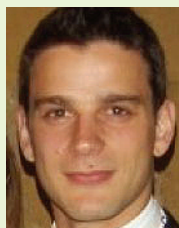


RNA Interference and Neurological Disorders



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RNA interference (RNAi) is a powerful mode of post-transcriptional gene silencing, the discovery of which earned Fire and Mello the 2006 Nobel Prize in Physiology or Medicine. Since their seminal work published in 1998, which led to the identification of double-stranded RNAs (dsRNAs) as being responsible for RNAi in a sequence-specific manner,¹ it has become clear that RNAi is an essential and ubiquitous process in all eukaryotic cells and organisms. It is especially critical in stem cells and during development, and it is now appreciated that dysregulation of RNAi function is a central feature of pathological processes, including cancer and neurological disease. Moreover the powerful ability of RNAi to silence genes has therapeutic potential.

RNAi biology

RNAi refers to the sequence-specific silencing of messenger RNA (mRNA) transcripts directed by short, 21-23 nucleotide antisense RNA species. The genes responsible for initiating RNAi are part of an evolutionary conserved cellular pathway that processes endogenous triggers of RNAi, termed microRNAs (miRNAs), into mature sequences capable of directing the silencing of sequence-matched mRNA targets (Figure 1). Over 900 human miRNAs have now been identified within the genome, and several have been shown to silence the expression of anywhere from tens to hundreds of mRNA transcripts at a time.² Thus the natural RNAi pathway within cells represents a powerful post-transcriptional gene regulation network that helps to maintain and enhance complexity arising from transcription of the genome, whilst the dysregulation of this network can have disease-causing potential.

miRNAs and neurological disease

Given their ability to enhance the complexity of the transcriptome, it is perhaps no surprise that the central nervous system (CNS) is enriched in miRNA expression. Here they display tight spatial and temporal expression patterns, and have now been shown to have key roles in multiple aspects of neurobiology including neuronal-lineage determination, synaptogenesis and neurogenesis

among others.³ Further to this, abnormalities at almost every stage of miRNA processing have now been linked to disease, and several neurological disorders are among those now identified as linked to miRNA dysregulation. Profiling of miRNA expression in post-mortem tissue samples from patients with neurological disorders such as Alzheimer's disease (AD),⁴⁶ Huntington's disease (HD),⁷ Parkinson's disease (PD),⁸ schizophrenia,⁹ and autism¹⁰ have identified miRNAs that demonstrate either increased or reduced expression relative to control tissues (Table 1); implicating their involvement in the disease process. Similarly, a plethora of dysregulated miRNAs have also been identified in pre-clinical neurological disease models. Importantly, such patterns of miRNA dysregulation may now hold promise as novel diagnostic or therapeutic biomarkers for neurological diseases, as has already been shown for several cancers. However it is unclear at present whether alterations in the expression of miRNAs across these disorders are causative of the disease phenotypes, or merely consequences of other primary defects. This will be important to determine in future since it could reveal novel therapeutic strategies involving for example the use of miRNA inhibitors or mimics to modulate miRNA activity.

In addition, the 3'UTRs of mRNAs typically contain target seed-matched sequences for miRNAs and are known to be a common site for genetic variation. Disease-linked single nucleotide polymorphisms (SNPs) have now been reported in specific 3'UTR miRNA target sites in cases of Tourette's syndrome,¹¹ PD,¹² TDP43-positive frontal temporal dementia¹³ and even in individuals displaying aggressive behavior¹⁴ (Table 1). These SNPs can lead to either reduced miRNA silencing of the mutated transcript by the targeting miRNA, or tighter regulation by the miRNA due to strengthening of the miRNA-binding site. Conversely, SNPs in miRNA transcripts themselves have also been reported for non-CNS disorders and this could be an additional mechanism found to link miRNAs to neurological disease in future either through direct effects on the processing of the miRNA or directly on target mRNA silencing.

RNA interference is of already central biological importance and it promises to make a major clinical impact, particularly in the neurosciences

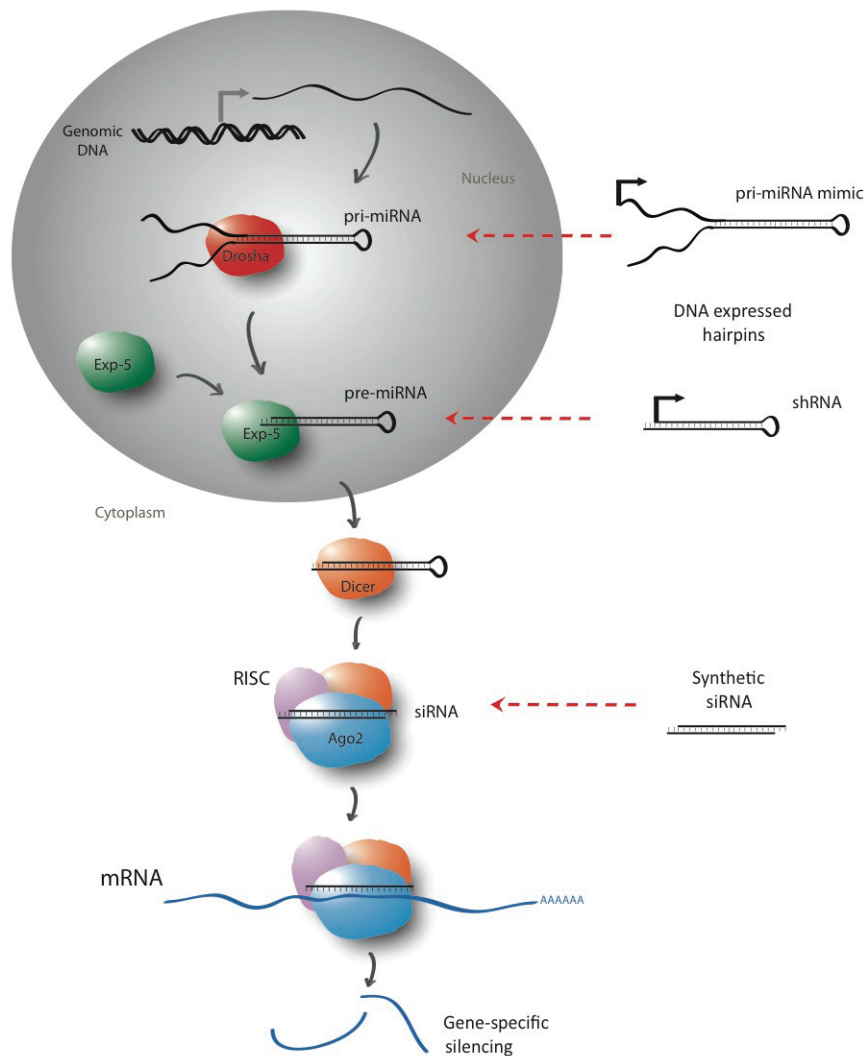


Figure 1. Mechanism and Exploitation of the RNAi pathway.

In the endogenous RNAi pathway, primary miRNA (pri-miRNA) sequences are transcribed from genomic DNA. Complementary base pairing between separated regions of the pri-miRNA sequence allows characteristic stem-loop secondary structures to form which are subsequently recognised by the ribonuclease enzyme, Drosha. The resulting cleavage within the stem produces a pre-miRNA precursor, with characteristic two nucleotide overhangs at the 3' end. These overhangs serve as a recognition signal for nuclear export by Exportin-5, and then for further processing by Dicer. Removal of the loop-region of the pre-miRNA by Dicer results in production of a dsRNA duplex, or short interfering RNA (siRNA) which initiates the formation of the Argonaute (AGO) protein-containing RNA-induced silencing complex (RISC). Within RISC a thermodynamic selection process selects one of the two siRNA strands as the active guide strand, referred to as the mature miRNA, that will be used to direct target mRNA silencing whilst the other strand is discarded. RISC subsequently scans the retained mature miRNA across mRNA sequences, and in particular the 3' untranslated regions (3'UTRs), searching for sequence homology to the mature miRNA. When identified, the AGO proteins within RISC initiate target mRNA silencing through translational repression in the case of incomplete homology, or alternatively through target mRNA degradation if homology to the mature miRNA sequence is complete. Gene silencing based therapy can exploit several points of the endogenous RNAi pathway, including expressed triggers of RNAi such as pri-miRNAs and shRNAs, as well as siRNA forms.

Manipulation of RNAi pathway for neurological disease therapy

Therapeutic exploitation of the RNAi mechanism described above is also a prominent focus of research. For several neurological diseases it is likely that silencing genes that are over-expressed or which harbour pathogenic mutations would be therapeutically beneficial, especially where mutations result in gain-of-function. Silencing of pathogenic human transgenes in mice leads to phenotypic improvements in both HD¹⁵ and spinocerebellar ataxia 1 (SCA1)¹⁶ mouse models. In the HD models, RNAi treatment that reduces

mutant human and wild-type mouse Huntington mRNA transcripts concomitantly by ~60% has been shown to lead to improved motor coordination over sham-treated littermates, and importantly to an increase in life expectancy.¹⁵ Similarly, cerebellar degeneration was reduced and an ataxic behavioural phenotype improved following RNAi treatment leading to reduced ataxin-1 in the SCA1 mice.¹⁶ However, the non-allele specific silencing used in these studies would only be applicable to those diseases in which the function of the normal wild-type gene is non-essential. For several neurological diseases, a func-

tioning copy may be important, if not a necessity. In these cases, allele-specific silencing of the mutant gene only would be desirable. Though extensive screening is required to identify RNAi triggers capable of discriminating between wild-type and mutant transcripts, allele-specific therapies have been demonstrated in neurological disease models of SCA3, frontotemporal dementia, SCA7 and HD.^{17,19}

The choice of RNAi trigger is also of importance. Gene-specific silencing can be achieved using any of the processed small RNA species produced in the natural RNAi pathway as a method to target a gene of interest (Figure 1). Whilst pri-miRNA mimics and shRNAs can be expressed from DNA-encoded plasmids to allow incorporation into viruses for long-lasting expression, chemically synthesised siRNAs are delivered as dsRNA duplexes that are targets for nuclease digestion and rapid clearance from the body, making their effects short-lived. However one concern that must be addressed before routine use of RNAi in the clinic is that all three approaches can direct off-target silencing of mRNA transcripts with near-complete base-pairing to the antisense species. Thus, delivery of the RNAi trigger should ideally be restricted to only the cell-type of interest where possible to limit this undesirable silencing.

The transient nature of a siRNA-based therapy lends itself to one-off treatments for infectious or relapsing diseases. For example, intracranial injections of siRNAs targeting the Japanese encephalitis virus or Nile river virus have prevented a lethal phenotype in viral challenged mice.²⁰ In contrast, one-off treatments using an shRNA or pri-miRNA mimic expressed from within a suitable viral vector could lead to long-term gene silencing more suitable for chronic neurological diseases such as PD and HD.¹⁵ However these DNA-encoded RNAi triggers should ideally be modified to allow inducible or even neuronal-specific expression as these characteristics would be particularly useful to limit off-target silencing of near complete base-paired transcripts to the RNAi trigger.

Finally, one of the biggest challenges in developing RNAi-based therapies for neurological disorders is that of delivery to the CNS. The most common viral approaches for RNAi delivery in pre-clinical models are intracranial injections of integrating lentiviruses or non-integrating adeno-associated viruses (AAVs). However, it is unclear whether this will become a preferred method of administering treatment in patients due to the invasive nature of administration. Clearly, identification of novel methods of traversing the blood-brain barrier (BBB) following systemic injection are required. Encouragingly, transvascular delivery of a siRNA complexed with a neuronal targeting-peptide across the BBB to the striatum, thalamus and cortex has been demonstrated in vivo.²⁰ Likewise, the use of

Table 1. Neurological disorders with reported miRNA abnormalities

| Disease | miRNA abnormality | Functional effect | Ref. |
|---------------------------------|--|--|------|
| Alzheimer's Disease | Decreased expression of miR-29a/b-1 cluster | miR-29a and miR-29b-1 regulate BACE-1 expression. Loss leads to abnormally high BACE-1 levels | 4 |
| Alzheimer's Disease | Decreased expression of miR-107 | miR-107 regulates BACE-1 expression. Loss leads to abnormally high BACE-1 levels. Loss seen in early stages of disease | 6 |
| Alzheimer's Disease | Decreased expression of miR-106b | miR-106b regulates APP expression | 5 |
| Huntington's Disease | Decreased expression of miR-9/9* | miR-9/9* is a bidirectional miRNA with one strand, miR-9, regulating the transcriptional regulator REST, and the other strand, miR-9*, regulating CoREST. In turn REST and CoREST negatively regulate miR-9/9* such that double negative feedback loop is seen | 7 |
| Parkinson's Disease | Decreased expression of miR-133b | miR-133b regulates maturation and activity of midbrain dopaminergic neurons in subsequent murine models through a negative feedback loop with transcription factor Pitx3 | 8 |
| Parkinson's Disease | Polymorphism in fibroblast growth factor 20 target site of miR-433 | Parkinson's disease-associated polymorphism leads to reduced miR-433 repression of target and subsequent downstream increase in α -synuclein expression | 12 |
| Schizophrenia | Decreased expression of miR-26b, miR-92, miR-24 and miR-30e | Unknown mechanisms. Upto 15 miRNAs decreased and 1 increased with microarrays. Confirmed 4 to be significant with qPCR | 9 |
| Autism | Dysregulated expression of 9 miRNAs | Unknown mechanisms. Upto 28 miRNAs dysregulated in initial analysis. Confirmed 9 to be significant with further analysis | 10 |
| Tourette's Syndrome | Polymorphism in Slit and Trk-like 1 target site of miR-189 | Tourette's-associated polymorphism leads to enhanced miR-189 repression of target site | 11 |
| TDP43-frontal temporal dementia | Polymorphism in progranulin target site of miR-659 | Common polymorphism leads to enhanced miR-659 repression of target and is associated with 3-fold increase in susceptibility to disease | 13 |
| Aggressive behaviour | Polymorphism in serotonin 1B receptor target site of miR-96 | Common polymorphism leads to enhanced miR-96 repression of target and is associated with increased aggression | 14 |

systemically injected, pegylated immuno-liposomes carrying shRNAs or siRNAs has impressive site-specific knockdown in induced in vivo models of intracranial brain cancer.^{21,22} A new range of nanotechnology vehicles are additionally showing promise such as gold nanorod nanoplexes incorporating siRNAs which cross an in vitro BBB model²³ and the use of self-derived exosomes that have been modified with targeting moieties in our laboratory. Finally, certain viruses such as AAV9 have been shown to cross the BBB, and it will be important to see how these may be harnessed for RNAi therapeutics in future.

Conclusion

Within the last decade our understanding of RNAi has revealed it to be of central importance in regulating genome activity. Not surprisingly for a biological process of such importance, RNAi dysfunction is now notable in many diseases, including neurological disorders. Moreover, our ability to exploit the power of RNAi silencing has moved rapidly to the point where RNAi-based therapeutics are already in clinical trials, and such approaches are well advanced in numerous pre-clinical neurological disease models. The future will inevitably reveal much more on the role of RNAi, and miRNAs in particular, in fundamental neurological processes and functions. It will also very likely see the first RNAi-based clinical trials in patients with neurodegenerative disease in the next five years. ♦

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