Inflammation and epilepsy: the contribution of astrocytes
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3.1 Expression Pattern of miR-146a in Experimental and Human Temporal Lobe Epilepsy

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Abstract.
Increasing evidence supports the involvement of inflammatory and immune processes in temporal lobe epilepsy (TLE). MicroRNAs (miRNA) represent small regulatory RNA molecules that have been shown to act as negative regulators of gene expression controlling different biological processes, including immune-system homeostasis and function. We investigated the expression and cellular distribution of miRNA-146a (miR-146a) in a rat model of TLE as well as in human TLE. miR-146a analysis in rat hippocampus was performed by polymerase chain reaction and immunocytochemistry at 1 week and 3–4 months after induction of status epilepticus (SE). Prominent upregulation of miR-146a activation was evident at 1 week after SE and persisted in the chronic phase. The miR-146a expression was confirmed to be present in reactive astrocytes. In human TLE with hippocampal sclerosis, increased astroglial expression of miR-146a was observed mainly in regions where neuronal cell loss and reactive gliosis occurred. The increased and persistent expression of miR-146a in reactive astrocytes supports the possible involvement of miRNAs in the modulation of the astroglial inflammatory response occurring in TLE and provides a target for future studies aimed at developing strategies against pro-epileptogenic inflammatory signalling.

Introduction

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Temporal lobe epilepsy (TLE) is a common and often medically intractable neurological disorder. TLE is often associated with hippocampal sclerosis (HS) which is histopathologically characterized by selective neuronal cell loss, gliosis and synaptic reorganization (Thom 2004, Wieser and Epilepsy 2004). Increasing evidence highlights the activation of inflammatory pathways in TLE and suggests that a persistent upregulation of inflammatory gene expression may contribute to the etiopathogenesis of TLE (Vezzani and Granata 2005, Vezzani, et al. 2008).

MicroRNAs (miRNA) represent an evolutionarily conserved class of endogenous ~22-nucleotide non-coding RNAs that act as small regulatory molecules involved in posttranscriptional gene repression (Cao et al., 2006; Tsai & Yu, 2009). Several miRNAs have been found in the human brain, and they are found to play a crucial role in a wide range of biological processes, including the regulation of the innate and adaptive immune response (Pedersen & David, 2008; Sonkoly et al., 2008; Pauley et al., 2009). Unique miRNA expression profiles have been recently reported in injured rat hippocampus after ischaemic stroke, intracerebral haemorrhage and kainic acid-induced acute seizures (Liu et al., 2009). In addition to the brain, miRNAs are also reported to be regulated in blood, suggesting the possible use of blood miRNAs as biomarkers for brain injury (Liu et al., 2009).

Attention has been focused on miRNA-146a (miR-146a), which can be induced by different pro-inflammatory stimuli, such as interleukin (IL)-1β and tumour necrosis factor alpha (TNF-α; Taganov et al., 2006; Sheedy & O’Neill, 2008), and is upregulated in various human pathologies associated with activation of inflammatory responses (Lukiw et al., 2008; Nakasa et al., 2008; Pauley et al., 2008; Sonkoly et al., 2008). Furthermore, miR-146a has been shown to critically modulate innate immunity through regulation of Toll-like receptor (TLR) signalling and cytokine responses (Taganov et al., 2006; Pedersen & David, 2008; Sheedy & O’Neill, 2008). Thus, miRNA, such as miR-146a, may represent a potentially interesting tool for therapeutic intervention in pathological conditions where inflammatory processes are key players in the disease biology. In order to understand the regulation and function of miR-146a in epilepsy, we investigated the dynamics of miR-146a expression during epileptogenesis in a rat model of TLE, as well as the expression and cellular distribution in hippocampal specimens of patients with TLE with HS.
**Material and Methods**

**Experimental animals**

Adult male Sprague Dawley rats (Harlan CPB laboratories, Zeist, The Netherlands) weighing 300-500 grams were used in this study which was approved by the University Animal Welfare committee. The rats were housed individually in a controlled environment (21±1°C; humidity 60%; lights on 08:00 AM - 8:00 PM; food and water available *ad libitum*).

**Electrode implantation and seizure induction**

Rats were anaesthetized with an intramuscular injection of ketamine (57 mg/kg; Alfasan, Cuyk, The Netherlands) and xylazine (9 mg/kg; Bayer AG, Germany), and placed in a stereotactic apparatus. In order to record hippocampal electroencephalograms (EEG), a pair of insulated stainless steel electrodes (70 μm wire diameter, tips were 80 μm apart) were implanted into the left dentate gyrus (DG) under electrophysiological control as previously described (Gorter et al., 2001). A pair of stimulation electrodes was implanted in the angular bundle. Rats underwent tetanic stimulation (50 Hz) of the hippocampus in the form of a succession of trains of pulses every 13 s. Each train had a duration of 10 s and consisted of biphasic pulses (pulse duration 0.5 ms, maximal intensity 500 μA). Stimulation was stopped when the rats displayed sustained forelimb clonus and salivation for minutes, which usually occurred within 1 h. However, stimulation never lasted longer than 90 min. Differential EEG signals were amplified (10 ×) via a FET transistor that connected the headset to a differential amplifier (20 ×; CyberAmp, Axon Instruments, Burlingame, CA, USA), filtered (1–60 Hz), and digitized by a computer. A seizure detection program (Harmonie, Stellate Systems, Montreal, Canada) sampled the incoming signal at a frequency of 200 Hz per channel. EEG recordings were also monitored visually and screened for seizure activity. Behaviour was observed during electrical stimulation and several hours thereafter. Immediately after termination of the stimulation, periodic epileptiform discharges occurred at a frequency of 1–2 Hz and they were accompanied by behavioural and EEG seizures (SE; status epilepticus). Most rats were monitored continuously from the cessation of SE to the time of death (24 h–1 week). The chronic epileptic group (3–4 months after SE) was monitored during and shortly after SE, and during 3–5 days before death in order to determine the frequency of spontaneous seizures. Sham-operated control rats were handled and recorded identically, but did not receive electrical stimulation. None of these rats needed to be reimplanted. Chronic epileptic rats had frequent daily seizures (range, 5–12). The time between the last spontaneous seizure and the time the animals were killed was < 5 h. The experimental protocols followed the European Communities Council directive 86/609/EEC and the Dutch Experiments on Animals Act (1997), and were approved by the Dutch Animal Welfare Committee (DEC).
Rat tissue preparation for RNA isolation

After decapitation, the hippocampus was removed and sliced into smaller parts (200-300 µm). CA3 region was dissected from the slices under a dissection microscope. All material was frozen on dry ice and stored at -80 °C until use. Rats were decapitated in the acute phase (one day after SE, n=3), in the latent period (1 week after SE, n=6; the rats in this group did not exhibit spontaneous seizures) and in the chronic epileptic phase (3-4 months after SE, n=3; only rats that exhibited daily seizures were included in this group). Rats that did not develop SE during stimulation (non-SE rats) were included as controls and were sacrificed 3-4 months after stimulation (n=3).

Rat tissue preparation for immunocytochemistry and in situ hybridization

Rats were disconnected from the EEG recording set-up and deeply anaesthetized with pentobarbital (Nembutal, intraperitoneally, 60 mg/kg). For immunocytochemistry, the animals were perfused through the ascending aorta with 300 mL of 0.37% Na2S solution, followed by 300 mL 4% paraformaldehyde in 0.1 m phosphate buffer, pH 7.4. Thereafter, the brains were removed, incubated for 72 h in 0.3 m EDTA, pH 6.7 (Merck, Amsterdam, The Netherlands) and paraffin embedded. Paraffin-embedded tissue was sectioned at 6 µm, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel GmbH, Brunschweig, Germany) and used for in situ hybridizations and immunocytochemistry. Horizontal sections were analysed at a mid-level of the brain (5300–6100 µm below cortex surface). In situ hybridization was performed on two adjacent serial hippocampal sections from each group (control, n = 6; 24 h, n = 4; 1 week, n = 6; 3–4 months, n = 6). Two additional serial slices were used for the double-staining, combining in situ hybridization with immunocytochemistry (in the same slices) with different antibodies, as described below.

Human material

The human cases included in this study were obtained from the files of the Department of Neuropathology of the Academic Medical Center (AMC, University of Amsterdam) and the VU University Medical Center (VUMC). Ten patients underwent resection of the hippocampus for medically intractable TLE. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. All samples were obtained and used in a manner compliant with the Declaration of Helsinki. Two neuropathologists reviewed all cases independently. In six cases a pathological diagnosis of HS (without extra-hippocampal pathology) was made. The HS specimens include four cases of classical HS (grade 3, mesial temporal sclerosis type 1a) and two cases of severe HS (grade IV; mesial temporal sclerosis type 1b; Wyler et al., 1992; Blumcke et al., 2007). Four non-HS cases, in which a focal lesion (ganglioglioma not involving the hippocampus proper) was identified,
were also included to provide a comparison group to HS cases. Control hippocampal tissue was obtained at autopsy from five patients without history of seizures or other neurological diseases. Brain tissue from a patient with viral encephalitis was also used for in situ hybridization (as positive control for miR-146a expression). All autopsies were performed within 12 h after death. Table 1 summarizes the clinical features of TLE and control cases. Hippocampus from control patients (n = 5) and surgical hippocampal tissue block from patients with HS (n = 6) was snap frozen in liquid nitrogen and stored at −80°C until further use [RNA isolation for reverse transcriptase polymerase chain reaction (RT-PCR)]. Additional hippocampal tissue from the same patients and from four non-HS cases was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6 μm, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel GmbH, Brunswick, Germany) and organosilane-coated slides (SIGMA, St Louis, MO, USA), and two slices were used for in situ hybridizations and immunocytochemistry. Two additional slices were used for the double-staining, combining in situ hybridization with immunocytochemistry (in the same slices) with different antibodies, as described below. Additional immunocytochemistry (single-labelling) was performed for complement factor H (CFH) in both control and HS hippocampal tissue.

**RNA isolation**

For RNA isolation, frozen material was homogenized in Trizol LS Reagent (Invitrogen, Carlsbad, CA, USA). After addition of 200 μg glycogen and 200 μL chloroform, the aqueous phase was isolated using Phase Lock tubes (Eppendorf, Hamburg, Germany). RNA was precipitated with isopropyl alcohol, washed with 75% ethanol and dissolved in water. The concentration and purity of RNA were determined at 260/280 nm using a nanodrop spectrophotometer (Ocean Optics, Dunedin, FL, USA).

**Real-time quantitative PCR analysis (qPCR).** cDNA was generated using Taqman MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions. miRNA (miR-146a and the U6B small nuclear RNA gene, rnu6b) expression was analysed using Taqman microRNA assays (Applied Biosystems), which were run on a Roche Lightcycler 480 (Roche Applied Science, Basel, Switzerland) according to the instructions of the manufacturer. Data analysis was performed with the software provided by the manufacturer.

**Statistical analysis**

Statistical analyses were performed with spss for Windows (spss 11.5, SPSS, Chicago, IL, 182
USA) using two-tailed Student’s t-test, and to assess differences between more than two groups a non-parametric Kruskal–Wallis test followed by Mann–Whitney U-test were used. A value of \( P < 0.05 \) was considered significant.

**In situ hybridization.** In situ hybridization for miR-146a was performed using a 5’ fluorescein-labelled 19mer antisense oligonucleotide containing locked nucleic acid and 2'OME RNA moieties (FAM – AacCcaTggAauTcaGuuCucA, capitals indicate LNA, lower case indicates 2'OME RNA). The oligonucleotides were synthesized by Ribotask ApS, Odense, Denmark. The hybridizations were done on 6-μm sections of paraffin-embedded materials described previously (Budde et al., 2008). The hybridization signal was detected using a rabbit polyclonal anti-fluorescein/Oregon green antibody (A21253, Molecular Probes, Invitrogen) and a horseradish peroxidase-labelled goat anti-rabbit polyclonal antibody (P0448 Dako, Glostrup Denmark) as secondary antibody. Signal was detected with chromogens 3-amino-9-ethyl carbazole (St Louis, MO, USA) or Vector NovaRed (Vector Laboratories, Burlingame, CA, USA), and the nuclei were stained with haematoxylin. Slides were sealed with glycerol-gelatin (St Louis, MO, USA). As control for non-specific binding, other similarly modified oligonucleotides were used. These probes were specific for other human transcripts (miR-338, MIMAT0004701; miR-218, MIMAT0000275; miR-204, MIMAT0000265; miR-134, MIMAT0000447). These oligonucleotides showed different staining patterns (no expression in glial cells). Additionally negative control assays were performed without probes and without

<table>
<thead>
<tr>
<th>Total or mean (Range or percentage)</th>
<th>Male/female</th>
<th>HS (n = 6)</th>
<th>Non-HS (n = 4)</th>
<th>Control (n = 5)</th>
</tr>
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<tbody>
<tr>
<td>Mean age at surgery (Mohaupt, et al.)</td>
<td>31.5 (19-50)</td>
<td>35.1 (21-43)</td>
<td>44.3 (26 –52)</td>
<td></td>
</tr>
<tr>
<td>Seizure type</td>
<td>CPS (100%); SGS (17%)</td>
<td>CPS (100%); SGS (17%)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Seizure frequency (CPS/months)</td>
<td>10.6 (3-50)</td>
<td>10.1 (3-30)</td>
<td>NA</td>
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<tr>
<td>Duration of epilepsy (Mohaupt, Karas, Babiychuk, Sanchez-Freire, Monastyrskaya, Iyer, Hoppeler, Breil and Draeger)</td>
<td>11.8 (9 – 20)</td>
<td>12.8 (9 – 18)</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

HS, hippocampal sclerosis; CPS, complex partial seizures; SGS, secondary generalized seizures; HS, hippocampal sclerosis; NA: not applicable.
primary antibody (sections were blank). For the double-staining, combining immunocytochemistry with in situ hybridization, sections were first processed for immunocytochemistry as previously described (Aronica et al., 2001a, 2003) with glial fibrillary acidic protein (GFAP; polyclonal rabbit; DAKO, Glostrup, Denmark; 1:4000), neuronal nuclear protein (NeuN; mouse clone MAB377; Chemicon, Temecula, CA, USA; 1:2000), HLA-DR [anti-human leukocyte antigen (HLA)-DP, DQ, DR (mouse clone CR3/43); DAKO, Glostrup, Denmark; 1:400], CFH (polyclonal goat; Quidel, San Diego, CA, USA; 1:100) or the biotinylated lectin Ricinus Communis Agglutinin I (RCA 120; Vector Laboratories, Burlingame, CA, USA; 1:500, for the visualization of microglial cells on rat tissue), using Fast Blue B salt (St Louis, MO, USA) or Vector Blue substrate (Vector Laboratories) as chromogen. After washing, sections were processed for in situ hybridization as described above. Images were captured with an Olympus microscope (BX41, Tokyo, Japan) equipped with a digital camera (DFC500, Leica Microsystems-Switzerland, Heerbrugg, Switzerland).

To analyse the percentage of double-labelled cells positive for miR-146a and GFAP, or for the microglia marker (HLA-DR, human; lectin, rat), digital photomicrographs were obtained from five hippocampal samples. Images of three representative fields (CA3 and DG) per section were collected (Leica DM5000B). Images were analysed with a Nuance VIS-FL Multispectral Imaging System (Cambridge Research Instrumentation, Woburn, MA, USA). Spectra were acquired from 460–660 nm at 10-nm intervals, and Nuance software (version 2.4) was used for analysis, as previously described (Boer et al., 2008; van der Loos, 2008). The total number of cells stained with miR-146a and GFAP (or HLA-DR or lectin), as well as the number of cells double-labelled, were counted visually and percentages were calculated (expressed as mean ± SEM) of cells co-expressing miR-146a and GFAP (or HLA-DR or lectin) in two regions

![Figure 1. Quantitative real-time PCR of miR-146a expression in rat and human hippocampus](image)

(A) Fold change (compared with expression in control rats, n = 6) in CA3 at 24 h (n = 4), 1 week (n = 6) and 3–4 months (n = 6) after status epilepticus (SE) or after stimulation (non-SE; n = 4). (B) Expression levels of miR-146a in human (autopsy) control hippocampus (n = 5) and human hippocampal sclerosis specimens (HS; n = 6). In both rat and human tissue the miR-146a expression was normalized to that of the U6B small nuclear RNA gene (rnu6b). The error bars represent SEM; statistical significance: *P < 0.05 compared with control.

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of prominent gliosis (CA3 and DG of rat, at 1 week post-SE, and of human hippocampus). Sections incubated without the primary Ab or with pre-immune serum were blank, and when processed for in situ hybridization showed only the in situ hybridization signal.

RESULTS

miR-146a expression by real time qPCR
miR-146a expression was studied using qPCR in both rat and human hippocampal tissue. miR-146a expression in rat CA3 region was significantly increased at 1 week (latent phase) and 3–4 months (chronic phase) post-SE, compared with non-SE values (Fig. 1A). Increased miR-146a expression was also observed in human TLE HS specimens compared with control hippocampus (Fig. 1B). In both rat and human tissue the miR-146a expression was normalized to that of the U6B small nuclear RNA gene (rnu6b).

miR-146a expression by in situ hybridization in rat hippocampus
To determine the temporal–spatial expression and cellular distribution of miR-146a, we performed in situ hybridization using LNA- and 2’OMe RNA-modified oligonucleotides in tissue samples of control rats and rats that were killed at different time points after SE (1 day, 1 week and 3–4 months post-SE). In control hippocampus miR-146a was confined to neuronal cells, including pyramidal cells of CA1 and CA3 regions, as well as granule cells and hilar neurons of the DG (Fig. 2A, C, E and G). No detectable staining was observed in resting glial cells. At 1 day post-SE, miR-146a showed a similar pattern as control hippocampus, with predominant neuronal staining; occasionally expression was observed in cells with glial appearance in the areas of neuronal damage (CA1, CA3, hilus; not shown). At 1 week post-SE (Fig. 2B, D, F and H–J), prominent upregulation of miR-146a expression was detected within the different hippocampal regions in glial cells. Strong and diffuse glial miR-146a expression was particularly observed in the inner molecular layer of the DG and in the hilar region (Fig. 2I). Pyramidal neurons of CA1 and CA3 regions and granule cells of DG also displayed strong miR-146a expression. In the chronic phase (3–4 months post-SE) the hippocampus showed a pattern similar to that observed at 1 week post-SE, with both neuronal and glial expression, which was mainly localized in regions of prominent gliosis, such as the hilar region (Fig. 2J). Co-localization studies indicated that miR-146a was induced in glial cells in this region and that expression was confined to astrocytes, whereas no detectable expression was observed in lectin-positive cells of the microglial/macrophage lineage (Fig. 2J and inserts a/b). The percentage of cells positive for miR-146a and co-expressing GFAP was quantified in both CA3 and DG at 1 week post SE (76 ± 2, CA3; 70 ± 4, DG). No co-localization with lectin was observed in both regions.
Figure 2. In situ hybridization analysis of miR-146a expression in hippocampal tissue of control rats and after induction of SE. (A, C, E, G) Control hippocampus showing neuronal miR-146a expression in the different hippocampal subfields, including pyramidal neurons of CA3 (C; insert in C) and CA1 (E; insert in E) regions, granule cells of the dentate gyrus (DG; G) and hilar neurons (G; insert in G: high-magnification of hilar neurons). gcl, granule cell layer. (B, D, F, H) Hippocampus 1 week post-SE showing increased miR-146a expression within the different hippocampal regions, including CA3 (D; insert in D: high-magnification of CA3), CA1 (F; insert in F: high-magnification of CA1) and DG (H). miR-146a expression was observed in both neuronal and glial cells; arrows in D, F (and insert in D) indicate positive pyramidal neurons of CA3 and CA1; positive glial cells (arrowheads in D, F and insert in D) were particularly abundant in regions of prominent gliosis (CA1, CA3 and the inner molecular layer, iml, of the DG). Insert in (H) shows a high-magnification of positive glial cells in the DG (iml). (I and J) In situ hybridization analysis of miR-146a expression in the hilar region of the hippocampus 1 week (I) and 3–4 months post-SE (J) showing increased expression in glial cells (arrows; insert in I). Sections are counterstained with haematoxylin. Inserts in (J): in situ hybridization and immunohistochemistry analysis showing in (a) absence of miR-146a (red) expression in lectin-positive microglial cells (blue) and in (b) colocalization with the astroglial marker glial fibrillary acidic protein (GFAP; purple) in astrocytes. Scale bars: 1250 μm (A and B); 70 μm (C–J); 35 μm (inserts in C, D, F and G); 20 μm (insert in E); 17 μm (inserts in H and I); 11 μm (insert in J). LT, long-term, 3–4 months after SE.
Figure 3. In situ hybridization of miR-146a expression in the hippocampus of control and patients with TLE with hippocampal sclerosis (HS). (A and C) Control hippocampus showing miR-146a expression in pyramidal neurons of CA1 (A), in granule cells of the dentate gyrus (DG; C) and hilar neurons (C and insert in C). gcl, granule cell layer. (B, D–F) HS showing increased miR-146a expression in CA1 (B) and DG (D–F). (B) miR-146a expression is observed in residual pyramidal neurons (arrows in B, CA1), as well as in glial cells (arrowheads). (D) Increased miR-146a expression is observed in the inner molecular layer (iml) of the DG. (E) High-magnification of DG with positive neuronal (gcl) and glial cells (iml; arrows). (F) High-magnification of the hilar region of the DG showing positive glial cells (arrows; insert). (G–J) Double in situ hybridization and immunohistochemistry analysis in HS, with microglial [human leukocyte antigen (HLA)-DR, G–H] and astroglial markers [glial fibrillary acidic protein (GFAP), I], and with complement factor H (CFH, J). (G) miR-146a expression in residual CA3 pyramidal neurons (red), but absence of expression in HLA-DR-positive microglial cells (blue). (H) (hilus) miR-146a expression in a glial cell (red; HLA-DR-negative), but the absence of expression in a HLA-DR-positive microglial cell (blue). (I) Co-localization (purple) of miR-146a in a GFAP-positive astrocyte. (J) miR-146a expression (red) in glial cells expressing CFH (blue). Scale bars: 80 μm (A and B, and E and F); 120 μm (C and D); 40 μm (G); 30 μm (H); 15 μm (I); 20 μm (J); 40 μm (insert in F).
miR-146a expression by in situ hybridization in hippocampal sclerosis

The cellular distribution of miR-146a in human hippocampus was investigated using in situ hybridization. Differences in the expression level, as well as in the cell-specific distribution, were found in specimens from patients with HS (Fig. 3). In control hippocampus, we observed miR-146a expression in neuronal cells, including pyramidal cells of CA1 and CA3 regions, as well as granule cells and hilar neurons of the DG (Fig. 3A, C and E). No detectable staining was observed in resting glial cells. In all the HS specimens examined, miR-146a expression was increased in the different subfields of the hippocampus; abundant miR-146a-positive glial cells with typical astroglia morphology were observed in the areas of prominent gliosis (Fig. 3B, D and F). Pyramidal neurons of CA1–CA4 regions and granule cells of DG also displayed miR-146a expression. Non-sclerotic hippocampus (non-HS) displayed a pattern of expression similar to that observed in control autopsy hippocampus. Double-labelling confirmed miR-146a expression in GFAP-positive reactive astrocytes, whereas no detectable expression was observed in HLA-DR-positive cells of the microglial/macrophage lineage (Fig. 3G–I). The percentage of cells positive for miR-146a and co-expressing GFAP was quantified in both CA3 and DG in HS specimens (76 ± 5, CA3; 78 ± 5, DG). No co-localization was observed with HLA-DR in both regions.

Similar cellular distribution with miR-146a expression, confined to neurons and reactive astrocytes, was also observed in tissue specimens from a patient with viral encephalitis and prominent gliosis (not shown). Because upregulation of miR-146a has been shown to be associated with a downregulation of CFH in Alzheimer’s disease (AD) brain tissue (Lukiw et al., 2008), CFH expression was evaluated with double-labelling in miR-146a-positive cells. CFH was expressed in miR-146a-positive cells with glial morphology (Fig. 3J). In control hippocampus only neuronal expression was observed (not shown).

DISCUSSION

The miR-146a has been recently indentified as a potentially endogenous regulator of TLR and cytokine receptor signalling, suggesting a link between miRNAs and human inflammatory diseases (Taganov et al., 2006; Pedersen & David, 2008; Sheedy & O’Neill, 2008; Otaegui et al., 2009). An upregulation of miR-146a has also been shown in human AD brain, suggesting that the misregulation of specific miRNAs could contribute to the inflammatory pathology observed in AD brain (Lukiw et al., 2008). Until now, however, the expression of miR-146a at the cellular level in both rat and human hippocampus has not been previously assessed. The present study, which reveals that miR-146a is highly expressed in the hippocampus, is the first to focus on the cellular distribution of miRNA in a rat model of TLE, as well as in hippocampal tissue from patients with TLE.
miR-146a expression during epileptogenesis in the rat.

We detected an upregulation of miR-146a during epileptogenesis and in the chronic epileptic phase in the rat hippocampus of the TLE model. The results of both qPCR and in situ hybridization analyses indicated a prominent expression at 1 week after SE, which corresponds to the time of maximal astroglial and microglial activation and upregulation of several other genes involved in the immune response (Aronica et al., 2000, 2001b; Hendriksen et al., 2001; Gorter et al., 2006). miR-146a was still significantly upregulated in the chronic phase. In situ hybridization analysis of miR-146a in rat hippocampus showed expression in both neuronal and glial cells. Double-labelling experiments showed miR-146 expression in astrocytes. Previous experimental evidence in rodent models of seizures has demonstrated that reactive glial cells express high levels of pro-inflammatory cytokines, such as IL-1β and TNF-α (for review, see Vezzani et al., 2008). The analysis of the spatio-temporal expression of IL-1β in TLE models indicates that astrocytes mostly represent a source of this cytokine during epileptogenesis, as well as in the chronic epileptic tissue, (Vezzani et al., 2008). Because IL-1β represents a major pro-inflammatory cytokine involved in the induction of miR-146a (Taganov et al., 2006; Nakasa et al., 2008; Sheedy & O’Neill, 2008), it is possible that expression of miR-146a in astrocytes may represent an attempt to modulate the inflammatory response triggered by this cytokine. Accordingly, recent studies identify miR-146a as a key regulator in a feedback system whereby induction of nuclear factor kappa-B (NFkB) through a myeloid differentiation factor 88 (MyD88)-dependent pathway may upregulate the miR-146a, which in turn could downregulate the levels of two key adapter molecules, IL-1RI-associated protein kinases-1 (IRAK1) and -2, and TNF receptor-associated factor 6 (TRAF6) downstream of TLR and cytokine receptors, reducing the activity of this inflammatory pathway (Taganov et al., 2006; Hou et al., 2009). These observations are particularly interesting considering the known proconvulsant action of IL-1β mediated by the IL-1 receptor type 1, as well as the recently reported role of TLR-signalling pathways in epilepsy (Vezzani et al., 2008; Maroso et al., 2009), and suggest that miR-146a induction could function in fine-tuning the response to cytokines in TLE during epileptogenesis.

miR-146a expression in TLE patients with HS.

The upregulation of miR-146a observed in the chronic epileptic phase in the post-SE model of TLE was confirmed in human HS specimens of patients undergoing surgery for pharmacologically refractory TLE. In situ hybridization analysis of miR-146a in human control hippocampus and HS specimens demonstrated expression in neuronal cells. In contrast (as observed in the post-SE rat hippocampus), the expression in glial cells was detected only in tissue of patients with HS, particularly in regions with prominent gliosis. Expression of
the miR-146a was observed in neurons and in reactive astrocytes in HS tissue. Neurons constitute an additional source of pro-inflammatory cytokines (including IL-1β), potentially contributing to the inflammatory pathology observed in TLE (Ravizza et al., 2008). Thus, the neuronal expression of miR-146a may also represent an attempt to regulate this inflammatory pathway. A physiological mechanism of defence against activation of inflammatory pathways during epileptogenesis is represented by induction of inhibitory factors, such as CFH (Boon et al., 2009), an important repressor of inflammatory signalling. This factor inhibits excessive activation of the complement cascade, which is prominently activated in both experimental and human TLE (Aronica et al., 2007). Interestingly, CFH has been identified as a target of miR-146a. For instance, in AD brains, upregulation of miR-146a has been linked to downregulation of CFH (Lukiw et al., 2008). Thus, the possible repression of the bioavailability of inhibitory molecules, such as CFH, has to be taken into account with respect to the interpretation of the ultimate effects of changes in the expression of miR-146a. However, CFH gene expression has been shown to be induced during epileptogenesis in the post-SE model (Aronica et al., 2007). In addition, expression of CFH protein was observed in miR-146a-positive glial cells in the chronic epileptic phase in HS specimens. In conclusion, our observations demonstrate an upregulation of miR-146a with prominent expression in astrocytes during epileptogenesis in a rat model of TLE as well as in human TLE. Understanding the role of miR-146a epilepsy-associated pathologies may be relevant for the development of new therapeutic strategies whereby glial function is targeted. Whether a misregulation of specific miRNAs, such as miR-146a, could contribute to epileptogenesis remains to be explored. Overexpression and loss of function studies in vitro, as well as in animal models, will help to further identify the exact role of miR-146a in the modulation of the inflammatory response and associated pathogenic signalling in epilepsy.

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REFERENCES


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