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Posttranscriptional control of neuronal development by microRNA networks

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Abstract

The proper development of the nervous system requires precise spatial and temporal control of gene expression at both the transcriptional and translational levels. In different experimental model systems, microRNAs (miRNAs) – a class of small, endogenous, noncoding RNAs that control the translation and stability of many mRNAs – are emerging as important regulators of various aspects of neuronal development. Further dissection of the *in vivo* physiological functions of individual miRNAs promises to offer novel mechanistic insights into the gene regulatory networks that ensure the precise assembly of a functional nervous system.

Introduction

Recent progress in understanding the different classes of small, noncoding RNAs has led to the discovery of novel gene regulatory mechanisms. This knowledge greatly enhances our ability to decipher the complex molecular networks that control the precise spatial and temporal patterns of gene expression crucial for various aspects of animal development. One class of regulatory RNAs is microRNAs (miRNAs), small RNAs of ~22 nucleotides (nt) processed from genome-encoded transcripts of 70–80 nt [1–5]. The first two miRNAs shown to be involved in the control of developmental timing in *Caenorhabditis elegans* were lin-4, discovered in 1993 [6], and the evolutionarily conserved let-7, discovered in 2000 [7]. Since then, hundreds of miRNAs have been cloned from different species, and a dozen or so have been found to regulate several developmental processes by inhibiting translation or destabilizing target mRNAs [8–11]. Moreover, each miRNA is predicted to target hundreds of mRNAs, suggesting that many protein-coding genes are potentially regulated by this pathway [8–11].

Many miRNAs are developmentally regulated and show tissue-specific expression patterns, including dozens expressed only in the nervous system [12–15]. These miRNAs may play important roles in neuronal development, neuronal function, or both [16,17]. This review summarizes recent exciting findings about the roles of miRNAs and some miRNA pathway proteins in neuronal development, from early neurogenesis and cell-fate specification to neuronal differentiation and synaptic development of postmitotic neurons. The potential link between the miRNA pathway and human neurological disorders is also discussed.

Dicer in neuronal development

One of the key enzymes in miRNA biogenesis is Dicer, a member of the RNase III family of nucleases that cleave double-stranded RNAs [18]. In the canonical miRNA pathway,

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primary miRNAs (pri-miRNAs) are mostly transcribed by RNA polymerase II and contain 5′ cap structures and poly(A) tails. Pri-miRNAs are processed by the RNase III Drosha in the nucleus to produce ~70 nt precursor miRNAs (pre-miRNAs) with a hairpin structure [19] which are transported to the cytoplasm by exportin-5 in a Ran-GTP-dependent manner [20]. Dicer is required to cleave pre-miRNAs and generate an ~22 nt miRNA duplex that is incorporated into the RNA-induced silencing complex (RISC) [18,21,22]. In the absence of Dicer, most, if not all, miRNAs are not produced properly. Besides its functions in many other developmental processes [23–26], Dicer is essential for proper brain morphogenesis: maternal-zygotic *dicer* zebrafish mutant embryos fail to produce mature miRNAs and exhibit gross morphological defects in the nervous system. Remarkably, these defects were largely rescued by injection of microRNA-430 (miR-430) alone [27]. Although this study did not detect any dramatic disruption of major signaling pathways involved in early patterning, subtle defects remain to be examined in detail.

A more informative approach is to remove Dicer activity in specific neuronal cell types. In mice, for instance, deletion of Dicer in postmitotic midbrain dopamine neurons causes a progressive loss of those cells, suggesting an essential role of miRNAs in the differentiation or maintenance of dopamine neurons [28]. A good candidate for this process is miR-133b, which is enriched in midbrain and deficient in patients with Parkinson's disease and in animal models of that disorder. Loss of miR-133b activity seems to increase dopamine release slightly in cell cultures [28], suggesting a subtle role in dopamine neuron maturation and function.

The essential role of Dicer in the maintenance of the mature nervous system is also demonstrated by the finding that loss of Dicer-1 in *Drosophila* dramatically enhances neurodegeneration caused by a mutant form of the spinocerebellar ataxia type 3 (SCA3) protein; this effect may be explained, at least in part, by the absence of *bantam*, an miRNA that prevents apoptosis during development by suppressing the proapoptotic gene *hid* [29]. Consistent with this notion, selective genetic ablation of Dicer in Purkinje cells leads to cerebellar degeneration and ataxia [30]. Moreover, in *miR-8* mutant flies, the increased expression of the transcriptional regulator Atrophin increases apoptosis in the nervous system [31]. Thus, miRNAs are important in maintaining the structural integrity of mature neurons through fine-tuning the expression levels of key target genes.

Dendritic spines and postsynaptic densities are enriched in Dicer, raising the possibility that it also participates in synaptic development and plasticity [32]. However, the underlying mechanisms may be complicated. Besides the canonical miRNA pathway, Dicer is also required to generate mirtrons—novel ~22 nt small RNAs processed from short intronic hairpins [33,34]. Unlike miRNAs processed by the canonical miRNA pathway, mirtrons are generated independently of Drosha. The involvement of Dicer in other processes, such as heterochromatin assembly and processing of other endogenous double-stranded RNAs, also remains to be further elucidated in various model systems [35]. Therefore, neurodevelopmental defects as a result of the global loss of Dicer activity need to be interpreted with caution. For this reason, using loss-of-function approaches to understand the functions of specific miRNAs in different aspects of neuronal development may prove to be more informative.

Fragile X mental retardation protein 1

Another protein implicated in miRNA biogenesis is the fragile X mental retardation protein 1 (FMRP). Loss of FMRP function causes fragile X syndrome, the most common form of inherited mental retardation in humans [36]. FMRP is an evolutionarily conserved RNA-binding protein with two ribonucleoprotein K homology (KH) domains and an arginine- and glycine-rich domain (RGG box) that preferentially bind *in vitro* to tertiary RNA structures

named the ‘kissing complex’ [37] and the ‘G quartet’ [38], respectively. Although hundreds of mRNAs associate preferentially with FMRP-containing complexes [39,40], systematic identification of the RNA species that directly bind to FMRP in the native environment in neurons remains a major challenge.

Although the exact function of FMRP at the molecular level still remains unknown, it appears to be associated with the miRNA pathway. In concurrent attempts to identify new components in purified RISC [41] and in a complex associated with the *Drosophila* homolog of the FMRP (dFMR1) [42], it was found that dFMR1 and the RISC subunit Argonaute 2 (Ago2) form a complex in *Drosophila* S2 cells. Ago2- and dFMR1-associated complexes also contain Dicer and miR-2b [41,42]. Moreover, FMRP, the mammalian Argonaute family protein elF2C2 and 20 nt small RNAs with unknown identity were found in the immunoprecipitated mRNP complexes from human tumor cell lines [43]. These findings suggest a role for dFMR1 in the miRNA pathway but dFMR1 does not appear to be an essential component in RISC [41,42].

Mutant mice or flies lacking FMRP or dFMR1 are viable and have a grossly normal nervous system. Nonetheless, the loss of these proteins causes various defects in spine and synapse formation [44–47], axonal and dendritic growth and branching [48–51] and neurogenesis [52]. Although FMRP is a multifunctional protein involved in several different stages of RNA metabolism, its effects on neuronal development are probably mediated at least in part through the miRNA pathway. Consistent with this notion, dFMR1 interacts with Mel31B, a P-body protein required for miRNA-mediated repression of translation [53]. Further dissection of the exact role of dFMR1 and FMRP in RISC will help explain the link between the miRNA pathway and human mental disorders.

miRNAs in early neurogenesis

Genetic analysis of individual miRNAs has begun to shed light on their specific functions in different aspects of neuronal development, from early neurogenesis to synaptic formation. The *Drosophila* peripheral nervous system is an excellent model system for dissecting genetic programs underlying early neurogenesis. Clusters of ectodermal cells that express proneural genes give rise to sensory organ precursors (SOPs) through lateral inhibition, a process that requires the actions of the Notch signaling pathway (Figure 1) [54]. Enhancer of Split complex (E[spl]-C) and the Bearded complex (Brd-C) are major Notch target genes whose 3′ UTRs contain potential target recognition sequences for several miRNAs [55]. Indeed, ectopic expression of miR-7 and a few other miRNAs increases SOP production, likely through downregulation of Notch target genes, although the effects of these miRNAs on SOP specification have not been examined by loss-of-function approaches [56].

Drosophila miR-9a is 100% conserved at the nucleotide level with vertebrate miR-9a, which is specifically expressed in the brain [12–14], raising the possibility that it may play an important role in brain development. The physiological function of *miR-9a* in neuronal development was revealed in *Drosophila* by both loss- and gain-of-function analyses [57]. *miR-9a* mutant flies are viable and fertile, but a small number of mutant embryos or adults show extra sensory neurons and sensory bristles as a result of increased production of SOPs during early neurogenesis. Conversely, ectopic expression of the *miR-9a* precursor suppressed SOP specification [57]. miR-9a is highly expressed in epithelial cells, including those adjacent to SOPs in proneural clusters, indicating that miR-9a inhibits neuronal fate in non-SOP cells to ensure the precise specification of neuronal precursors during development (Figure 1).

During SOP specification, expression of the zinc finger transcription factor Senseless (Sens) must be downregulated in non-SOP cells in the proneural cluster [58]. The *sens* 3′ UTR

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contains three computationally predicted miR-9a binding sites [59]. Indeed, in transfected HEK 293 cells, reporter gene expression was suppressed by miR-9a through the sens 3′ UTR [57]. More importantly, *miR-9a* and *sens* showed strong genetic interactions. For instance, loss of one copy of *sens* significantly rescued the SOP defects in *miR-9a* mutants, indicating that the *sens* 3′ UTR is a key, physiologically relevant *in vivo* target of miR-9a in non-SOP cells during early neurogenesis [57]. Although each miRNA is predicted to target hundreds of mRNAs, it is possible that only the alterations in the expression levels of a few key target mRNAs are relevant to a specific biological process.

It is likely that miR-9a also regulates early neurogenesis in vertebrates, as it is specifically expressed in proliferating neural precursors in zebrafish [14] and in mouse embryos and adult mice [60,61]. In fact, miR-9a appears to contribute to the *in vitro* differentiation of embryonic stem cells [62]. Whether miR-9a also functions to suppress the random activation of neurogenic genes during mammalian neurogenesis is unknown. Loss-of-function studies in genetically altered mice should offer novel insights into this important question. miR-9a is 100% conserved at the nucleotide level from flies to humans, but the mechanism of its action and its targets may not be evolutionarily conserved. Indeed, the microRNA let-7 regulates different genetic pathways in different organisms, probably through distinct downstream target mRNAs [63]. The key target mRNAs of miR-9a in mammalian neurogenesis are determined.

Lsy-6 and miR-273 in cell-fate specification

A remarkable feature of the nervous system is the diversity of its neurons, which differ in dendritic morphologies, axonal targeting specificities, neurotransmitters and other cell-fate-specific characteristics. Although transcriptional control of neuronal cell identity is well established, miRNAs are also involved in this important step of neuronal development.

In *C. elegans*, for example, two morphologically similar chemosensory neurons – ASE left (ASEL) and ASE right (ASER) – express different chemoreceptors that correlate with the functional differences between the two neurons [64,65]. lsy-6, the first miRNA shown to be involved in neuronal development, is expressed in ASEL but not in ASER (Figure 2). Genetic analysis showed that lsy-6 is required to specify ASEL identity: loss of lsy-6 leads to loss of the ASEL-specific chemoreceptor Gcy-7 and ectopic expression of the ASER-specific chemoreceptor Gcy-5 in ASEL neurons [66]. lsy-6 exerts its effects by binding to the 3′ UTR of *cog-1*, an Nkx-type homeobox gene, which leads to downregulation of Cog-1 expression [66]. The specific expression of lsy-6 in ASEL but not in ASER neurons is controlled by the zinc finger transcription factors Lsy-2 [67] and Die-1 [68]. Interestingly, miR-273 is expressed at a much higher level in ASER than in ASEL neurons, and overexpression of miR-273 in ASEL suppresses Die-1 expression, although the effects of loss of miR-273 have not been examined yet [68]. Thus, downregulation of Die-1 in ASER is probably a result of the action of miR-273. Moreover, miR-273 expression is activated by Cog-1. Suppression of Cog-1 expression in ASEL by lsy-6 accounts for the lower expression of miR-273 in those neurons than in ASER neurons [69]. These findings indicate that specific miRNAs and transcription factors form a double-negative feedback loop to maintain the cellular identities of ASEL and ASER neurons (Figure 2) [70]. In this case, the direct effect of miRNAs on target gene expression does not have to be dramatic, but they can still function as developmental switches as a result of their involvement in feedback loops that can amplify and maintain different expression levels of key transcription factors in these two types of neurons.

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miR-124a in neuronal differentiation

miR-124a is 100% conserved at the nucleotide level from worms to humans and is expressed throughout the embryonic and adult central nervous systems of different species [12–15]. It is estimated that miR-124a is the most abundant miR in the brain, accounting for 25%–48% of all brain miRNAs [12]. Thus, it may play an important role in neuronal differentiation or function.

In mouse brain, miR-124a seems to be largely restricted to differentiating and mature neurons, with much less expression in neural progenitors [60]. This expression profile seems to be controlled by RE1 silencing transcription factor (REST), a transcription repressor that inhibits miR-124a expression in nonneuronal cells and neural progenitors but is absent from the *miR-124a* locus in mature neurons [71].

Ectopic expression of miR-124a in HeLa cells leads to the suppression of a large number of nonneuronal transcripts [72]. Some of these transcripts are elevated in cortical neurons treated with antisense 2′-O-methyl oligonucleotides complementary to miR-124a [71], suggesting that these mRNAs are endogenous targets of miR-124a. Therefore, neuronal differentiation may require both derepression of REST and downregulation of some mRNAs by miR-124a. As the most abundant miRNA in the brain, miR-124a likely regulates many target mRNAs and therefore may even play a role in maintaining the homeostasis of differentiated neurons. For instance, target mRNAs encoding small C-terminal domain phosphatase 1 (SCP1) [73], laminin γ1 and integrin β1 [74] are downregulated in differentiated neurons (Figure 3). Moreover, downregulation of the RNA-binding protein PTBP1 by miR-124a during neuronal differentiation leads to a global neuron-specific alternative splicing pattern [75].

The exact developmental consequences of the loss of endogenous miR-124a in differentiating neurons are unclear. In the chick neural tube, neither inhibition nor overexpression of miR-124a altered neuronal fate, as assessed with cell-specific markers [74]. However, another study reported a seemingly opposite result from the same assay system [73]. The reason for this discrepancy is unknown. Further investigation will be required to determine whether endogenous miR-124a affects other aspects of neuronal differentiation, such as dendritic/axonal growth as in the case of miR-132 [76], or synaptogenesis, as in the case of miR-134 [77].

miR-7 in photoreceptor differentiation

Another well-established system for studying cell differentiation is the *Drosophila* eye, which consists of ~800 ommatidia. In each ommatidium, the R8 photoreceptor neuron differentiates first and recruits other progenitor cells to differentiate into seven other photoreceptor neurons and support cells. A key factor that ensures the timely differentiation of retinal cells is the ETS-domain transcription repressor Yan, which is expressed in progenitor cells and suppresses their differentiation into photoreceptors [78]. Signaling through the epidermal growth factor leads to activation of the RAS-ERK pathway and rapid degradation of Yan [78]. The absence of Yan in differentiating cells is essential for the specification of photoreceptor neuronal fate.

Yan controls miR-7 transcription, and high-level Yan expression in progenitor cells represses miR-7 expression. In differentiating cells, miR-7 expression is elevated as a consequence of Yan degradation, and miR-7 further represses Yan expression by binding to sequences in its mRNA 3′ UTR. Thus, miR-7 and Yan form a reciprocal negative feedback loop and show a mutually exclusive expression pattern (Figure 4). Indeed, ectopic expression of miR-7 promotes photoreceptor neuron differentiation [79]. Interestingly, *miR-7* loss-of-function mutants have no obvious defects in eye development [79],

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suggesting that in this particular case, miR-7 does not function as an absolute switch in the feedback loop. ERK-mediated phosphorylation and degradation likely play a major role in downregulating Yan in differentiating photoreceptor neurons, whereas miR-7 ensures its complete depletion. Such a negative feedback loop also operates between miR-133b and the paired-like homeodomain transcription factor Pitx3 during the maturation of midbrain dopamine neurons [28], raising the possibility that it is a common module in gene regulatory networks.

miR-134 in synaptogenesis

Since the discovery of polyribosomes near spines in distal dendrites of dentate granule neurons in 1982 [80], a large number of mRNAs have been found in dendrites [81]. The functional significance of local protein synthesis in dendrites was demonstrated by its requirement in brain-derived neurotrophic factor (BDNF)-induced synaptic plasticity in the hippocampus and by experiments in different experimental systems [81]. Because miRNAs, as well as several protein factors that either positively or negatively influence mRNA translation and stability, are often associated with active polyribosomes, some miRNAs are expected to be present in dendrites and help control local protein synthesis. Therefore, these miRNAs may contribute to synapse formation and synaptic function by regulating the local translation of their target mRNAs.

Indeed, miR-134, a brain-specific miRNA, localizes near synaptic sites in dendrites of hippocampal neurons and regulates the size of dendritic spines [77]. miR-134, but not let-7c, negatively regulates the width of dendritic spines but not their density or dendritic branching. Reduced miR-134 activity with 2′-O-methylated antisense oligonucleotide decreased spine width by 7.6%. Considering the heterogeneity and the dynamic nature of dendritic spines on cultured neurons, this phenotype is relatively subtle and suggests a modulatory role for miR-134 in spine formation. Future genetic knockout of miR-134 will no doubt reveal the full extent of miR-134 function in this important process.

How does miR-134 exert its effect on spine shape? The 3′ UTR of *Limk1*, one of the BDNF-induced genes that regulate actin polymerization and microtubule disassembly [82], contains one miR-134 binding site and acts as the major downstream mediator of miR-134 function. miR-134 negatively regulates the translation of *Limk1* mRNA in dendrites through its 3′ UTR in a manner that is dependent on the miR-134 binding site. Moreover, the effect of miR-134 overexpression on spine shape can be rescued by overexpression of Limk1, whose mRNA is not regulated by miR-134. Interestingly, BDNF treatment relieves miR-134-dependent inhibition of *Limk1* translation, which seems to be mediated by the mammalian target of rapamycin (mTOR) pathway [77]. Exactly how BDNF does so and how miRNAs regulate local translation in dendrites remain to be determined [83]. It is largely unknown what regulates the association and dissociation of miRNAs and their target 3′ UTRs in neurons. However, recent advances in our understanding of the actions of miRNAs in other cell types [84–87] raise the possibility that regulation of translation initiation, elongation, polyadenylation or mRNA stability by different miRNAs in response to extrinsic factors or neuronal activity may operate locally near synapses as well.

Concluding remarks

It has become increasingly clear that miRNAs modulate gene expression levels during multiple steps of neuronal development in diverse organisms, from early neurogenesis to synaptogenesis. In a few cases, miRNAs are involved in feedback loops with some key transcription factors and seem to function as molecular switches in neuronal development. In many other cases, the effects of a specific miRNA are relatively subtle, suggesting that miRNAs ensure the precision of gene expression and the accuracy of these

neurodevelopmental events. This unique function is no less important than other molecular regulators whose misexpression often leads to robust developmental defects. However, our current understanding of miRNA function in the nervous system is still in its infancy, and the number of miRNAs that have been analyzed by loss-of-function approaches remains very small (Table 1). Additional genetic analysis with more sensitive functional assays will undoubtedly reveal the full extent of miRNA function in neuronal development and may offer novel insights into human neurological disorders as well.

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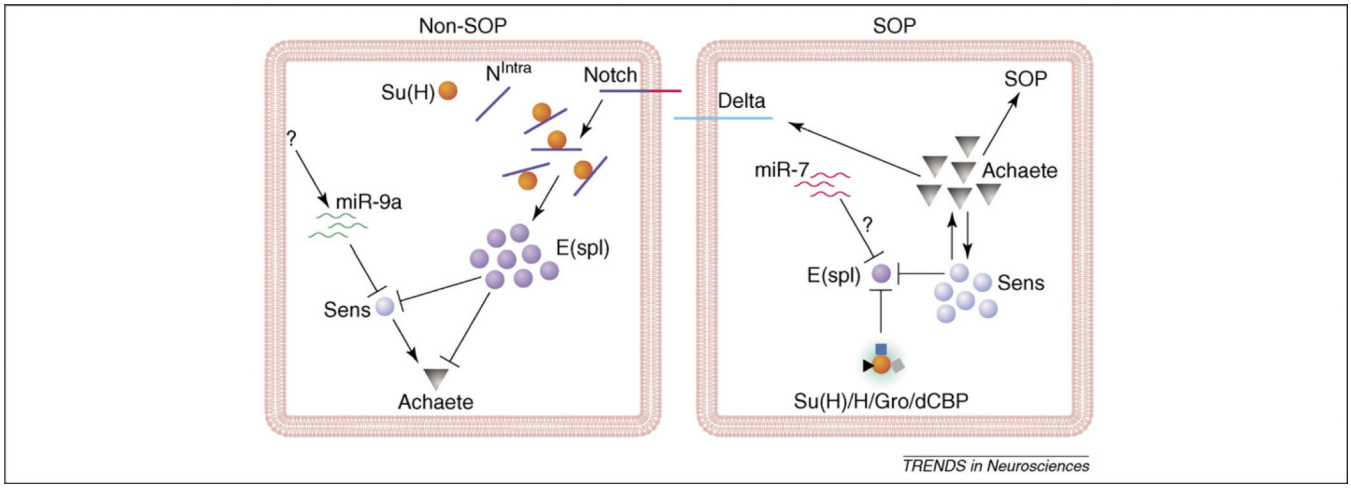

Figure 1.

The roles of miRNAs in the specification of SOPs. In non-SOP cells in the proneural cluster, enhanced Notch signaling leads to the association between Su(H) and Notch intracellular domain (N^{Intra}), which in turn activates the transcription of E(spl). E(spl) suppresses the expression of Sens and proneural genes. To ensure a low level of Sens expression in non-SOP cells, miR-9a suppresses Sens through its 3′ UTR. In SOPs, the lack of Notch signaling leads to the formation of a repressor complex containing Su(H), which inhibits E(spl) expression. Sens expression is high and maintains proneural gene expression that endows the SOP fate. The absence of miR-9a in SOPs is partially responsible for the high level of Sens expression. miR-7 and other miRNAs may be involved in the suppression of E(spl).

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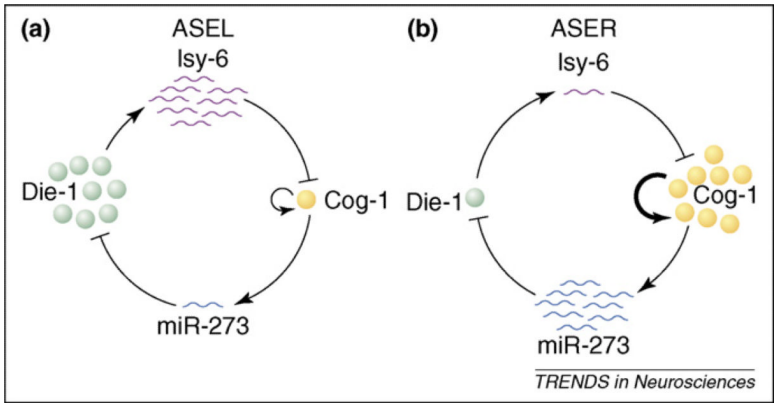

Figure 2.

Schematic representation of the double negative feedback loops between miRNAs and transcription factors. (**a**) In ASEL sensory neurons in *C. elegans*, a high level of lsy-6 suppresses Cog-1, which controls the expression of miR-273. (**b**) In ASER, a high level of miR-273 suppresses Die-1, a transcription factor required for lsy-6 expression.

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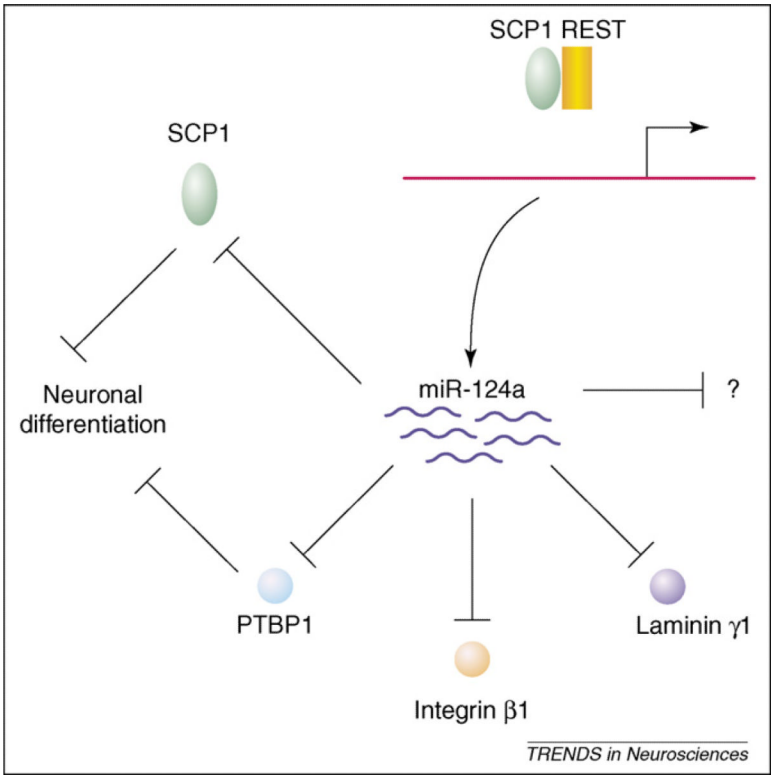

Figure 3.
The role of miR-124a in neuronal development and its regulation by REST. The upregulation of miR-124a expression during neuronal differentiation requires the derepression by the REST-SCP1 complex. As the most abundant miRNA in the brain, miR-124a regulates the expression of many target mRNAs. Yet, the developmental consequences of lack of miR-124a *in vivo* remain to be further examined.

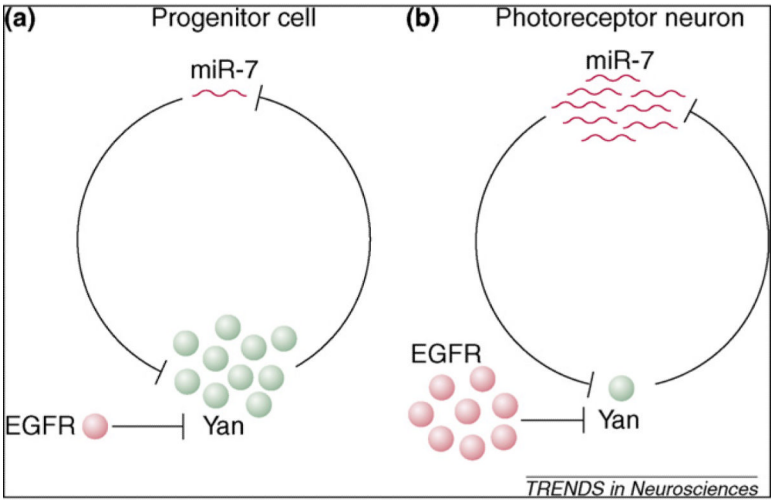

Figure 4.

A negative feedback loop between an miRNA and a transcription factor in *Drosophila*. (**a**) In progenitor cells in the *Drosophila* eye, high-level expression of the transcription factor Yan suppresses miR-7 expression. (**b**) During photoreceptor differentiation, transient activation of the epidermal growth factor receptor (EGFR) signaling pathway leads to the degradation of Yan and the expression of miR-7, which further downregulates the level of Yan through binding to its 3′ UTRs.

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Table 1

The functions of different miRNAs in the development and maintenance of the nervous system

miRNAs	Species	Approaches	Functions	Targets	Refs
lsy-6	*C. elegans*	LOF and GOF *in vivo*	Required to specify ASEL sensory neuron identity	Cog-1	[66,70]
miR-273	*C. elegans*	GOF *in vivo*	Expressed in ASER and suppresses ASEL identity	Die-1	[68–70]
miR-7	*Drosophila*	LOF and GOF *in vivo*	Ensures complete depletion of Yan photoreceptor differentiation	Yan	[79]
miR-430	Zebrafish	Genetic rescue	Required for clearance of maternal mRNAs and brain morphogenesis	??	[27]
miR-134	Rodent	LOF and GOF in culture	Modulates the size of dendritic spines in cultured neurons	LimK1	[77]
miR-9a	*Drosophila*	LOF and GOF *in vivo*	Ensures the precise specification of SOPs in *Drosophila*	Senseless	[57]
miR-124a	Vertebrates	LOF and GOF *in vivo*[a] and in culture	Promotes neuronal differentiation (?)	Laminin γ1, integrin β1, SCP1, PTBP1	[71–75]
miR-132	Rodent	LOF and GOF in culture	Regulates neuronal morphogenesis and circadian clock	P250GAP, etc.	[76,88]
miR-9a	Rodent	LOF and GOF in culture	Involved in neural lineage differentiation from embryonic stem cells	??	[62]
miR-133b	Rodent	LOF and GOF in culture	Regulates the maturation/function of midbrain dopamine neurons	Pitx3	[28]
Bantam	*Drosophila*	LOF and GOF *in vivo*	Prevents neurodegeneration in a *Drosophila* model of SCA3	??	[29]
miR-8	*Drosophila*	LOF and GOF *in vivo*	Required for neuronal survival	Atrophin	[31]
miR-219	Rodent	LOF *in vivo*[b]	Regulates circadian period length in mice	SCOP, etc.	[88]

Abbreviations: GOF, gain of function; LOF, loss of function; SCA3, spinocerebellar ataxia type 3.

[a]Antisense 2′-O-methyl oligonucleotides were used to inhibit the activity of endogenous miR-124a in the developing chick neural tube.

[b]Cholesterol-modified oligonucleotides (antagomirs) were used to repress miR-219 *in vivo*.