Published in final edited form as:
Trends Cell Biol. 2013 January ; 23(1): 30–36. doi:10.1016/j.tcb.2012.08.013.

MicroRNAs and Neurodegeneration: Role and Impact

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Abstract

Neurodegenerative diseases are typically late-onset progressive disorders that affect neural function and integrity. Although most attention has been focused on the genetic underpinnings of familial disease, mechanisms are likely shared with more predominant sporadic forms, which can be influenced by age, environment and genetic inputs. Previous work has largely addressed the roles of select protein-coding genes; however, disease pathogenesis is complicated and can be modulated through not just protein-coding genes, but also regulatory mechanisms mediated by the exploding world of small non-coding RNAs. Here, we focus on emerging roles of miRNAs in age-associated events impacting long-term brain integrity and neurodegenerative disease.

Keywords

microRNA; neurodegenerative disease; aging; miRNA biogenesis; dicer; small RNAs

miRNAs and neurodegeneration

Neurodegenerative diseases are a group of typically late-onset, progressive disorders that lead to cognitive and/or movement disorders. Some of the most studied include Alzheimer's disease AD), Parkinson's disease (PD) Amyotrophic Lateral Sclerosis (ALS), and polyglutamine (polyQ) disorders such as Huntington's disease (HD) and the spinocerebellar ataxias (SCAs) [1–5]. These diseases share features such as the abnormal accumulation of protein, which includes plaques and tangles in AD, Lewy bodies in PD, bunina bodies in ALS, and nuclear and cytoplasmic accumulations in polyQ disease. In these diseases, key proteins accumulate, the genes of which are ones in which familial mutations can be found. Mechanisms that affect disease pathogenesis involve multiple fundamental cellular pathways, including protein folding and clearance processes. Thus, understanding the pathogenic mechanisms requires studying a broad spectrum of basic cellular machineries.

miRNAs are ~20–24 nucleotide (nt) small RNAs that regulate the translation or levels of target mRNA transcripts (Reviewed in [6–9]). Hundreds of miRNAs have been discovered from plants to animals that impact a variety of biological processes. miRNAs are generated through cleavage of a primary transcript in the nucleus (pri-miRNAs) by the Drosha/DGCR8 (Drosha/Pasha in *Drosophila*) microprocessor to generate a precursor miRNA (pre-miRNA) (Figure 1). The pre-miRNA is exported to the cytoplasm where Dicer cleaves it to

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release the double-stranded miRNA duplex. One of these strands preferentially associates with the Ago complex to form miRNA Silencing Complex (miRISC), while the other strand is usually degraded. In animals, miRNAs recognize their targets primarily through complementarity with the seed sequence at nucleotides 2–8 of the 5' end of the miRNA. Hundreds of mRNA targets could exist per miRNA family, and it is possible that most protein-coding genes are targets of miRNAs.

Three main approaches have been taken to study the effects of miRNAs on long-term brain integrity and neurodegenerative disease. First is the disruption of proper miRNA biogenesis followed by examination of the effect on the brain over time. Second is the identification of individual miRNAs that target specific disease genes and the impact. Third is the examination of the impact of disease-associated proteins on the miRNA pathway, such as miRNA biogenesis or mRNA-silencing function (Figure 2). Following, we will describe recent advances in each approach that reveal critical roles of miRNAs on brain integrity.

Disrupting the miRNA biogenesis pathway causes neurodegeneration

A range of approaches — including cloning of miRNAs, miRNA microarrays, and small RNA deep-sequencing analyses — have revealed expression of select miRNAs in the developing mammalian brain and primary neuronal cultures (for example, [10–13]). Analysis of the expression pattern of miRNAs using *in situ* hybridization with locked nucleic acid (LNA) probes in zebrafish identified their tissue-specific patterns [14]. Such patterns indicated a potential role of miRNAs in neuronal development and function. Subsequent studies revealed roles of specific miRNAs, such as miR-124, in these processes [15–17].

A functional link between miRNAs and neurodegeneration was discovered when studying the effect of global disruption of miRNA biogenesis on neuronal development. Mutants of Dicer in the mouse die early, before neurulation, precluding the ability to assess function in the brain [18]. However, disruption of zebrafish Dicer revealed an essential role in brain morphology and neural differentiation [19]. Injecting a miR-430 duplex rescued the defects in brain morphology, indicating the importance of this specific miRNA. Subsequently, conditional disruption of Dicer in different neuronal populations or cell lines revealed the effect of the miRNA pathway on proliferation, migration and differentiation, as well as long-term neural integrity [20–25]. For example, depleting Dicer in embryonic stem (ES) cells reduces the ability of the cells to differentiate into midbrain dopaminergic neurons [24]—a major neural population compromised in PD. Transfecting the small RNA fraction from embryonic mouse midbrain cells rescues the defect, assigning the role to small RNAs. Consistent with this, deleting Dicer in midbrain dopaminergic neurons in the mouse causes progressive loss of the cells, concomitant with disruption of locomotion, reminiscent of PD [24].

In another example, loss of Dicer from mouse cerebellar Purkinje cells did not impair cellular morphology or function at young ages (8–10 weeks). However, by 13 weeks of age, Purkinje cells, which are the cell type compromised in many ataxias, had progressively degenerated. Intriguingly, the older mice also developed slight tremors and mild ataxia that worsened with age [20]. Disruption of Dicer in spinal motor neurons mimics clinical and pathological features of ALS, a disease associated with loss of motor neurons, indicating a possible impact of the miRNA pathway in pathogenesis of this disease [26]. Interestingly, some key proteins associated with this disease have been shown to modulate miRNA biogenesis or function (below). Deletion of Dicer from glial cells such as astrocytes and oligodendrocytes can cause neural degeneration in the mouse [27, 28]. In addition, conditional loss of Dicer in Schwann cells in mouse revealed its importance for axonal

integrity [29]. In humans, dicer protein levels have been found decreased in temporal lobe epilepsy patients with hippocampal cell loss (sclerosis), with about half of the miRNAs in the tissue reduced in levels [30].

In *Drosophila*, knockdown of Dicer1 is also associated with dopaminergic neural loss and climbing defects [31]. Loss of Dicer1 also enhances toxicity of human pathogenic neurodegenerative disease proteins Ataxin-3 (associated with spinocerebellar ataxia) and Tau (associated with AD and frontotemporal dementia (FTD)) [32]. Intriguingly, depleting Dicer from human HeLa cells also enhances toxicity of disease-associated pathogenic Ataxin-3 protein, and is rescued by adding back the small RNA fraction indicating a role for miRNAs. This study identified a specific miRNA, bantam, that modulates Ataxin-3 and Tau toxicity. Supporting the role of miRNAs in polyQ disease pathogenesis, study of *Drosophila* miR-34 reveals a potent neuroprotective function in mitigating toxicity of pathogenic forms of Ataxin-3 [33]. Beyond Dicer, haploinsufficiency of DGCR8, a component of the microprocessor complex that cleaves pri-miRNAs to generate pre-miRNAs, leads to neuronal dysfunction in the mouse [34–36].

These studies, which target disruption of components of the miRNA biogenesis pathway, strongly suggest that miRNA activity impacts long-term brain integrity. Note, however, that the identification of individual miRNAs involved is a crucial component of such work. One reason for this is the potential effect of disrupting miRNA biogenesis on the proper expression of many related or unrelated proteins. Another reason is that disrupting the major components in miRNA biogenesis may cause dysfunction or degeneration independent of an effect on miRNAs. For example, Drosha, another component of the microprocessor, is reported to regulate neurogenesis by controlling Neurogenin 2 expression independent of its role in miRNA processing [37]. This function entails Drosha binding and cleavage of a hairpin structure in the 3'UTR of Neurogenin 2 mRNA. Recent studies have also shown that DGCR8 has a much broader impact on RNA processing beyond just miRNAs [38].

Individual miRNAs target disease genes

Efforts to profile miRNAs in tissue from patients with neurodegenerative disease has identified miRNAs that are misregulated in the brain, some of which have been shown to directly target transcripts of familial disease genes. Recent reviews discuss the roles of individual miRNAs on the common neurodegenerative diseases [39–41]. In general, discovering specific miRNAs that target the 3’UTR of key disease genes, and assessing the expression pattern and level of those miRNAs can uncover the extent to which they may impact the level of the disease protein, and thus impact pathogenesis. Here, we highlight a few examples to illustrate the impact of specific miRNAs on select diseases.

AD is the most common neurodegenerative disease, and although predominantly sporadic, analysis of familial situations has identified critical genes for its etiology [2]. The pathological features of AD are the deposit of intracellular neurofibrillary tangles containing Tau protein and extracellular plaques containing Amyloid-beta (Aβ) peptides in the brain. Increased production and impaired clearance of Aβ is a likely cause of Aβ accumulation. Various Aβ peptides are produced upon the cleavage of amyloid precursor protein (APP) by β-site APP-cleaving enzyme 1 (BACE1) and γ-secretase. Some of these processing events promote amyloid formation whereas others do not [3].

Studies in AD highlight the intricate and complex loops of miRNA regulation that can occur. miR-29a/b are downregulated in a subset of AD patients that show elevated BACE1 protein expression, which is predicted to promote amyloidogenic peptide formation [42]. The 3’UTR of BACE1 contains a miR-29a/b target site, and miR-29 targets BACE1. The BACE1 3'UTR also contains sites for other miRNAs, including miR-107, miR-124, and

miR-195 [43–45]. miR-107 is downregulated in AD, and targets cofilin, a component of rod-like actin structures in the AD brain. miR-15a, which belongs to miR-107/103 family, is also downregulated in AD patients [46]. Interestingly, the miR-15 family can target extracellular signal-regulated kinase 1 (ERK1), which is a Tau kinase, and this could potentially lead to abnormal Tau phosphorylation *in vivo*, another pathological hallmark of AD [47]. Other miRNAs implicated in AD pathology include miR-16, miR-101, miR-106a, miR-520c, and miR-153, which target APP [48–50]. Overall, these findings highlight the critical impact of select miRNAs on regulation of the expression of central proteins in AD pathogenesis and progression.

ALS is characterized by the degeneration of motor neurons in the brain and spinal cord, sharing a clinical and pathological spectrum with FTD, the second most common dementia [4]. The RNA binding proteins TDP-43 and FUS are both implicated in pathogenesis of ALS and FTD. TDP-43 is mutated in a subset of ALS patients, and TDP-43 knockdown in human cells leads to aberrant expression of some miRNAs [51]. In mouse, miR-206 deficiency accelerates disease progression in a model of ALS [52], which, together with the effect of Dicer loss in mimicking ALS pathogenesis [26], reinforces the importance of proper regulation of miRNAs and the miRNA pathway to ALS pathogenesis.

An example of discovering a miRNA-target loop that is conserved in flies and humans was revealed with studies of the miR-8 miRNA and one of its targets (Atrophin 1) in flies [53]. The Atrophin-1/Dentatorubral-pallidoluysian atrophy (DRPLA) protein is mutated and accumulates in the polyQ disease DRPLA. The DRPLA protein binds an orthologous protein RERE *in vitro*, and RERE overexpression causes mislocalization of the DRPLA protein [54]. miR-200b and miR-429 in human potentially target the RERE transcript [53]. Intriguingly, *Drosophila* miR-8, which has the same seed sequence as miR-200b and miR-429 in humans, targets the *Drosophila* Atrophin 1 mRNA. Further, miR-8 deletion in flies (which would lead to increased Atrophin 1 protein levels) causes a mild increase of apoptosis in larval brains and climbing defects in adults with age, reminiscent of disease features. These findings indicate a potentially conserved role of the miR-8/miR-200 family in neurodegeneration, contributing to the pathogenesis of DRPLA.

miRNAs can target pathways that impact brain integrity and disease

Many miRNAs may become misregulated in neurodegenerative disease, some of which may have a causal role in pathogenesis. However, it is often unclear what causes miRNA misregulation. One example of a miRNA with a suggested known mechanism is miR-133b. miR- 133b is downregulated in the midbrain of PD patients and in a dopaminergic neuron deficiency model mouse (Aphakia strain) [24]. The Aphakia mouse has a mutation in Pitx3, a transcription factor in which single nucleotide polymorphisms (SNPs) are associated with PD in some reports [55–57]. Pitx3 overexpression leads to upregulation of pre-miR-133b in differentiating ES cells, while miR-133b directly inhibits Pitx3 through its 3'UTR [24]. This suggests a negative feedback loop in which Pitx3 activates miR-133b expression, while miR-133b in turn represses Pitx3 expression. This study raises the possibility that an observed misregulation of miR-133b in PD may be related to the SNPs linked to PD in Pitx3, in at least some, PD situations although there are negative association reports [58–60] Although the knock-out mouse of miR-133b showed no obvious defects in midbrain dopaminergic neurons during development or aging, or in the expression of neuronal genes, including Pitx3, [61], the existence of the other miRNAs in miR- 133 family could explain the lack of effects. The role of miR-133b-Pitx3 loop in PD pathogenesis may limited to a subset of PD patients.

miR-34 defines a miRNA family that is highly conserved in human, fly and *Caenorhabditis elegans*. In human, the miR-34 family (miR-34a, miR-34b, miR-34c) is misregulated in many cancer types, and regulation of expression of these miRNA by p53 to impact apoptosis and cell cycle control is well defined [62, 63]. miRNA profiling in the adult mouse nervous system revealed enrichment of miR-34a in spinal cord and brainstem regions (medulla oblongata and pons) [11], and miR-34a increases with age in cortex and hippocampus by *in situ* hybridization [64, 65]. miR-34a is also enriched in the cerebral cortex of an Alzheimer's mouse model [66] and miR-34c increases with age in mouse and human hippocampus, AD patients, and mouse AD models [67]. Targets include SIRT1, whose regulation by miR-34c is associated with memory impairment in mice [67], and is inversely correlated with miR-34a expression in cortex and hippocampus with age [65]. miR-34c also functionally inhibits translation of Bcl-2 in cell studies, an anti-apoptotic protein whose function may also include modulating the processing of APP [66, 68]. Further, miR-34b is elevated in the plasma of Huntington's disease (HD) patients [69].

In contrast, in PD patients miR-34b/c is downregulated at early (pre-motor) stages in brain samples [70]. This study suggests that deficiency in miR-34b/c may promote mitochondrial dysfunction, concomitant with a decrease in Parkin and DJ1, two genetic loci associated with recessive parkinsonism. Overall, these studies highlight misregulation of the miR-34 family in neurodegenerative disease, although it is not clear if misregulation is a cause or consequence of disease pathogenesis.

In *Drosophila*, miR-34 is a brain-enriched, adult-onset miRNA. Deleting mir-34 causes early onset loss of motor behavior, susceptibility to stress, brain degeneration, and a shorter lifespan [33]. In addition, miR-34 upregulation mitigates polyQ degeneration. One miR-34 target is Eip74EF, a critical gene for the development of the animal [33]. This miRNA-target loop supports the idea of antagonistic pleiotropy; the target gene is beneficial in early life, but deleterious in later life, thus down regulation of the gene in the adult may protect the animal against its deleterious functions in the adult stage [71, 72]. Here, a single miRNA couples age- associated physiology of the animal (climbing, stress) with age-associated brain gene expression and long-term maintenance of the brain [33]. In *C. elegans*, miR-34 impacts lifespan through regulation of autophagy genes [73]. Given that alterations of miR-34 in mouse and human brain are associated with age and disease, although the precise role (to protect or alternatively promote loss of brain integrity and age-related functions) may be distinct or depend on precise targets, miR-34 is an intriguing a molecular link that may coordinate these age-associated biological processes in multiple organisms.

Disease proteins themselves may impact miRNA biogenesis and/or function

Beyond miRNAs affecting the level of key disease proteins or pathways, disease proteins themselves may directly affect miRNA biogenesis or miRISC target-mRNA silencing. The human Huntingtin (HTT) protein, whose CAG repeat expansion causes HD, interacts with Ago2 protein in cellular P-bodies (sites of mRNA decay) [74]. HTT depletion impairs miRNA target silencing [74], and in HD animal models, many miRNAs are misregulated [75]. This may be due, at least in part, to upregulation of Repressor Element 1 Silencing factor (REST), a transcription factor that is upregulated in HD neurons and can repress hundreds of neural genes [76, 77]. Interestingly, REST binding motifs are found in close proximity to a subset of miRNA genes in the human genome, including miR-9/miR-9*, miR-29a/b, miR-124, and miR-132 [78–81]. In addition, REST and its cofactor coREST have functional miR-9 and miR-9* target sites, respectively [82], and miR-9 is misregulated in HD patients [83]. Thus, REST may be required for the expression of many miRNA genes,

although the response may depend on context, such as the differentiation state of the cells [84].

TDP-43 mislocalization and mutation is associated with ALS, and TDP-43 loss-of-function as well as gain-of-function activities may contribute to disease [4, 85]. In human cells, expression of some miRNAs is affected by TDP-43 knockdown [51]. Interestingly, TDP-43 interacts with Drosha and Dicer complexes (which function in the generation of pri-miRNAs and pre-miRNAs, respectively), and binds select pri-miRNAs and pre-miRNAs in the nucleus and the cytoplasm, respectively, through the terminal hairpin loops [86]. Furthermore, nuclear TDP-43 facilitates Drosha-dependent cleavage of select pri-miRNAs, while cytoplasmic TDP-43 promotes Dicer- dependent cleavage of select pre-miRNAs. Interesting questions for the future include the mechanisms by which TDP-43 affects the processing of only a subset of pri- and pre- miRNAs, and the precise impact of these processing defects on disease pathogenesis.

PolyQ expansions in Ataxin-2 (Atx2) are associated with spinocerebellar ataxia-2 (SCA2), parkinsonism and ALS [87, 88]. As with other disease situations, it is possible that aspects of loss-of-function as well as gain-of-function contribute to disease. In flies, Atx2 is required for the silencing activity of select miRNAs; in Atx2-deficient cells, several miRNA reporters (although not all) become upregulated [89]. The detailed mechanisms by which the Atx2 protein impacts miRNA silencing, and whether a role of Atx2 in miRNA silencing is relevant to diseases associated with altered Atx2, remain to be addressed.

These findings suggest roles of disease-relevant proteins in miRNA biogenesis or miRNA target silencing. Given the potential of these proteins to affect these general aspects of miRNAs, and the predicted large number of mRNA targets of miRNAs, it is readily conceivable that disruption of these processes might lead to misregulated expression of a large number of proteins. Such gross disruption has the potential to profoundly impact long-term neural function and integrity.

Concluding remarks

Recent technological advances in high-throughput small RNA profiling *in vivo* have identified changes in the small RNA population in neurodegenerative disease or with age. So far, functions of only a handful of these miRNAs have been revealed, and an important question will be identifying the roles of the many other miRNAs that change in disease or in an age-associated manner. In addressing this question, studies of *C. elegans* have provided crucial insights into miRNA function; not all miRNAs that change with age lead to modulated lifespan upon altered activity [90], and many miRNAs are not required for development or viability [91, 92]. Redundancy among different miRNAs is one possibility. Alternatively, the changes of such miRNAs could be a result, rather than cause, of age-associated physiological events. Another possibility is that such miRNAs might be required only in a perturbed environment or genetic background to confer ‘robustness’ on gene expression [93–95]. Considering this, sensitizing the background when studying loss-of-function of miRNAs [94, 96], or examining stressful conditions might uncover novel roles. Another important question is what initiates misregulation of miRNAs in age or disease, especially for those miRNAs that impact age- associated events or the onset or progression of neurodegeneration. We have only limited examples of such mechanisms, such as where the disease-relevant genes themselves seem to trigger the misregulation of miRNAs.

Profiling of miRNAs by deep-sequencing analyses reveals that, in addition to simple up- or downregulation, miRNAs show potential differential isoform accumulation in the HD context [83], or clearly with age such that — in the case of *Drosophila* miR-34 — only the shorter 21 nucleotide form accumulates [33]. Although the biological meaning of this

processing is not yet defined, it is intriguing that production of different isoforms of miRNAs is regulated by a specific exonuclease in flies [97, 98]. This indicates that miRNA 3'end trimming may be a biologically critical process. Future careful analyses promise to reveal the unidentified function of many miRNAs and the impact of controlling miRNA isoform accumulation with age and/or disease.

As with *Drosophila* miR-34, studying the expression and function of individual miRNAs throughout adult lifespan might reveal a coordinated role of miRNAs in various aspects of age- associated processes from lifespan to long-term brain integrity. To this end, *Drosophila* and *C. elegans* remain key model organisms with their relatively shorter lifespan and ease of observing defects at the organismal level. Identifying more miRNAs that are modulated with age and detailed studies of individual miRNAs in multiple systems will bring more insights into the impact of miRNAs in age-associated processes and brain disease.

Therapeutic potential of miRNAs

Based on disease-associated changes in miRNA levels, such as the miR-34 family, one could potentially use changes in miRNA expression as biomarkers of aging and age-associated processes such as neurodegenerative disease [69, 99]. In addition, with the identification of miRNA with functions like miR-34, and the successful delivery of disease-modulating miRNAs, it is tantalizing to target the miRNA itself *in vivo* to mitigate disease onset or progression. Spinal and bulbar muscular atrophy (SBMA) is a polyQ disease with expansion in the *Androgen Receptor (AR)* gene [1]. miR-196a and miR-196b were identified as miRNAs that decrease the level of both the normal and CAG-repeat expanded *AR* transcripts [100]. Interestingly, these miRNAs directly target the mRNA of CUGBP Elav-like family member 2 (CELF2), whose protein positively regulates the *AR* mRNA level through a site on the transcript that is distinct from the CAG repeat — that is, a CTG repeat. Silencing of the CELF2 mRNA in spinal cord motor neurons by delivering miR-196a via an adeno-associated viral vector (AAV) improves the motor function of SBMA model mice. These findings, together with other examples, highlight the complex pathways and loops-within-loops of gene regulation that are impacted by miRNAs. They also offer promise that targeting and tweaking miRNAs through a variety of approaches could have therapeutic potential for neurodegenerative diseases.

Acknowledgments

We thank V. Lekova and N. Liu for helpful comments. We apologize to those colleagues whose work could not be cited due to space limitations. Research in the Bonini laboratory is supported by grants from the NIH and The Ellison Medical Foundation. NMB is an investigator of the HHMI.

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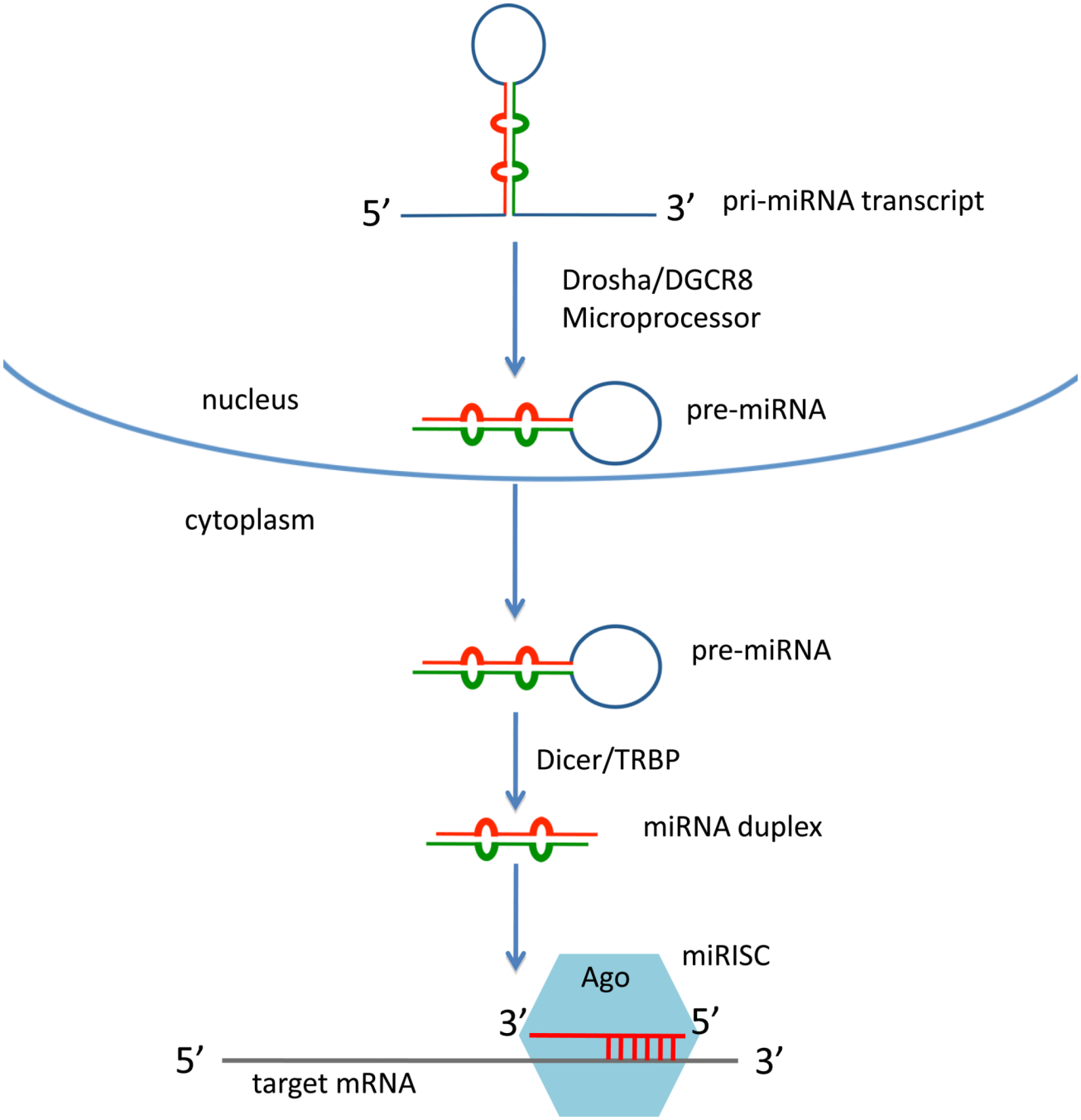

Figure 1. The miRNA biogenesis pathway

The biogenesis of a miRNA starts with the transcription of the primary (pri-miRNA) by RNA Polymerase II. The pri-miRNA is cleaved by the microprocessor complex (Drosha/DGCR8 in vertebrates, Drosha/Pasha in *Drosophila*) to generate the precursor miRNA (pre-miRNA). The pre-miRNA is transported to the cytoplasm, then cleaved by the Dicer/TRBP (Dicer/Loqs in *Drosophila*) complex to generate the miRNA duplex. After incorporation into miRISC and strand selection, the mature miRNA strand induces translational repression and/or mRNA cleavage, leading to reduction of the protein.

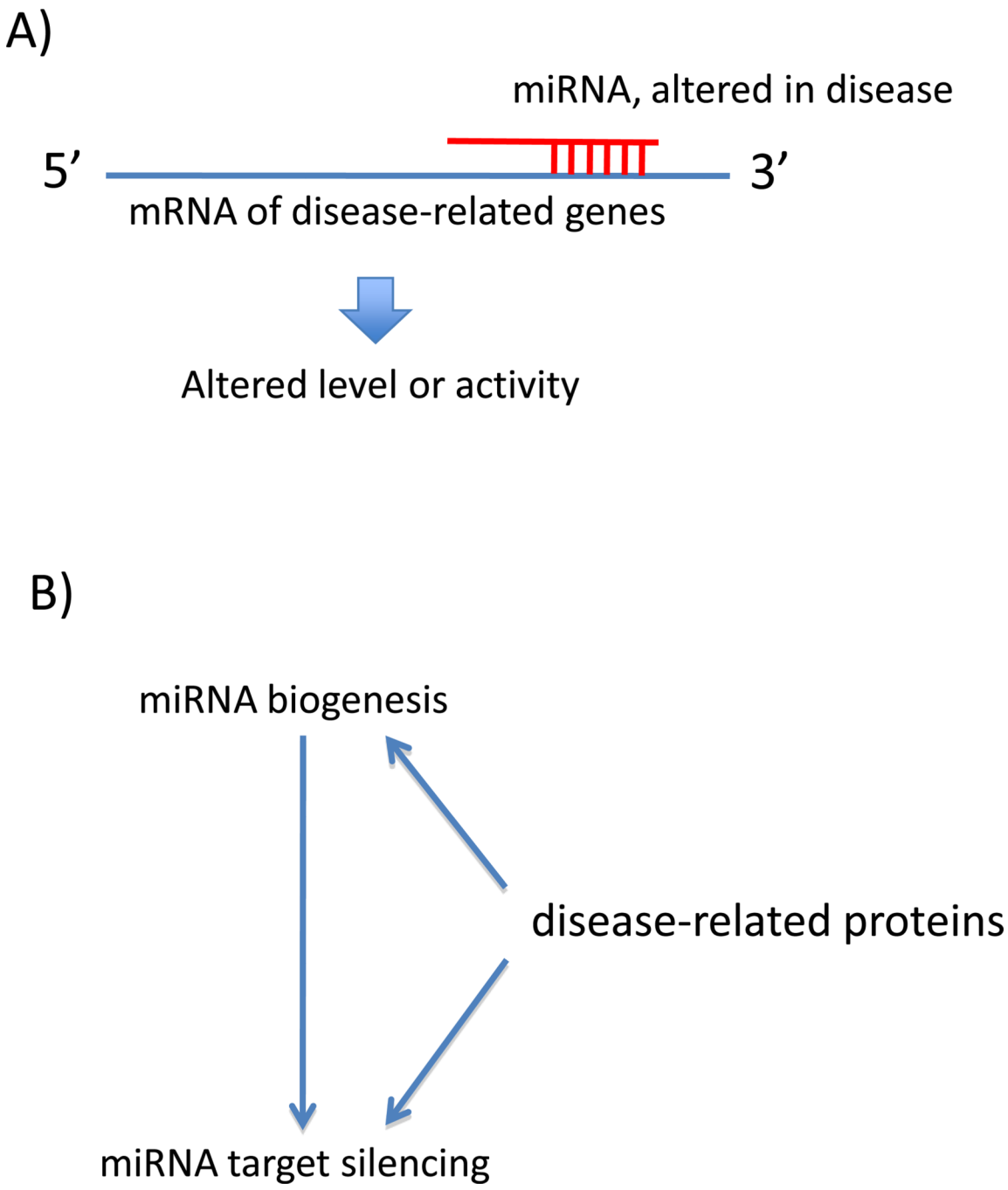

Figure 2. Ways by which miRNA pathways impact neurodegenerative disease

(A) miRNAs, which can be altered in disease, may directly target disease-related transcripts, to alter their translation or level.

(B) Evidence also suggests that some disease-related proteins, such as HTT and TDP-43, may directly affect miRNA biogenesis or miRNA target silencing activity.