (second vEEG recording). Animals were housed individually during EEG monitoring, but the cage allowed contact with neighboring animals in order to minimize their stress. EEG signals were amplified, filtered and digitized as described in the previous paragraph for AB, using a data acquisition software (TWin EEG and TWin Look, Grass Technologies, West Warwick, RI, USA). All EEG recordings were visually screened, and seizures were confirmed by trained human observers. An EEG seizure was defined as a high frequency (>8 Hz), high amplitude (>2x baseline) discharge

lasting for at least 5 s. ⁶ Rats were defined as having epilepsy if at least 1 unprovoked EEG seizure was detected.

These experiments were approved by the 1st Animal Ethics Committee in Warsaw.

Lithium-pilocarpine post-status epilepticus model

Rats (Envigo Laboratories S.r.l, Udine, Italy, weighing 250-300 g at the start of the experiment) were housed individually in a controlled environment (22-24°C, 55-65% humidity, 12 h light/dark cycle with lights on between 7:00-19:00, water and standard laboratory food available ad libitum).

Surgery was carried out to implant the electrodes for EEG recordings. Rats were secured to a stereotactic apparatus, with the nose bar positioned at -3.3 mm, under ketamine/xylazine (87 and 15 mg/kg i.p.) anesthesia. Anesthesia was then maintained using isoflurane (2% in air; 1.2 ml/min). A polyimide insulated stainless-steel bipolar electrode (Plastics One, Roanoke, VA, USA; 125 µm wire diameter) was implanted into the right dorsal hippocampus (3.9 mm posterior and 1.7 mm lateral from bregma, 3.5 mm ventral to the surface of the brain). A stainless-steel screw electrode was implanted contralaterally on the skull over the right motor cortex as reference electrode. Three stainless steel screws were inserted into the skull (above the left motor cortex, right somatosensory cortex, and right visual cortex) and served as anchors for dental acrylic. The socket contacts of all electrodes were placed in a multi-channel electrode pedestal that was attached to the skull with dental acrylic. Animals received an antibiotic prior to and after surgery (enrofloxacin, 5 mg/kg subcutaneous (s.c.)), to avoid possible infections and an analgesic drug (tramadol, 7 mg/kg s.c. daily) for 3 days after surgery.

After 2 weeks of recovery, rats were administered 127 mg/kg lithium chloride by gastric gavage. After 14-16 h, they received a subcutaneous injection of methyl-scopolamine (1 mg/kg, Sigma-Aldrich, Saint Louis, MO, USA), a muscarinic antagonist incapable of crossing the blood-brain barrier, to reduce the undesirable peripheral effects of pilocarpine. SE was induced 30 min later by administration of pilocarpine (37 mg/kg i.p., Sigma-Aldrich). In case an animal did not enter SE after 30 min, another half dose of pilocarpine was administered (18 mg/kg). Other half doses of pilocarpine (maximum 2 other doses) were administered every 30 min until SE was reached. Animals that did not develop SE within 30 min after the last pilocarpine administration were excluded from the experiment. To yield ~50% of animals with recurrent spontaneous seizures, SE was interrupted 1 h after onset by i.p. administration of a cocktail of drugs: diazepam (10 mg/kg), phenobarbital (25 mg/kg), and scopolamine (1 mg/kg). This cocktail was administered again after 3 h. Finally, after another 4 h, rats received an i.p. administration of diazepam and scopolamine only. To facilitate animal's recovery and reduce the weight loss that follows SE, hydration was promoted by daily s.c. administration of 0.9% saline (1 ml) and palatable food was provided to support feeding for the next 5 days.

⁷ Age-matched control animals had electrodes implanted but were not administered lithium-pilocarpine.

Rats were monitored by vEEG (EEG100C amplifier/MP150 Data Acquisition system, Biopac Systems, Goleta, CA, USA), paired with video cameras to record animal behavior. EEG signals were amplified (5000x), band-pass filtered (0.005 Hz), and then digitized at 0.5 kHz with 12 bit resolution using data acquisition software (Acq-Knowledge 5.0; Biopac). Animals were continuously vEEG recorded for 2 weeks beginning 3 days after SE and for another 2 weeks 2 months after SE. Video monitoring was performed between the two epochs of vEEG. All EEG recordings were visually screened, and seizures were confirmed by trained observers.

An EEG seizure was defined as a high-amplitude rhythmic discharge that clearly represented an atypical EEG pattern (i.e., repetitive spikes, spike-and-wave discharges, poly-spike-and-wave, or slow-waves; frequency, and amplitude modulation) lasting >10 s.³ Rats were defined as having epilepsy if at least 1 unprovoked EEG seizure was detected.

These experiments were approved by the Animal Ethics Committee of the University of Ferrara and the Italian Ministry of Health (authorization DM 371/2016-PR).

Lateral fluid-percussion model of post-traumatic epilepsy (PTE)

The animal cohort has been described in detail previously.⁸ Rats (Envigo Laboratories S.r.l., Udine, Italy, weighting 350–420 g at the start of the experiment) were housed individually in a controlled environment (22±1 °C, 50-60% humidity, 12 h light/dark cycle with lights on between 7:00-19:00, with water and standard laboratory food available ad libitum).

TBI was induced by LFP injury as described previously.⁸ The impact pressure was adjusted to produce severe TBI with an expected acute post-impact mortality of 20–30% within the first 48 h. To diagnose PTE, rats were continuously (24/7) monitored with vEEG for 4 weeks during the 6th post-injury month.⁹ Briefly, at 5 months post-TBI, rats were anesthetized and placed into a stereotactic frame, and three epidural stainless steel screw recording electrodes (E363/20 Plastics One Inc., Roanoke, VA, USA) were implanted into the skull. One electrode was positioned rostral and another caudal to the craniectomy. One electrode was located contralateral to the center of the craniectomy. Two screws inserted over the cerebellum served as ground and reference electrodes, respectively. Electrodes were attached to a plastic pedestal and fixed on the skull with dental acrylate. Video-EEG monitoring was started 1 week after electrode implantation and conducted using the Nervus EEG Recording System (Nervus magnus M40 Amplifier, Taugagreining, Iceland). EEG was

acquired at 2 kHz with high-pass filter of 0.5 Hz. No notch or low-pass filters were used. The time-locked video was recorded using a WVBP330/GE video camera (Panasonic, Japan). A WFL-II/LED15 W infrared light (Videor Technical, GmbH, Germany) was used during the lights-off period to allow for continuous video-monitoring.

Seizures were detected from vEEG recordings by browsing the files visually as well as by using a semi-automatic seizure detection algorithm.⁹ A seizure was defined as a high-amplitude rhythmic discharge with frequency and amplitude modulation that clearly represented an abnormal EEG pattern (repetitive spikes, spike-and-wave discharges, poly-spike-and-wave, or slow-waves) and lasted at least 10 s. Behavioral severity of electrographic seizures was scored according to Racine.¹⁰ Rats were defined as having epilepsy if at least 1 unprovoked electrographic seizure occurred during EEG recording.¹¹

All animal procedures were approved by the Animal Ethics Committee of the Provincial Government of The Southern Finland (license number ESAVI/5146/04.10.07/2014).

Blood Collection

In each animal (all models), tail vein blood was sampled during the latent phase (before the occurrence of spontaneous recurrent seizures) at 2 days (D2) and 9 days (D9) after SE or TBI. Plasma sample preparation was performed as described previously.¹² Rats were briefly anesthetized by isoflurane (5% induction, 1–2% maintenance). A 24G butterfly needle was used to draw blood from the lateral tail vein into 2 BD Microtainer K₂EDTA-tubes (#365975, di-potassium ethylenediaminetetraacetic acid, BD Biosciences, Franklin Lakes, NJ, USA; 500 µl/tube). To obtain plasma, tubes were centrifuged at 1300x g for 10 min at 4°C within 1 h after blood sampling. Plasma aliquots of 50 µL were carefully collected, pipetted into 0.5 ml

Protein LoBind tubes (#022431064, Eppendorf, Hamburg, Germany), and stored between -70 and -80°C.

Following vEEG monitoring (see previous paragraphs) samples were allocated in two groups: those from animals that developed epilepsy (*i.e.*, spontaneous recurrent seizures) and those from animals that did not develop epilepsy. Ultimately, we obtained and analyzed samples from 8 epileptic (Epi) and 8 Non-Epileptic (Non-Epi) animals per each SE model, plus 7 Epi and 9 Non-Epi from TBI animals, plus 4 paired sham-operated controls per model, for a total of 80 animals and 160 plasma samples.

Small-RNA sequencing from plasma

Library preparation and sequencing. Five frozen 50- μ l plasma aliquots (total 250 μ l) per blood sample were sent to Amsterdam, where they were pooled for RNA extraction. Absence of hemolysis in the plasma samples was visually inspected by an experienced researcher before pooling the aliquots and further confirmed by measurement of hemoglobin absorbance at 414 nm using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA). Samples were considered hemolyzed if the A414 value was ≥ 0.25 .¹²

RNA was extracted from 200 μ l plasma using an miRNeasy Mini Kit (#217004, QIAGEN, Hilden, Germany). Small RNA sequencing was conducted by GenomeScan (Leiden, the Netherlands). Small RNA library preparation was performed using the Illumina TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, CA, USA). Briefly, small RNA was isolated from purified RNA by size selection after adapter ligation. The excised product was used for PCR amplification. The quality and yield after sample preparation was measured with a Fragment Analyzer. The size of the resulting products was consistent with the expected size of approximately 150 bp. Single-end sequencing with a read length of 51 nucleotides was

performed on the Illumina HiSeq 4000. To increase read depth each sample was sequenced on two flow-cells giving two replicates for each sample.

Quantification of miRNAs. Read quality of the raw data was assessed using the FastQC v11.8 software (Babraham, Cambridgeshire, UK) and the Trimmomatic v0.36 was used to filter low-quality base calls and any adapter contamination.¹³ Low-quality leading and trailing bases were removed from each read, and a sliding window trimming using a window of 4 and a phred33 score threshold of 15 was used to assess the quality of the read body. Any reads <17 nucleotides were discarded. After quality control, the replicates for each sample were merged and aligned to the rat reference genome (Rno6) using Bowtie2 with the—very-sensitive-local settings. Using the featureCounts program from the Subread package, the number of reads aligned with the known rat miRNAs in accordance with miRBase22 was calculated.^{14, 15}

Quantification of isomiRs. IsomiR expression levels were assessed using isomiRage.¹⁶ The reads that passed quality control were aligned to a custom reference genome that included the sequences of all canonical mature miRNAs in accordance to miRbase v22¹⁴ and all possible isomiR variants. The isomiR variants were generated by including all the possible combinations of one, two, or three bases extending the 5'- or the 3'-end of known miRNA sequences plus the sequences obtained by trimming canonical miRNA from their 3'-end down to a length of 18 bp. Alignment to the custom genome was performed using Bowtie v1.1.2, and no mismatches were allowed.¹⁷ Only the best alignment was reported for each read. The number of reads that aligned to each isomiR were summed to give the unnormalized isomiR expression matrix.

The adopted nomenclature of isomiRs used through this report is the following: (1) an isomiR sequence followed by _miRNA (e.g., rno-miR-145-3p_miRNA), refers to the canonical sequence of the isomiR; (2) an isomiR sequence followed by a combination of nucleotides and

_3prime (e.g., rno-miR-423-3p_A_3prime) refers to the 3' prime modification added to the canonical miRNA sequence; (3) an isomiR sequence followed by a combination of nucleotides and _5prime (e.g., rno-miR-3556a_TAC_5prime) refers to the 5' prime modification added to the canonical miRNA sequence; (4) an isomiR sequence followed by _trimX (e.g. rno-miR-10a-5p_trim4) refers to the trimming of X nucleotides starting from the 3' prime end of the canonical sequence of the miRNA.

Differential expression analysis. The raw miRNA and isomiR count matrixes were passed to R. MiRNAs or isomiRs that had a read count of ≥ 1 in any of the samples in the comparisons were included in the analysis. Normalization and differential expression analyses were performed using the R package DESeq2.¹⁸ The false discovery rate was controlled for using the Benjamini-Hochberg correction, with miRNA and isomiR expression changes with an adjusted p-value < 0.05 being considered differentially expressed.

Identification of classifiers. For each comparison, a stably expressed reference miRNA or isomiR was selected based on a covariance < 0.3 . Next, the expression of each miRNA or isomiR was divided by the selected reference gene to create a ratio. This ratio was used as input for a Receiver Operating Characteristic (ROC) curve analysis and an area under the curve (AUC) was calculated. The resulting AUC showed the ability of a miRNA or isomiR based ratio to distinguish between two groups. The analyses were carried out in R studio, using the package "pROC".¹⁹

To assess the robustness of each miRNA/isomiR classifier, a permutation analysis was performed. For each permutation, samples were randomly assigned to groups and ROC analysis was performed for the classifier of interest. The AUC for each permutation was recorded, and this was repeated 10000 times to produce a test statistic distribution. Finally, the p-value was calculated by observing where the original AUC fell within this distribution.

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