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Review Article

miRNAs in brain development

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ABSTRACT

MicroRNAs (miRNAs) are small, non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. In the brain, a large number of miRNAs are expressed and there is a growing body of evidence demonstrating that miRNAs are essential for brain development and neuronal function. Conditional knockout studies of the core components in the miRNA biogenesis pathway, such as Dicer and DGCR8, have demonstrated a crucial role for miRNAs during the development of the central nervous system. Furthermore, mice deleted for specific miRNAs and miRNA-clusters demonstrate diverse functional roles for different miRNAs during the development of different brain structures. miRNAs have been proposed to regulate cellular functions such as differentiation, proliferation and fate-determination of neural progenitors. In this review we summarise the findings from recent studies that highlight the importance of miRNAs in brain development with a focus on the mouse model. We also discuss the technical limitations of current miRNA studies that still limit our understanding of this family of non-coding RNAs and propose the use of novel and refined technologies that are needed in order to fully determine the impact of specific miRNAs in brain development.

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Introduction

MicroRNAs (miRNAs) are single-stranded, endogenously expressed non-coding RNAs of 22–24 nucleotides. They are important regulators of gene expression acting on the posttranscriptional level [6]. In animals, the majority of miRNAs is transcribed by RNA polymerase II, which leads to the generation of a primary miRNA transcript (pri-miRNA; see Fig. 1). This primary transcript resembles protein-coding transcripts in its structure, containing typically a stem-loop structure, a 5′ cap end and a 3′ poly (A) tail [16]. The stem-loop structure of the pri-miRNA is recognised and bound by DiGeorge syndrome critical region 8 (Dgcr8), which recruits the RNAse III enzyme Drosha to form a multiprotein complex. This complex, referred to as microprocessor [23,24], cleaves the pri-miRNA to a precursor miRNA transcript (pre-miRNA) of 50–70 nucleotides [4]. After this nuclear processing, the pre-miRNA is exported through nuclear pore complexes into the cytoplasm through the action of Ras-related nuclear protein-guanosine triphosphate and the nuclear export protein Exportin-5 [36,52]. In the cytoplasm, the pre-miRNA is subsequently cleaved by the RNA III nuclease Dicer, forming mature miRNA duplexes. The miRNA duplexes are denaturated and one miRNA strand is selected, stabilised and bound to Argonaute (Ago) proteins that constitute a part of the RNA-induced silencing complex (RISC). The miRNAs guide the RISC to their targets, and bind to miRNA response elements on the 3′-untranslated region (3′UTR) of mRNAs. Depending on sequence complementary, the binding leads to the degradation or translational repression of mRNA [7].

In addition to the canonical pathway, several alternative pathways for the generation of miRNAs have been identified. For example, miRtrons are miRNAs transcribed from introns, which are processed through intron splicing. These miRtrons skip Drosha cleavage and are immediately transported to the cytoplasm [4]. Furthermore, an alternative biogenesis pathway has been identified for miR-451. The hairpin structure of the pri-miRNA transcript for miR-451 is cleaved by Ago2 in the cytoplasm, thereby bypassing Dicer processing [4].

Numerous miRNAs are brain-enriched or even specific for the brain [39] and many show a high or specific expression during brain development [20,31]. To investigate the function of miRNAs during brain development, most studies have focused on the depletion of core components in the miRNA biogenesis pathway or generation of separate KOs for single miRNAs. In this review, we want to give an overview of investigations on the role of miRNAs during mouse brain development.

The effect of miRNA biogenesis enzymes on brain development

To prevent the production of the majority of mature miRNAs, mice carrying deletions of the different enzymes in the miRNA biogenesis pathways have been generated. Pioneers in this field were Bernstein and co-workers in 2003, who replaced one exon in the Dicer gene with a neomycin-cassette. Homozygous KO mice of Dicer were not viable and died around E7.5 before gastrulation. The survival until this time point was most likely due to the presence of maternal Dicer protein [8].

Since homozygous deletion of Dicer results in embryonic lethality, conditional KOs have been used to study the role of miRNAs during brain development. Cre-mediated deletions of Dicer can be made in any tissue, at any time point, where the Cre is driven by a tissue-specific or even cell-specific promoter, and/or introduced using a viral vector. In many conditional Dicer KO-studies in the brain, proliferation defects and/or apoptosis have been observed, suggesting the importance of miRNAs in cell survival [13,17,26,30,38,44]. Specific Cre-deletion of Dicer in postmitotic dopaminergic neurons has been shown to result in progressive apoptotic loss of dopaminergic neurons in the midbrain. Depletion of this neuronal population was complete at six weeks of age, as demonstrated in tests for reduced locomotion capacity [30]. Two other examples include conditional Purkinje

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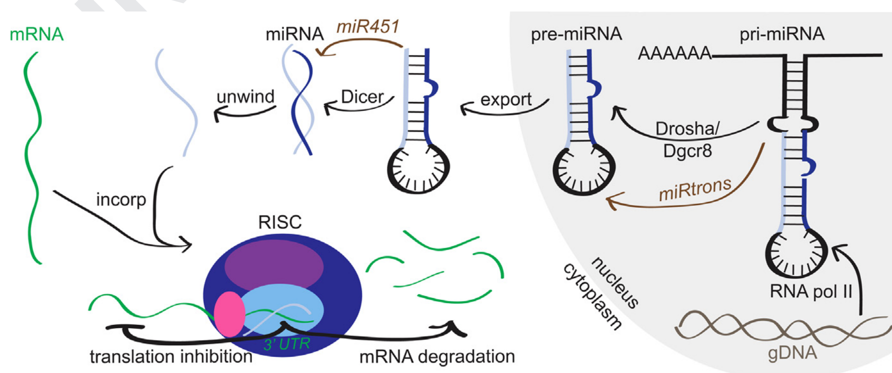

Fig. 1 – miRNA biogenesis and function. In the nucleus, pri-miRNAs are transcribed by RNA polymerase II (RNA pol II). The canonical miRNA pathway is thereafter followed by Drosha/Dgcr8 cleavage into pre-miRNA. miRtrons are known to skip this step and are instead processed through intron splicing and lariat debranching. After exportation of the pre-miRNA into the cytoplasm, the canonical pathway continues with cleavage by the RNA III nuclease Dicer into mature miRNAs. For the miRNA miR-451, this cleavage is performed by Ago2 rather than Dicer. After unwinding of the mature miRNA, one strand is incorporated into the RNA-induced silencing complex (RISC) and recruits its target mRNAs. This binding is dependent on sequence complementarity in the 3′-untranslated region (3′ UTR) of the mRNA. Successful RISC processing results in translation inhibition or degradation of the target mRNA.

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cell Dicer deletion in the cerebellum that led to death of this cell type [44], and selective Dicer inactivation in excitatory forebrain neurons, which resulted in reduced brain size due to postnatal apoptosis [17]. Moreover, Dicer ablation in olfactory progenitor cells has resulted in altered terminal differentiation and loss of maintenance of the progenitor pool [13]. However, it has been demonstrated that the loss of Dicer is not always accompanied by cell death. For example, deletion of Dicer in striatal neurons does not lead to apoptosis, and the cells survive over the lifetime of the animal. Still, the animal exhibits a variety of phenotypes, such as reduced brain size, smaller neurons and front and hind limb clasping [15].

It is apparent that Dicer is required for appropriate brain development in mice. The question remains what happens if Dicer is deleted in neural stem cells? Ablation of Dicer in neuroepithelial cells, and in primary neural stem and progenitor cells during development using Emx1 driven Cre, results in hypotrophy of the postnatal cortex with reduced cortical thickness and defective cortical layering due to apoptosis. These neurons also display impaired differentiation and the mice die soon after weaning. Interestingly, the neuroepithelial cells and their progenitors were unaffected during early stages of neurogenesis (before E14.5) with regards to viability, cell cycle progression and differentiation [18]. This raised the hypothesis that progenitor cells are less dependent on miRNA regulation than their differentiated progeny [3,18]. Deletion of Dicer at embryonic day 14 in a subset of radial glia (RG) progenitors generated an increased number of functional cortical neurons. The observation continued in the postnatal animal, suggesting that this was a result of prolonged cortical neurogenesis. One explanation might be that the mosaic presence of Dicer$^{+/+}$ RG cells provides sufficient support to prevent other defect in the Dicer$^{-/-}$ RG population. Also, it is possible that some miRNAs persists after the deletion [42]. Another study used Emx1 and Nestin to drive Cre and thereby deleted Dicer in neural progenitor cells [29]. No surviving Nestin-Cre Dicer KO mice were born due to severe CNS developmental defects, but the Emx1-Cre-Dicer mice survived until P30. Severe embryonic abnormalities were observed in both KO mice, and the strains displayed diverse cortical defects. This was most likely due to incomplete deletion of Dicer in the Nestin-Cre lines before E18.5. Therefore, it was suggested that normal early progenitor development was enabled by miRNAs that were processed prior to Dicer ablation. They also concluded that Dicer has a critical function in early and late neural stem/progenitor development [29]. This is in contrast to the theory that progenitor cells are less dependent on miRNAs than their differentiated progeny [18].

Taken together, Dicer KOs are embryonically lethal, and cell-type specific Dicer deletion during development has severe consequences for brain development. Further important proteins in the miRNA biogenesis pathway are those of the microprocessor complex. Dgrc8 recognises the pri-miRNA stem loop and recruits Drosha for the cleavage into pre-miRNA. KOs of the proteins in the microprocessor complex differs slightly from the Dicer KO with regards to anatomical, morphological and cellular abnormalities [28,41,49]. Dgcr8 KOs are embryonically lethal, however with a less severe phenotype than Dicer KOs [5]. Interestingly, haploinsufficiency of Dgcr8 results in a phenotype characterised by behavioural alternations and neuronal dysfunction further confirming the important role of miRNAs in the brain [21,48].

Although Dicer KO studies indicate an important role for miRNAs in brain development, conclusions based on the observed phenotypes are hard to interpret. This is due to the fact that Dicer itself has other functions than its involvement in miRNA biogenesis [27]. The disruption of Dicer could result in cellular consequences independent of miRNAs. Also, the existence of alternative miRNA biogenesis pathways independent of Dicer or Drosha complicates the removal of the entire miRNA population after deletion of these enzymes [51]. Therefore, additional studies are required to determine the function of miRNAs by manipulating individual miRNAs and miRNA families.

The effect of specific miRNAs on brain development

To understand the function of a specific miRNA in brain development, KOs of an individual miRNA or miRNA family must be achieved. However, this approach is often complicated, since one miRNA can be transcribed from multiple loci throughout the genome, and within other genes. For example, the miRNA Let-7 family that is highly expressed in the brain contains twelve different members that would make a KO extremely challenging. Moreover, miRNAs are often transcribed in clusters. The deletion or disruption of one miRNA gene could therefore disturb the expression of another unrelated miRNA in the same cluster. In addition, miRNA families share the same seed sequence and have overlapping targets. It is therefore likely that other members would compensate for the deletion of a family member. Despite these difficulties, there are some reports of miRNA KO mice that have enabled loss-of-function studies for specific miRNAs during brain development. Below, we describe the role of miR-124, miR-9 and the miR-17-92-cluster in brain development by focusing on KO-studies of these miRNAs. A summary on the effects of these KOs, together with that of Dicer can be found in Fig. 2.

miR-17-92 in cortical development

The miR-17-92 cluster is transcribed from a polycistronic gene, forming one primary transcript that is later processed to multiple mature miRNAs. The cluster is highly conserved throughout

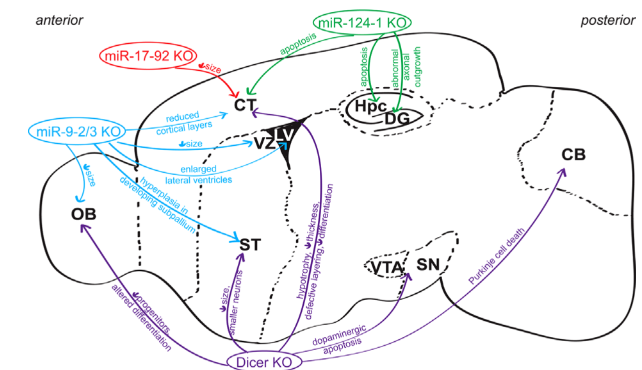

Fig. 2 – Dicer and miRNA knockouts, and their effect on brain development. A summary figure on the outcome of KOs for Dicer, miR-9-2/3, miR17-92 and miR-124-1. The figure gives an overview of effects on the olfactory bulb (OB), cortex (CT), striatum (ST), ventricular zones (VZ), lateral ventricles (LV), hippocampus (Hpc), the dentate gyrus (DG) of the Hpc, the cerebellum (CB) and the dopaminergic populations of the ventral tegmental area (VTA) and substantia nigra (SN).

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vertebrates [40], and together with its paralogues miR-106a-363 and miR-106b-25, the cluster consists of fifteen different miRNAs that belong to the miRNA subfamilies miR-17, miR-18, miR-19 and miR-92 [9]. The miR-17-92 cluster is an oncogene and is often upregulated in various cancers. PTEN is one of its targets, thus regulating proliferation [14]. Also, it regulates oligodendroglial cell number during development by regulating their proliferation [10]. Interestingly, knockdown of miR-17-92 in oligodendrocytes is similar to conditional Dicer loss in this cell type [10]. Furthermore, it mediates proliferation of neural progenitor cells after stroke in mouse [35].

Bian et al. investigated the role of the miR-17-92 cluster and its paralogues in cortical development by generating single (miR-17-92) and paired (miR-17-92 combined with one of the two paralogues) Emx1-Cre KO mice. This study showed that KOs of this miRNA family led to a slight decrease in cortical size. Moreover, they found an upregulation of miR-17-92 target genes *Pten* and *Tbr2*, leading to the loss of RG cells in the cortex and an increase of immediate progenitors. Bian and co-workers therefore suggested an important role for miR-17-92 cluster in regulating the balance between RG cell proliferation, their transition into immediate progenitors, and thus a role in neuronal generation during corticogenesis [9].

miR-9 in telencephalon development

miR-9 is a miRNA that is highly conserved throughout mammalian species. In rodents, it is specifically expressed in the brain with its major expression in neuronal precursors [2,19]. Moreover, it has been shown to be upregulated upon neuronal differentiation. miR-9 is transcribed from three different loci (miR-9-1, miR-9-2, miR-9-3) that produce the same mature miRNA sequence. Shibata and co-workers generated a miR-9-2/3 double KO (single mutations do not effect neurogenesis). The KO displayed retardation with respect to growth. The cerebral hemispheres, the olfactory bulb and also other parts of the brain were found to be of smaller size. Moreover, mutant mice displayed reduction in cortical layers and ventricular zones. The lateral ventricles were enlarged. Shibata et al. also found that the proliferating zone in the subpallium, which gives rise to different neuronal cell types [22] appeared hyperplastic after E12.5. In contrast, differentiated structures were underrepresented. Mutant mice died within one week after birth, demonstrating the crucial role of miR-9 during development in the brain [45].

miR-124 in brain development

miR-124 is one of the highest expressed miRNA in the murine brain and is highly-conserved throughout mammals. The expression of miR-124 is neural-specific and increases upon differentiation into neurons from neural stem cells [1,11,32,37]. Permanent loss of miR-124 function in neural stem cells results in decreased neurogenesis and a switch to gliogenesis [1]. Overexpression in neural progenitor cells, glioma cells, embryonic stem cells or HeLa-cells results in forced neural differentiation, concluding that miR-124 is a neuronal fate determinant [1,32–34,46,47,50].

miR-124 is transcribed from three different loci (miR-124-1, miR-124-2 and miR-124-3) that produce the same mature sequence. The miR-124-1 stem loop is transcribed from an exon within the gene retinal non-coding RNA3 (Rncr3). This gene is responsible for high expression levels of miR-124 in the brain, its nuclear localisation, its consensus sequence and represents its major source.

To investigate the role of miR-124-1 during brain development, miR-124-1 KO mice were generated by deleting Rncr3. In this study, it was found that KO mice had a 60–80% reduction in miR-124 expression. A majority (60%) of KO mice died around postnatal day 20. Rncr3 KO mice showed a significant loss of cone cells, cell apoptosis in hippocampus and cortex, and a decreased brain size. Moreover, axons in the dentate gyrus of Rnc3-depleted mice displayed abnormal outgrowth. Severe neuronal damage was further indicated by a front and hind limb clasping response of KO mice in the tail suspension assay. This neuronal impairment has also been observed in mouse models that mimic neurodegenerative disorders. In summary, the KO of Rncr3 indicates an important role for miR-124 during murine brain development [43].

Discussion and conclusion

Dicer KO studies strongly suggest a crucial role for miRNAs in rodent brain development. However, many questions still remain to be answered. First of all, no definitive conclusion about miRNAs can be drawn by the KO of Dicer due to the fact that Dicer has other regulatory functions in the cell besides miRNA processing. This indicates that the loss of miRNAs may only partially be responsible for the severe phenotypes in these KO mice. Second, several miRNAs are generated by alternative biogenesis pathways, bypassing the processing by Dicer and are in that way not affected by the KO of this enzyme. To address the latter issue, KOs of several enzymes involved in miRNA biogenesis pathway would be necessary to guarantee the complete loss of miRNAs, such as the paired knockout of Dicer/Dgcr8 or Dicer/Ago2. Although such studies would give an overall view on the role of all miRNAs in brain development, it may be more desirable to investigate the function of single miRNAs.

In this review, we discuss studies on KOs of three separate sets of miRNAs that are highly conserved among species and that are expressed at high levels in the brain; miR-124, miR-9 and the miRNA cluster miR-17-92. All three KO studies resulted in severe consequences on brain development, including malformations, cell apoptosis and embryonic lethality. The severity of these KOs demonstrates the indispensible role of miRNAs during murine brain development. However, miR-9 and miR-124 are transcribed from three different loci of the genome, which may complicate the outcome of these studies. Both Sanuki et al. and Shibata et al. only generated partial knockouts (miR-9-2/3 and miR-124a-1), making it difficult to form a definitive conclusion about the role of miR-9 and miR-124 in brain formation. To solve this issue, the entire miR-9 and miR-124 family, respectively, would have to be deleted.

Furthermore, all three studies identify mRNA target genes of the investigated miRNAs, which are directly involved in the development of the KO phenotypes. However, miRNAs are able to regulate hundreds of genes. Therefore, it is likely that the detected target genes are only a small fraction of the whole network of genes that is dysregulated in miRNA KOs and therefore involved in the generation of the KO phenotype.

Taken together, there are still technical limitations making complete miRNA KOs challenging. However, the presented studies demonstrate that certain miRNAs may be indispensible for brain

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development. In the future, it will be interesting to conduct KO studies and use techniques such as translating ribosome affinity purification [25] or Ago2-crosslinking immunoprecipitation [12] followed by high throughput sequencing, to investigate specific miRNA-mRNA target interactions and subsequent changes in gene expression.

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