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MicroRNA and epilepsy: Profiling, functions and potential clinical applications

David C. Henshall

Abstract

Purpose of Review—To provide a synthesis of recent profiling studies investigating microRNA changes in experimental and human epilepsy and outline mechanistic, therapeutic and diagnostic potentials of this research area for clinical practice.

Recent Findings—A series of studies in experimental and human epilepsy have undertaken large-scale expression profiling of microRNAs, key regulatory molecules in cells controlling protein levels. Levels of over 100 different microRNAs were found to either increase or decrease in the hippocampus, of which more than 20 were identified in more than one study, including higher levels of miR-23a, miR-34a, miR-132 and miR-146a. Altered levels of enzymes involved in microRNA biogenesis and function, including Dicer and Argonaute 2, have also been found in epileptic brain tissue. Functional studies using oligonucleotide-based inhibitors support roles for microRNAs in the control of cell death, synaptic structure, inflammation and the immune response. Finally, data show brain injuries that precipitate epilepsy generate unique microRNA profiles in biofluids.

Summary—MicroRNA represents a potentially important mechanism controlling protein levels in epilepsy. As such, microRNAs might be targeted to prevent or disrupt epilepsy as well as serve as diagnostic biomarkers of epileptogenesis.

Keywords

Epileptogenesis; Hippocampus; Seizure; Status epilepticus; Temporal lobe epilepsy

Introduction

Epilepsy is a chronic neurologic disorder characterized by recurring seizures which result from abnormal and synchronous firing of neurons in the brain. Improved understanding of the mechanisms involved in the transformation of a normal brain to one capable of producing recurring seizures, and maintenance of the epileptic state thereafter, is essential if we are to identify the pathways involved and develop novel treatments or a cure. The use of gene expression profiling, beginning in the early 2000s, gave researchers unrivalled insight into the scale of gene expression changes in the epileptic brain [1]. However, a previously unrecognized layer of control – post-transcriptional interference with messenger RNA

Correspondence: David C. Henshall, Ph.D. Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, 123 St Stephen's Green, Dublin 2, IRELAND, Ph: +353 1 402 8629, Fx: +353 1 402 2447, dhenshall@rcsi.ie.

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(mRNA) translation by microRNAs (miRNA) – is now understood to be a major determinant of protein levels in cells.

The present review focuses on recent profiling work that has defined the “miRNAome” of epilepsy and some of the probable targets, including genes regulating neuronal microstructure, cell death, gliosis and inflammation – information that enables us to better understand the pathogenesis of epilepsy. As researchers begin to manipulate miRNAs *in vivo* and uncover the emerging potential of biofluid-detected miRNAs as biomarkers, miRNAs are increasingly recognised as an important new focus of research into molecular diagnostics, pathophysiology and treatment of epilepsy.

MicroRNAs: Biogenesis and mechanism of action

MiRNAs are an endogenous class of small non-coding RNA that function as an additional layer of gene expression control, regulating protein levels in cells [2] (Figure 1A). For comprehensive reviews on the biogenesis, mechanism and functions of miRNAs, the reader is referred elsewhere [3-5]. Briefly, transcription of miRNAs results in a primary transcript which is then processed via sequential cleavage by the RNases Drosha and Dicer to form the mature miRNA (~19-25 nt) (Figure 1B) [6]. To function, the mature miRNA is uploaded to an RNA induced silencing complex (RISC) which contains the protein Argonaute 2 (Ago2). The RISC-loaded miRNA then forms sequence-specific base-pairing over a minimum 7-8 nt “seed” region of the mRNA, usually within the 3' untranslated region (UTR) (Figure 1C) [7]. This leads to mRNA degradation or translational inhibition thereby reducing protein levels of the target by anywhere from 2 to 10 fold [8]. In rare cases up-regulation of translation has been reported [9].

Suitability of miRNAs as targets in epilepsy

Most previous efforts to disrupt epileptogenesis based on targeting single (protein-coding) genes has failed in pre-clinical trials [1]. MiRNAs are attractive alternatives for several reasons. Individual miRNAs can have several targets within the same cell and impact more than one pathway. Indeed, over 60% of all proteins are predicted targets of miRNAs [10]. This establishes these small molecules as “meta-controllers” of gene expression in the brain [11]. Second, the processes under miRNA control include several central to epileptogenesis, including neuronal death, gliosis, inflammation and neuronal microstructure [12-13]. Third, the field of RNA-based therapeutics has advanced dramatically in recent years with many innovative medicines now in clinical trials [14-15]. Together, miRNAs offer potent new approaches to interrupt pathogenic pathways not previously possible.

Changes to miRNAs following epileptogenic insults

Symptomatic (acquired) epilepsy is often a result of an earlier brain insult, including prolonged seizure, stroke, infection and trauma. Data from expression profiling studies reveal that acute brain injuries generate unique miRNA responses [16]. However, a sub-set of miRNAs may also be conserved between different neurologic insults [17] (Figure 2a). The implication is that miRNAs influence patho-mechanisms that are shared between epileptogenic injuries. Are there also common miRNA profiles in established epilepsy?

Converging on the conserved: a sub-set of “epilepsy” miRNAs?

Four studies have profiled miRNA expression in experimental epilepsy, each using status epilepticus as the epileptogenic trigger and focusing on the period ~2 months after the initial insult when animals were actively seizing (Table 1). Altogether, changes to over 100 different miRNAs were identified [18-21], providing compelling evidence that epilepsy is associated with widespread changes to miRNA expression. Comparing the number of miRNAs regulated with those called “present”, it appears just under 20% of brain-expressed miRNAs are altered in epilepsy. This is a substantial number and even without proteomics data we can speculate that a significant portion of proteins in the injured tissue would be affected by these miRNA changes.

Among regulated miRNAs, 14 are increased in at least two studies and 9 decreased (Table 1). This represents a possible “core” miRNA response in epilepsy. Some of these appear novel to epilepsy and were not found after acute neurologic injury, including status epilepticus [17]. Interestingly, more than half of the commonly up-regulated miRNAs were found in different models and/or brain regions, indicating the conserved miRNAs are not simply those identified between the most similar models or regions sampled; they are central to epilepsy rather than model-related artefacts. Overall, there appears more consistency among up-regulated miRNAs compared to down-regulated; 3 of the 14 consistently up-regulated miRNAs were detected in at least 3 profiling studies. A number of the studies which focused just on individual miRNAs also detected changes to some of the same miRNAs. This includes miR-21, miR-132, miR-146a and miR-155 [22-25]. Up-regulation of miR-134 was detected in one profiling study [18] and two other studies [25-26••]. Taken together, these findings reveal a core group of miRNAs with possible important roles in the establishment or maintenance of the epileptic state.

Factors underlying the conserved miRNA responses in epilepsy

What factors underlie such conserved miRNA changes in epilepsy? Several miRNAs, including miR-21 and miR-132, and 7 of the 9 commonly down-regulated miRNAs also change within 48 h of status epilepticus [13•]. These may reflect permanent changes induced by the initial status epilepticus or those responsive to ictal activity. Since cell death and gliosis are common to each of the models profiled, levels of miRNAs constitutively expressed in neurons are likely to be lower while glia-expressed miRNAs may be higher. However, the evidence for such a simple interpretation of the data is weak. Few of the conserved miRNAs in epilepsy are those enriched in either neurons or glia [27]. Instead, common aspects of the pathogenesis of epilepsy and/or the impact of recurring seizures may converge on a relatively limited number of miRNA-controlled processes. Results may also reflect the actions of specific transcription factors controlling miRNAs, which are increasingly understood [28].

Comparison of experimental to human epilepsy

Profiling studies were recently reported using resected hippocampus from temporal lobe epilepsy (TLE) patients [29•-30] along with work on individual miRNAs in human epilepsy [22-26••]. Significant changes were found for just under 100 different miRNAs and a number of the same miRNAs changed expression in the same direction as in the experimental work.

This includes up-regulation of miR-146a and miR-132, as well as miR-9 and miR-99a [18], miR-27a and miR-203 [19] and miR-135a [21]. From those down-regulated, experimental models also detected miR-30a/b, miR-138, miR-324 and miR-330 [20] and miR-187 [21]. Nevertheless, this represents only ~20% of the total regulated in human epilepsy. For the remainder, it is possible that chronic drug treatment, etiology and technical factors are introducing variance in miRNA profiles between experimental models and patient samples. There is a risk that such disconnect could later result in failure to translate from pre-clinical to clinical. Additional studies, particular in human material are required and further cross-comparisons needed to resolve inter-species discrepancies. Profiling studies using mouse models are also needed to validate the rat work.

Targets and pathways under miRNA control in epilepsy

Generally, mRNA transcripts with long 3'UTRs, which include many involved with development and cell differentiation, are under more potent control by miRNAs than those with short 3'UTRs. The implication is that some, but not all, pathogenic processes in epilepsy are under miRNA control. Several of the profiling studies have explored pathways potentially affected by the miRNA changes. These include apoptosis, synaptic functions, inflammation and the immune response (Table 1). Thus, major pathways linked to epileptogenesis and chronic epilepsy are predicted to be under miRNA control [1,13,31].

Determining the functions and significance of epilepsy-associated miRNAs

A number of options are available to manipulate miRNA *in vivo* and learn whether they are important for seizures or the pathophysiology of epilepsy. The main techniques include use of genetic and oligonucleotide-based approaches [16,32]. Levels of specific miRNAs can be increased using miRNA “mimics” or decreased using antagomirs, which work by binding to miRNAs and inhibiting their function [16]. Transgenic and knockout mice also exist for a few miRNAs and studies of mice lacking Dicer show that failure of a functioning miRNA system contributes to neurodegeneration and seizures [33]. This is noteworthy since loss of Dicer was reported in both experimental epilepsy and a sub-set of TLE patients with hippocampal sclerosis [30]. Finally, mice lacking or over-expressing individual miRNAs have also been developed, including for some of the conserved miRNAs in epilepsy [34-35]. We await assessment of such mice to support the *in vivo* contribution of these miRNAs to epilepsy. The number of experimentally validated targets of miRNAs in the brain remains limited and databases that integrate miRNA expression and their targets in specific cells are not yet complete.

The only functional studies in epilepsy have used antagomirs, which have been deployed against miR-34a [19,36], miR-132 [37], miR-134 [26••] and miR-184 [38]. Pre-treatment of rodents with antagomirs targeting these miRNAs was reported to reduce (miR-34a, miR-132, miR-134) as well as increase (miR-184) neuronal death caused by status epilepticus. These studies did not pursue the mechanism by which antagomirs produced the effects, which is presumably via recovery of certain proteins otherwise repressed by the miRNA. While it has been demonstrated that antagomirs increase protein levels of previously de-repressed targets [26••], no studies have proven the antagomir effect is dependent on one or more specific targets of the miRNAs.

Arguably, the most complete evidence for miRNA involvement in epilepsy comes from studies of miR-134 [26••]. Expression of miR-134 was found to be increased in experimental and human epilepsy and silencing miR-134 resulted in a strong reduction in evoked and spontaneous seizures and long-lasting neuroprotection [26••]. One of the best understood targets of miR-134 is Limk1, a protein involved in the control of dendritic structure [39]. Dendritic spines are critical points of contact for excitatory transmission in the CNS and targeting the miRNA also altered spine density [26••]. The study also confirmed RISC-loading of the miRNA and recovery of protein targets in antagomir-treated animals. It is expected that further functional studies will reveal additional miRNAs central to the pathogenesis of epilepsy which might represent future therapeutic targets.

For the other conserved miRNAs in epilepsy we do not have functional data. Astrocyte-expressed miR-146a is thought to negatively regulate inflammation by targeting members of the IRAK and TRAF families [40]. The functions of miR-23a, which was up-regulated in all profiling studies on experimental epilepsy, include control of apoptosis, inflammation and transcription factors involved in differentiation (up to date listings of well-validated miRNA targets can be found on miRTarBase). For miR-34a, roles have been proposed in promoting apoptosis and silencing miR-34a during status epilepticus resulted in protection of the hippocampus, perhaps through promoting anti-apoptotic signalling [19].

Improving successful identification of causally-important miRNAs in epilepsy

Although we now have reasonably comprehensive descriptions of miRNA expression changes in epilepsy, studies tend to rely on bioinformatic tools to infer which targets/ pathways are impacted. Without experimental validation, such predictions are likely error-prone. Indeed, up- and down-regulated miRNAs may target the same genes in epilepsy [21]; without protein data the outcome is not possible to predict. The recent study by Bot et al. offered an improved approach, combining mRNA data with miRNA to support functional targeting [20]. Ago2 pull-down studies can provide critical direct evidence that a miRNA is “functional”. Indeed, levels of a miRNA can increase after seizures without a change in the RISC content [37]. Identification of the mRNA within the RISC and quantitative proteomics will also generate more meaningful data on the most important miRNAs. Most profiling studies use a fold-change cut-off but this may not be appropriate for very abundant miRNAs where this would potentially miss changes in the order of thousands of copies per cell. Notably, the most abundant miRNAs in the hippocampus, such as members of the let-7 family, miR-124 and miR-9 [41-42] often do not appear in the miRNA lists in epilepsy studies (see Table 1) and researchers should be encouraged to re-mine their data with this in mind.

Analysis of miRNAs specific to the “latent” period is essential to identify those involved in epileptogenesis. Gorter and colleagues recently found distinct miRNA profiles during this period implying that a set of epileptogenesis-associated miRNAs likely exists that could form the basis of future targeting efforts [21]. The predicted functions of the targets of these include control of axon guidance and several signalling pathways previously implicated in epileptogenesis [21]. Analysis of time points 24 h or more after status epilepticus will also likely identify miRNAs involved in epileptogenesis [13•]. Since antagomirs have already

proved effective for blocking miRNAs *in vivo*, efforts might be directed towards targeting the novel up-regulated miRNAs from these datasets.

Clinical translation: Therapeutics and miRNAs as biomarkers of epilepsy

There is growing interest in targeting miRNAs in a range of diseases, including CNS disorders [16•]. Blocking liver-expressed miR-122 is effective in combating hepatitis C infection and miravirsin, an antagomir targeting miR-122 and the first to enter clinical trials, was found to be safe and effective in humans [43•]. There will of course be major challenges with targeting and delivery of miRNAs for CNS disorders [14-15]. Foremost, antagomirs are too large to cross an intact blood brain barrier. We need more data on the effects they produce in animals and we may need a means to target miRNA manipulations to specific cell types to avoid off-target effects. If these problems can be overcome, miRNA-based treatments could be deployed as anti-epileptogenic or disease-modifying treatments.

A second potential clinical application of miRNA research is to use biofluid profiles as molecular diagnostics [44-45]. For example, using injury-induced patterns of miRNA expression to support diagnoses, prognosis and inform optimal treatment. Because of the chemistry of miRNAs and their manner of transport in biofluids – enclosed in microparticles and complexed to Ago2 – they are stable and can be reliably detected in serum or plasma. Data show that blood levels of certain miRNAs are altered following epilepsy-precipitating injuries, including status epilepticus [17,21] as well stroke, intracerebral hemorrhage and trauma [17,46-47]. Liu and colleagues identified 5 up-regulated and 7 down-regulated miRNAs common to different neurologic insults in blood at 24 h [17] (Figure 2b). One of these, miR-152, is among the conserved miRNA in epilepsy (see Table 1). The recent study by Gorter and colleagues also identified miRNAs in plasma whose levels were altered during either epileptogenesis or in chronic epilepsy [21]. These discoveries, if validated in humans, could lead the way to simple diagnostic tests that could support patient treatment decisions and prognosis.

Conclusions

There is growing evidence supporting miRNA changes in the pathophysiology of epilepsy. The recent profiling work provides important new data toward a complete description of the molecular mechanisms involved in disease pathogenesis. A sub-set of epilepsy miRNA are emerging and as researchers turn their attention to establishing the *in vivo* functions of these miRNAs we may obtain a range of novel treatment targets. The pace of discovery seems set to continue; three new papers on miRNA and seizure models recently appeared in the same month [20-21,48] and the European Commission recently funded a consortium to investigate the role miRNA in the pathogenesis, treatment and prevention of epilepsy. Perhaps uniquely, miRNA research has the potential to deliver therapeutics and diagnostics that link directly to how these regulatory molecules influence disease pathogenesis in epilepsy.

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key points

- Epilepsy is associated with large-scale changes to microRNA levels in the brain
- More than 20 microRNAs show consistent changes across animal models. Several of these are also altered in resected tissue from patients with intractable epilepsy.
- *In vivo* manipulation of individual microRNAs supports functional roles in the control of inflammation, cell death and hyper-excitability.

Figure 1. How microRNAs work

(A) Simplified concept of how miRNAs work. The normal translation of a messenger RNA (mRNA) into protein can be reduced by miRNA binding. Protein depicted is a surface representation of human Ago-2 (with permission from Schirle et al (2012) [2]). (B) Canonical pathway for production of mature miRNAs. Transcription by pol II or III produces a primary transcript before two stages of processing that results in production of the mature miRNA. Scheme is a simplification of the process. Drosha refers to the microprocessor complex which comprises several other proteins including DGCR8 and RNA-associated proteins. (C) Mechanism by which miRNAs target mRNAs. MiRNA are up-loaded to the RISC complex which contains Ago-2. Targeting of the miRNA to the mRNA usually occurs within the 3' untranslated region, and features a “seed” region of 7-8 nt, followed by additional binding. The result is degradation of the mRNA or translation inhibition. MiRNAs also function in non-RISC related processing, for example acting as “decoys”. The miRNA sequence in C is mmu-miR-34a-5p.

Figure 2. Conserved brain and blood miRNA responses following epileptogenic brain injuries
(A) Profiling data show unique as well as common up- and down-regulated miRNAs 24 h after different epileptogenic insults to the brain. **(B)** Blood profiling data show each insult produces unique as well as shared miRNA expression responses in blood suggesting a set of common miRNAs that could represent biomarkers of epileptogenic brain injury. Boxes list the conserved miRNAs. Note, -3p and -5p denote mature miRNAs that originate from opposite arms of the same miRNA. Data are adapted with permission from Liu et al. [••17].

Table 1

miRNA profiling in experimental epilepsy

Reference	Platform	Epilepsy model	Time point(s)	# Profiled	Regulated	Common miRNAs ^a	Common pathways ^b
[18]	Microarray (μParaflo)	Li-PILO in rats	2 months	349	23 (18% ^c) 18 up, 5 down	Up: miR-21, miR-23a, miR-23b, miR-24, miR-27a, miR-27b, miR-34a, miR-126, miR-132, miR-146a, miR-140, miR-152, miR-210, miR-212	Cell death/apoptosis Axon guidance Development Immune response Inflammation Excitatory/inhibitory neurotransmission and the synapse Transcriptional regulation
[19]	Microarray (Agilent)	Li-PILO in rats	2 months	350	24 9 up, 15 down		
[20]	Microarray (Exiqon)	Electrical stimulation in rats (amygdala)	7-90 days ^d	All	66 (23% ^c) 9 up, 57 down		
[21]	Microarray (Exiqon)	Electrical stimulation in rats (angular bundle)	3-4 months	All	42 (13% ^c) 37 up, 5 down	Down: miR-33, miR-138, miR-139, miR-187, miR-190, miR-218a, miR-301a, miR-551b, miR-935	

^a Box lists the commonly regulated miRNAs (same direction in two or more studies).

^b Identified using bioinformatics (non-experimentally validated).

^c Based on number called present

^d Although this study profiled each time point, the major changes were only at the 7 and 30 day times

Key: PILO, pilocarpine.