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TOPICAL REVIEW

MicroRNAs – small RNAs with a big influence on brain excitability

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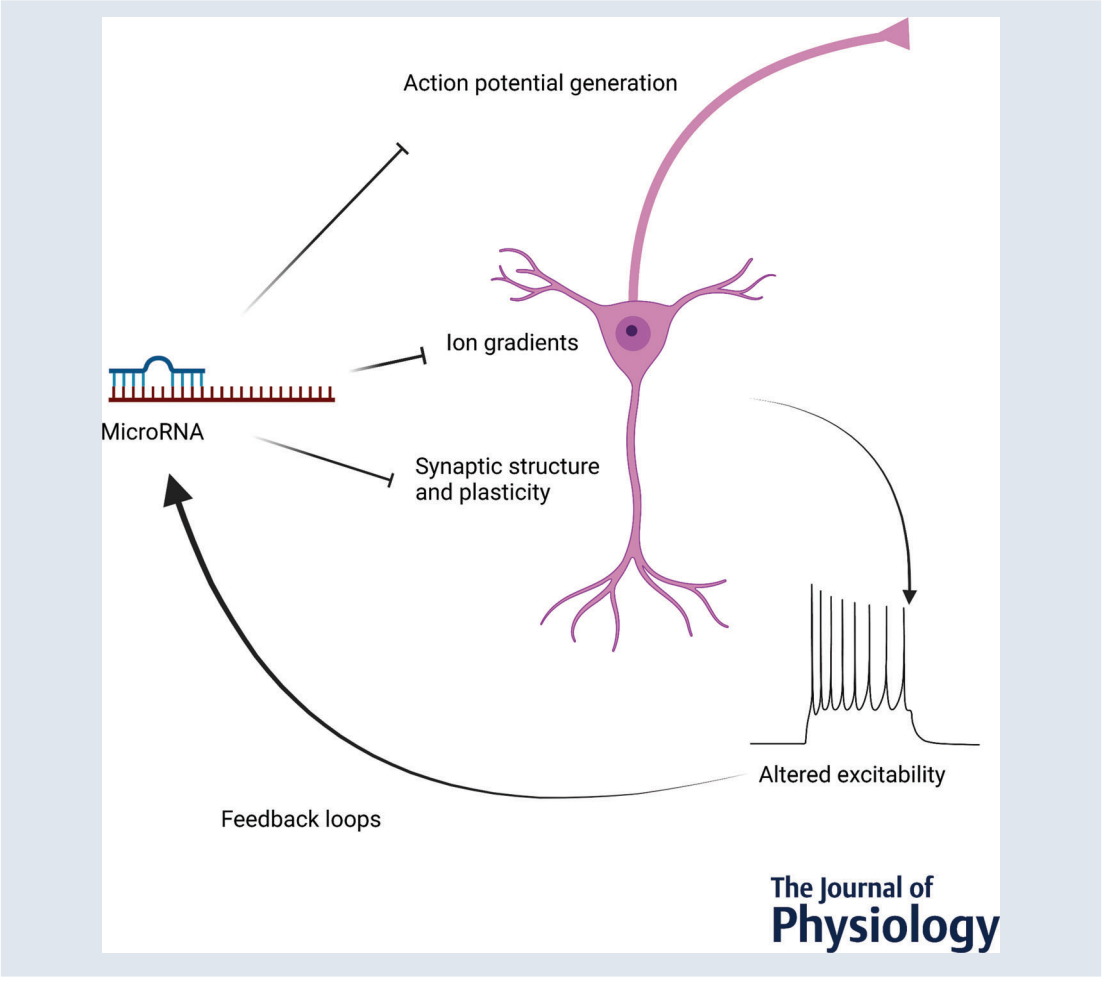

Gareth Morris completed his PhD at the University of Birmingham in 2014, studying network oscillations in epilepsy. He since completed postdoc positions at the UCL Institute of Neurology and the Royal College of Surgeons in Ireland, before winning a Marie Skłodowska-Curie Actions Individual Fellowship to study micoRNAs in epilepsy. In 2021 he started his own lab at University College London, developing a novel gene therapy approach with a Fellowship from Epilepsy Research UK.

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Abstract figure legend MicroRNAs are short non-coding RNAs which negatively regulate gene expression via targeting of complementary sequences, primarily in the 3′ untranslated region, of mRNA transcripts. There are hundreds of different microRNAs and each has a target pool which can comprise many different gene transcripts. Many microRNA–target interactions have been identified which can modulate neural excitability, with impacts on processes including synaptic transmission and plasticity, ion transporters and voltage-gated ion channels. The expression of many microRNAs is in turn regulated by neuronal activity, thereby forming feedback loops.

MicroRNA properties, biogenesis and general mechanism of action

MicroRNAs (miRNAs) are short non-coding RNA sequences, typically 21−23 nt in length, which suppress gene expression at the post-transcriptional level via complementary binding to the 3′ untranslated region (3′UTR) of targeted mRNAs (Bartel, 2004). MiRNA biogenesis is described in detail elsewhere (Bartel, 2004); in summary, relatively large primary miRNA (pri-miRNA) hairpin structures are transcribed from the genome using RNA polymerase II (though exceptions may exist, as ectopic miRNA expression with a pol III promoter can produce functional miRNAs *in vivo*; Chen et al., 2004). Pri-miRNAs are initially processed within the nucleus by the ribonuclease Drosha to form pre-miRNAs (Lee et al., 2003), which are then exported to the cytoplasm via exportin-5 (Yi et al., 2003). Pre-miRNAs are further cleaved by the cytoplasmic endonuclease Dicer (MacRae, 2006), leaving a mature double-stranded miRNA duplex. The breakdown of this duplex gives rise to the '−3p' and '−5p' forms of each miRNA. Depending on the biochemical stability of these strands, one of them will be favoured for functional interaction with argonaute-2 (Ago-2) via the RNA-induced silencing complex (RISC), and the other strand favoured for degradation.

MiRNA mechanism of action is also covered in detail elsewhere (Bartel, 2009), though a brief explanation will set this review in context. The RISC consists of a mature miRNA strand in complex with the endonuclease Ago-2. The RISC presents a 7−8 nt 'seed region' of the miRNA on the surface of the complex and this serves as a guide sequence, directing the RISC towards complementary mRNA sequences in the 3′UTR of targeted transcripts, where it suppresses gene expression via one of two mechanisms. Perfectly complementary binding between the miRNA and its target favours direct degradation of the mRNA by Ago2. The RISC can also target mRNA without completely perfect complementarity – in this case the mRNA is usually not degraded, but its translation is repressed via steric blocking. Since miRNAs can mediate their action without perfect sequence complementarity, this gives rise to an interesting property: one miRNA can influence the expression of many (often hundreds of) genes. Equally, one gene transcript can be targeted by many miRNAs. This likely places miRNAs as 'systems regulators' of gene pathways, and it is thought that their role is to buffer gene expression within a homeostatic window (Hobert, 2007). Indeed, there is extensive evidence that the expression of many miRNAs is dysregulated in disease. One particular focus has been epilepsy (Brennan & Henshall, 2020), where miRNAs have emerged as biomarkers (Brennan et al., 2020) and therapeutic targets (Morris, O'Brien et al., 2021; Venø et al., 2020) for seizure suppression. However, relatively little focus has been placed to date on how miRNAs might shape neural circuitry and excitation. Here, I review the literature and existing evidence for miRNA modulation of ion channel function and synaptic transmission, before offering thoughts on future perspectives in the field. Although this review focuses on the brain, similar mechanisms exist to regulate excitability in other tissues, including the heart (Yang et al., 2008).

MicroRNA interactions with ion channels

One obvious way in which miRNAs can shape neuronal excitability is through modulating the expression of specific ion channels (Gross & Tiwari, 2018), changing the biophysical and firing properties of individual neurons. One of the most well-studied examples of a miRNA–ion channel interaction is that between miR-324-5p and the *Kcnd2* gene transcript, encoding the voltage-gated potassium channel (VGKC) Kv4.2 (Gross & Tiwari, 2018; Gross et al., 2016; Tiwari et al., 2019). Kv4.2 belongs to the Shal family of VGKCs and mediates A-type potassium currents in the somatodendritic compartment of neurons (Jerng et al., 2004). These channels operate at subthreshold membrane potentials and are therefore implicated in signal integration via hyperpolarisation of the dendritic membrane. Indeed, functional Kv4.2 is reduced in models of temporal lobe epilepsy and leads to enhanced back-propagating action potentials

within the hippocampal circuit, likely reducing the threshold for seizure initiation (Bernard et al., 2004). As miR-324-5p represses the expression of *Kcnd2*, it follows that antisense inhibition of miR-324-5p leads to increased Kv4.2 functional expression and a reduction in neuronal excitability and seizures (Gross et al., 2016; Tiwari et al., 2019). Similarly, a recent study showed that miR-335-5p is able to reduce susceptibility to induced seizures via repression of the voltage-gated sodium channel subunit *Scn2a* (Heiland et al., 2022).

MiRNAs can also target ion channels associated with neuroplasticity. *Cacnb2* encodes the β_2-subunit of the L-type voltage-gated calcium channel (VGCC) Cav1.2 (Birnbaumer et al., 1998) and is a direct target of miR-499-5p (Ling et al., 2017; Martins et al., 2022). VGCC β-subunits enhance channel function via modulation of channel gating and kinetics, as well as enhanced trafficking of VGCCs from the endoplasmic reticulum to the cell surface (Bichet et al., 2000). Cav1.2 is the major VGCC isoform in neurons (Li et al., 2022) and so is a critical route for calcium influx in response to depolarisation, and therefore underlies key physiological processes which include NMDA receptor-independent plasticity (Moosmang et al., 2005), memory (Li et al., 2022; Moosmang et al., 2005) and activation of intracellular signalling pathways which can modulate gene expression (Dolmetsch, 2003).

MiRNA interactions with ion transporters

Electrochemical gradients must be established for charged ions to flow through the channels mentioned above and these gradients are largely established by ion transporter proteins, whose expression can also be modulated by miRNAs. One example is the targeting of *Slc12a2*, encoding the Na^+–K^+–$2Cl^-$ co-transporter NKCC1 (Virtanen et al., 2020), by miR-101 (Lippi et al., 2016). In immature neurons NKCC1 imports negatively charged chloride ions into the intracellular space, contributing to depolarising responses to GABAergic signalling. During postnatal development, NKCC1 expression decreases in tandem with increased expression of the K^+–Cl^- cotransporter KCC2 (encoded by *Slc12a5*) (Zhang et al., 2021). In contrast to NKCC1, KCC2 extrudes Cl^- into the extracellular space and therefore hyperpolarises E_{Cl}, and renders GABAergic transmission inhibitory. MiR-101 is expressed transiently during development and targets *Slc12a2*, reducing NKCC1 expression and contributing to the 'GABAergic switch' which is a critical aspect of brain network development (Lippi et al., 2016). Notably, this study used target site blockers to inhibit interactions between miR-101 and individual specific target transcripts, as opposed to antisense oligonucleotide miRNA inhibitors, which simultaneously prevent interaction between a miRNA and all of its target mRNAs. As a result, the authors demonstrated that miR-101 also limits the development of excitatory circuits by repressing the expression of kinesin family member 1A (*Kif1a*), decreasing the number of presynaptic boutons, and ankyrin 2 (*Ank2*), constraining the stabilisation of synapses. This exemplifies the assumed action of miRNAs to simultaneously mediate multiple unique molecular interactions (targeting *Slc12a2*, *Kif1a* and *Ank2*) which act in complement to give rise to a common phenotype (constraining circuit excitability during development).

MicroRNA interactions with synaptic transmission

Another mechanism by which miRNAs can modulate circuit function is synaptic scaling via interactions with genes associated with synaptic transmission. For example, *Gria1* encodes the GluA1 subunit of the AMPA subtype of ionotropic glutamate receptors (Ge & Wang, 2021). MiR-92a exhibits activity-dependent expression and binds to the 3′UTR of *Gria1* transcripts, thereby repressing GluA1 expression in response to neuronal activity (Letellier et al., 2014). This represents a miRNA-based molecular mechanism for synaptic scaling and homeostatic synaptic plasticity of neural circuit activity. Most AMPA receptor complexes contain both GluA1 and GluA2 subunits, and are permeable only to sodium and potassium. However, AMPA receptors lacking GluA2 subunits also become permeable to calcium (Cull-Candy & Farrant, 2021). These GluA1 homomers become more numerous in response to reduced neural activity and promote synaptic plasticity (Thiagarajan et al., 2005). *Gria2*, encoding GluA2, is also subject to homeostatic plasticity via miR-124 (Hou et al., 2015). MiR-124 shows activity-dependent expression owing to negative regulation by the transcription factor EVI1. When neural activity is reduced, EVI1 is downregulated leading to de-repression of miR-124, which in turn suppresses *Gria2*, leading to more calcium-permeable GluA1 homomers.

Similarly, miR-132 precursor expression is induced by cAMP-response binding protein (CREB) in response to neuronal activity (Vo et al., 2005). The subsequent increase in mature miR-132 has been shown to promote neurite outgrowth and this effect is associated with highly conserved repression of p250GAP (Vo et al., 2005). MiR-132 has also been shown to enhance short-term synaptic facilitation in response to paired-pulse paradigms (Lambert et al., 2010), although in this case the molecular mechanism is less well-defined. MiR-134-5p is another miRNA that is heavily linked to synaptic transmission via multiple simultaneous mechanisms. Notable targets include CREB and brain-derived neurotrophic factor, which have been linked to synaptic and long-term

plasticity in an Alzheimer's disease model (Baby et al., 2020), and *Limk1* (Jimenez-Mateos et al., 2012), which interacts with dendritic spine turnover. MiR-134-5p is also associated with activity-dependent dendritogenesis via fine-tuning of Pumilio2 (Fiore et al., 2009, 2014), which is also associated with homeostatic regulation of neuronal firing rates (Mulroe et al., 2022). This exemplifies the proposed role of miRNAs in modulating neural network excitability through multiple distinct molecular pathways acting in parallel. The various mechanisms discussed above by which miRNAs can modulate brain excitability are summarised in Fig. 1.

Limitations to our understanding: added complexity of miRNA–target interactions

The canonical mechanism of translational repression by miRNAs, as introduced above, is well characterised. However, there are many additional layers of complexity and as a result I suggest that it is near impossible to fully interpret the molecular and biophysical functions of a single miRNA. Firstly, one miRNA can target many (often hundreds of) transcripts, and one gene can be targeted by many different miRNAs. This likely gives rise to miRNA–target interactions (MTIs) which can be co-operative (multiple miRNAs simultaneously repressing the same gene), competitive (many different target binding sites competing for the same miRNA) or perhaps redundant (target suppression is saturated by one miRNA and so subsequent binding of a new one has no effect). For example, it has been shown that the level of miRNA-mediated transcript suppression depends on a number of contexts (Grimson et al., 2007) which include the presence of AU-rich nucleotide regions close to the site, co-operation between closely located sites for co-expressed miRNAs, auxiliary base pair complementarity outside of the seed region, and position within the 3′UTR (proximal to the stop codon but also at least 15 nt away from the stop codon). Moreover, the degree of complementarity between miRNAs and their targets determines if a given MTI favours transcript degradation or steric blocking of translation. Added to this, in certain conditions MTIs can cause degradation of the miRNA rather than the mRNA (known as target RNA-directed miRNA degradation; TDMD). This phenomenon appears to be most effective in neuronal cells and depends upon both the abundance of the miRNA and the number of TDMD sites in the targeted mRNA (la Mata et al., 2015). TDMD appears to be a

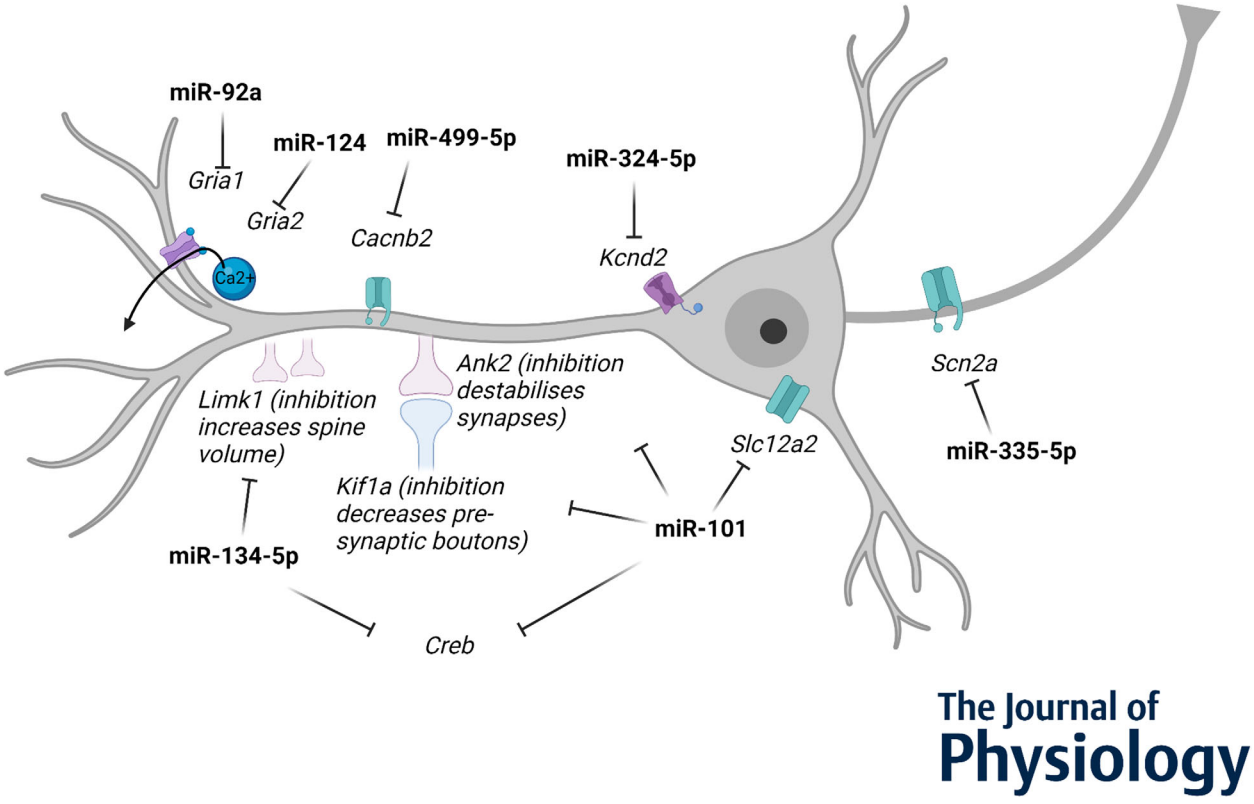

Figure 1. Key microRNA interactions which impact brain excitability
Various microRNAs target genes which are closely linked to brain excitability. Targets include ligand-gated ion channel subunits (*Gria1* and *Gria2*), genes involved in synaptic structure (*Limk1*, *Kif1a*, *Ank2*), ion transporters (*Slc12a2*) and voltage-gated ion channels/subunits (*Cacnb2*, *Kcnd2*, *Scn2a*).

separate process from canonical miRNA-mediated gene repression and uses different binding sites on the target transcript. Another layer of complexity is provided by miRNA interactions with other non-coding RNA systems. For example, *Ube3a1* is a variant of the E3 ubiquitin ligase *Ube3a*. *Ube3a1* encodes a truncated protein, but its transcript has a unique 3′UTR which appears to act as an endogenous sponge for miR-134 and in turn impacts dendritic growth (Valluy et al., 2015). Therefore, alternative transcripts of protein-coding genes may lead to endogenous mechanisms to compete with existing miRNA binding sites, with implications for neural circuit function. Similarly, miRNAs can target, and also be targeted by, other aspects of the epigenetic machinery, such as DNA methyltransferases and histone deacetylases (Yao et al., 2019). This gives rise to 'miRNA-epigenetic feedback loops' which can further modulate miRNA function. All of these features likely contribute to non-linear relationships between miRNAs, their mRNA targets and emergent behavioural phenotypes. Indeed, genetic titration of miR-218 expression revealed semi-log and exponential relationships between the miRNA and the expression of various target genes (Amin et al., 2021). *In silico* models of miRNA–mRNA network interactions may go some way to improve our understanding of miRNA (dys)function, though these must not rely on binary or linear relationships between the expression of miRNAs and their targets.

Species differences and challenges for translational research

Many functional miRNA studies to date have relied on rodent-based *in vitro* and *in vivo* models. These have led to extensive progress in understanding the possible functions of specific miRNAs, and many examples are discussed in this review. However, there are perhaps two key limitations in mapping these findings to human brain function: (1) there are an estimated 2600 mature miRNAs in the human genome (Plotnikova et al., 2019) and around 2000 in mice (Omariba et al., 2020), meaning that many human miRNAs are not captured in rodent models; and (2) where the 'same' miRNA does exist in rodents and humans, the sequences of the miRNA and of its target mRNAs may not be exactly conserved, which means that miRNAs likely have species-specific target pools. It is possible that the impact of species-specific sequences may be mitigated by the ability of miRNAs to operate with only partial target complementarity, adding complexity to the translation of studies between animal models and the human brain. To overcome this, studies in the epilepsy field have used surgically resected specimens in order to characterise MTIs in the human seizure focus (Heiland et al., 2022). A recent method (Morris et al., 2022) allows for the application of anti-miRs to non-diseased acutely resected human temporal neocortex (note – this tissue was removed during resective epilepsy surgery to give access to the seizure focus within the temporal lobe; whilst the neocortical tissue is assumed healthy, it was taken from the brain of a person with epilepsy and should be interpreted in this context). Induced pluripotent stem cell-based models can also be used to study MTIs in a human-derived preparation (e.g. Heiland et al., 2022), though these may not form physiologically realistic synaptic connections and networks (Morris, Rowell et al., 2021), possibly affecting the molecular pathways mediating miRNA function that are described in this article. Human models also have limitations (Richardson & Morris, 2022) and should be used carefully in parallel with animal models to better integrate findings between rodent and human brains. Finally, whilst this review is focused on how miRNAs influence brain excitability, it should be considered that miRNAs are ubiquitous and essential to cellular function in all tissues. From a therapeutic perspective, it is therefore key to ensure that any miRNA-based therapy is delivered to the correct tissue. For example, whilst miR-335-5p appears to reduce neuronal excitability and seizures via repression of *Scn2a*, it is also predicted to repress.

Concluding remarks

MiRNAs are small RNAs, but their influence over circuit excitability is vast. Direct mechanisms by which miRNAs can influence circuits include targeting ion channels, changing ionic gradients via modulating ion transporters, and roles in regulating synaptic connectivity. MiRNAs will inevitably influence circuit function via many more mechanisms which are not discussed here (e.g. interactions with glial cell processes; brain inflammation), and new MTIs are being validated on a regular basis – this review should not be considered an exhaustive list of all such interactions which modulate excitable properties in the brain. Many miRNAs exhibit activity-driven expression and are therefore well-placed to mediate homeostatic plasticity and play roles in neurological diseases. Given the sheer number of miRNAs and their targets, combined with context and species-specific complexities in MTIs, there remains much scope to better understand the role of miRNAs in shaping brain excitability and cellular functions in general.

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Additional information

Competing interests

G.M. reports the European Patent Application No. EP21198390.3 'Modulation of microRNA-335-5p for the treatment of sodium channelopathies.' with D. Henshall and M. Heiland, filed by the Royal College of Surgeons in Ireland.

Author contributions

Sole author.

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Supporting information

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