Timing Specific Requirement of microRNA Function is Essential for Embryonic and Postnatal Hippocampal Development

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Abstract

The adult hippocampus consists of the dentate gyrus (DG) and the CA1, CA2 and CA3 regions and is essential for learning and memory functions. During embryonic development, hippocampal neurons are derived from hippocampal neuroepithelial cells and dentate granular progenitors. The molecular mechanisms that control hippocampal progenitor proliferation and differentiation are not well understood. Here we show that noncoding microRNAs (miRNAs) are essential for early hippocampal development in mice. Conditionally ablating the RNAse III enzyme Dicer at different embryonic time points utilizing three Cre mouse lines causes abnormal hippocampal morphology and affects the number of hippocampal progenitors due to altered proliferation and increased apoptosis. Lack of miRNAs at earlier stages causes early differentiation of hippocampal neurons, in particular in the CA1 and DG regions. Lack of miRNAs at a later stage specifically affects neuronal production in the CA3 region. Our results reveal a timing requirement of miRNAs for the formation of specific hippocampal regions, with the CA1 and DG developmentally hindered by an early loss of miRNAs and the CA3 region to a late loss of miRNAs. Collectively, our studies indicate the importance of the Dicer-mediated miRNA pathway in hippocampal development and functions.

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Introduction

The hippocampus is a well studied brain structure due to its ability to process learning and memory functions. The adult hippocampus is located in the caudomedial edge of the mouse neocortex with a well-defined “C” shape structure that consists of the CA1, CA2, CA3 and the dentate gyrus (DG) regions. The subgranular zone (SGZ) in the adult hippocampal DG contains neural stem cells (NSCs), which can self-renew and give rise to both neurons and glia [1,2]. The presence of active neurogenesis in the adult hippocampus is likely related to cognitive functions and highlights adult NSCs as a promising means for stem cell based therapy for neurodegeneration disorders [3,4].

During brain development, the hippocampus is derived from the medial pallial domain adjacent to the cortical hem in the telencephalon [5,6]. The developing DG has distinct morphology compared to the hippocampal neuroepithelium and consists of migratory granular progenitors [5,6]. The cortical hem is a signaling center that releases multiple signaling molecules, which play an essential role in hippocampal development [7–11]. For example, Wnt3a is expressed in the cortical hem and its downstream gene Lef1 is expressed in the hippocampus. Previous studies have shown that Wnt3a and Lef1 mutant mice display severe hippocampal defects [12,13]. Transcription factors, such as Emx2, expressed in hippocampal neuroepithelial cells; and Lhx5, expressed in the cortical hem, are also important for morphogenesis of the developing hippocampus [14–16]. However, the molecular mechanisms that control precise and dynamic expression of the molecules essential for hippocampal development remain unclear.

A new post-transcriptional gene regulation mediated by micro-RNAs (miRNAs) has been demonstrated to have an important role in the development of the central nervous system (CNS). MiRNAs are approximately 22 nucleotide (nt) endogenous noncoding small RNAs [17,18]. MiRNA precursors are processed into mature miRNAs by the RNAse III enzyme Dicer [19,20]. A mature miRNA recognizes a complementary sequence in the 3′-untranslated region (3′-UTR) of its target messenger RNA (mRNA) and affects mRNA stability and/or silences protein translation [21,22].

Because miRNAs are processed by Dicer, genetic ablation of Dicer in specific tissues has revealed a global functional role of miRNAs in development. Regional specific deletions of Dicer expression in the CNS using different Cre lines result in smaller
cortical size and abnormal development of the midbrain and spinal cord [23–25]. Under a culture condition, cortical NSCs lacking Dicer also show survival defects and abnormal differentiation [26,27]. Dicer ablation in postmitotic neurons using the CaMKII-Cre line results in a smaller cortex and impaired dendritic branching in pyramidal neurons in the CA1 region [28,29]. Interestingly, mice with Dicer ablation in the adult brain using inducible CaMKII-CreERT2 line show enhanced learning and memory [30]. These studies, particularly the different timings of Dicer deletion, imply an important role of miRNAs in brain development, especially in hippocampal morphogenesis and functions.

To reveal miRNA functions in embryonic and postnatal hippocampal development, we here have examined hippocampal neurogenesis and formation by ablating Dicer expression in the brain at different embryonic time points using three Cre lines. We show that miRNAs are required for early proliferation of both hippocampal neuroepithelial cells and dentate granular progenitors. The differentiation of pyramidal neurons in the CA1 and CA3 regions and granule cells in the dentate gyrus is also affected by the different timings of the loss of miRNAs. Our results indicate an important temporal function of miRNAs in modulating morphogenesis of distinct regions in the developing hippocampus.

Results

miRNA function is required for normal hippocampal development

To examine whether miRNAs play a role in hippocampal development, we applied mouse genetic tools to ablate Dicer expression in developing brains using a Cre/loxP system. Floxed Dicer mice (Dicer<sup>lox/lox</sup>) with two loxP sites flanking exon 22 and exon 23, which encode the RNAase III domains of Dicer, were bred with three Cre lines: Emx1-Cre, Nestin-Cre and Nex-Cre lines (Fig. 1A). All three Cre lines are active in the developing hippocampus but commence at different time points, with Emx1-Cre active by embryonic day 9.5 (E9.5) and Nestin-Cre by E10.5 [31–33]. Nex-Cre line displays activity in the hippocampus by E13.5 [34]. Utilization of the Emx1-Cre and Nestin-Cre lines will ablate most progenitors, while the Nex-Cre line will affect postmitotic neurons. By utilizing these three Cre lines, we were able to examine temporal functions of miRNAs in hippocampal progenitor development and neurogenesis at different embryonic stages (Fig. 1B).

Mice with Dicer ablation using the Emx1-Cre line (called Emx1-Dicer cko) displayed a smaller cortex and strikingly, the morphology of the hippocampus (HP) was not clearly distinguishable at postnatal day 0 (P0) (Fig. 1C). Mice with Dicer ablation using the Nestin-Cre line...
(called Nestin-Dicer cko) died after birth [24]. In E18.5 brains, hippocampal morphogenesis occurred normally but the size was reduced, while the whole brain did not change in size significantly in Nestin-Dicer cko mice (Fig. 1D). Later ablation of Dicer using the Nex-Cre line (called Nex-Dicer cko) did not significantly affect hippocampal morphology. However, Nex-Dicer cko mice had a smaller brain compared to control litter mates at P22, and noticeably, the CA3 region in the hippocampus was thinner (Fig. 1E). These results indicate that miRNA functions are required for normal hippocampal development. The more severe defect of the Emx1-Dicer cko hippocampus suggests that earlier Dicer ablation may primarily affect hippocampal progenitor development.

Dicer deletion blocks miRNA biogenesis in the developing hippocampus

To demonstrate whether abnormal hippocampal development was caused by the absence of miRNA biogenesis, we examined expression of representative miRNAs in Dicer conditional knockout brains using in situ hybridization. In E16.5 control mice, while miRNAs Let-7a and miR-9 were highly expressed in the cortex and the hippocampus, their expression was undetectable in Emx1-Dicer cko brains (Fig. 2A, B). Similarly, Let-7a and miR-9 expression was depleted in the E14.5 Nestin-Dicer cko cortex and hippocampus, and was greatly reduced in the P22 Nex-Dicer cko hippocampus (Fig. 2C–F). Our results indicate that miRNA biogenesis is significantly blocked in the hippocampus of Dicer conditional knockout mice generated using all three Cre lines, confirming that the perceived hippocampal defects are mostly caused by the loss of miRNAs.

Dicer deletion affects expression patterns of early hippocampal markers

We next examined whether hippocampal morphogenesis defects are caused by altered expression of hippocampal markers [5,6]. In the developing brain, Wnt7b is expressed in the fimbria region adjacent to the hippocampus, while Wnt downstream gene Lef1 is expressed in the entire hippocampus [12]. In the E16.5 Emx1-Dicer cko fimbria region and hippocampus, where miRNA biogenesis was blocked at an early stage, expression of Wnt7b and Lef1 was greatly reduced compared to controls (Fig. 3A, B). In the wild type hippocampus, while Neuropilin 2 (NRP2) is expressed in
the entire hippocampus, homeobox gene **Prox1** is expressed highly in the DG [12]. In the E16.5 *Emx1-Dicer cko* hippocampus, **NRP2** expression did not display significant changes, but **Prox1** expression was greatly reduced in the DG (Fig. 3C, D). Similarly, in the P1 *Nex-Dicer cko* hippocampus, where miRNA biogenesis was blocked at a late stage, **Wnt7b** expression was reduced, but **Lef1** and **NRP2** expression did not change significantly (Fig. 3E-G). Even though the DG was smaller in the *Nex-Dicer cko* hippocampus, the intensity of **Prox1** expression in the DG was not changed (Fig. 3H).

We then examined expression of transcription factor **Lhx2**, which is expressed in the early hippocampus [5]. **Lhx2** expression was reduced in the E13.5 *Emx1-Dicer cko* hippocampus. The reduction of **Lhx2** expression was more profound in the E18.5 *Emx1-Dicer cko* mice, which exhibited a significantly reduced hippocampus size (Fig. 4A). Similarly, **Lhx2** expression was reduced in the E18.5 *Nestin-Dicer cko* hippocampus (Fig. 4B). Previous work has shown that reduced Lhx2 expression causes an expansion of the cortical hem, where Cajal-Retzius (CR) neurons are generated [8,35]. We thus examined expression of **Reelin**, a CR neuron marker. While **Reelin** expression did not show significant changes in the E13.5 *Emx1-Dicer cko* cortex, ectopic **Reelin** expression was detected in the E18.5 *Emx1-Dicer cko* cortex (Fig. 4C). However, **Reelin** expression was normal in the E15.5 and E18.5 *Nestin-Dicer cko* cortex (Fig. 4D). Our results indicate that early depletion of *Dicer* expression affects expression patterns of hippocampal markers, which may contribute to hippocampal morphogenesis defects. Moreover, altered expression of early markers in the hippocampus perhaps affects cortical hem development and results in abnormal production and migration of CR neurons in the cortex.

**Loss of miRNAs reduces early hippocampal and dentate granular progenitor numbers**

In the developing hippocampus, neurons in the CA1 and CA3 regions are derived from embryonic hippocampal neuroepithelial cells [5,6]. The dentate granular cells are derived from the presumptive dentate gyrus (DG) region adjacent to the hippocampal neuroepithelium (HN) (Fig. 5A). To further examine whether abnormal hippocampal morphology in *Dicer* knockout brains was caused by defects in progenitors, we examined proliferation and survival of hippocampal progenitors.

**Ki67** labels most progenitor cells in the G1, S, G2 and M-phase. We detected a great reduction of Ki67+ cell numbers in the HN region in both *Emx1-Dicer cko* and *Nestin-Dicer cko* brains at E15.5 (Fig. 5B-D). After a 30 min pulse of BrdU injection, which labels cells in the S-phase of the cell cycle, we detected fewer BrdU+ cells in the *Dicer* knockout HN (Fig. 5B-D). Moreover, the number of cells positive for phospho-histone H3 (PH3), which labels mitotic cells in the M-phase of the cell cycle, was greatly reduced in the HN of E15.5 *Emx1-Dicer cko* and *Nestin-Dicer cko* hippocampus (Fig. 5B-D).
Further analysis of dentate granular progenitor development revealed that similarly to the HN, numbers of Ki67+, BrdU+ and PH3+ cells were greatly reduced in the DG region when Dicer expression was deleted in Emx1-Dicer cko and Nestin-Dicer cko brains (Fig. 5B–E). Thus, the early loss of miRNA biogenesis using Emx1-Cre and Nestin-Cre lines causes a significant reduction of proliferating hippocampal neuroepithelial cells and dentate granular progenitors.

Dicer deletion causes a continuous loss of hippocampal progenitors

The CA1, CA3 and DG regions in the hippocampus are mostly formed at E18.5 (Fig. 6A). To test the continuous effects of Dicer deletion in hippocampal progenitor development, we examined the proliferation and survival of progenitor cells in the E18.5 hippocampus. Because the hippocampal morphology was severely disrupted in Emx1-Dicer cko brains by E18.5, mostly due to reduced proliferation and increased cell death (data not shown), the Nestin-Dicer cko mice were analyzed. Using a TUNEL assay to detect apoptotic cells, we observed an increase of TUNEL+ cells in the E18.5 Nestin-Dicer cko hippocampus, particularly in the CA1 and DG regions (Fig. 6B–D). Moreover, we detected decreased numbers of BrdU+ and Ki67+ cells in the CA1, CA3 and DG regions when Dicer was deleted (Fig. 6C–G). Our analyses suggest that the smaller hippocampus in E18.5 Nestin-Dicer cko brains is caused by a decrease of proliferating hippocampal and dentate granular progenitors, and an increase of apoptotic cells.

Dicer deletion causes early differentiation and abnormal maturation of hippocampal neurons

To further reveal the underlying mechanisms that cause abnormal hippocampal development in Dicer conditional knockout mice, we examined neuronal production. In the E18.5 hippocamp-

Figure 4. Abnormal production and migration of Cajal-Retzius neurons in Dicer conditional knockout mouse brains. (A, B) Lhx2 expression was reduced in the E13.5 (arrowheads) and E18.5 (arrows) hippocampus in Emx1-Dicer cko and Nestin-Dicer cko mice. (C, D) Reelin expression, which labels Cajal-Retzius neurons (arrows), was normal in the E13.5 Emx1-Dicer cko cortex, and E15.5 and E18.5 Nestin-Dicer cko cortices. However, ectopic Reelin expression was detected in the E18.5 Emx1-Dicer cko cortex (arrowheads).

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pus, Tbr2 expression was mostly detected in the DG region, which is likely in progenitors. Tbr2+ cells were greatly reduced in the E18.5 Nestin-Dicer cko hippocampus (Fig. 7A, B). At the same stage, the DG region was undetectable in the Emx1-Dicer cko hippocampus, indicating a severe hippocampal defect (Fig. 7A). Ectopic Tbr2+ cells were detected in the cortical progenitor zone along the hippocampal region in both Nestin-Dicer cko and Emx1-Dicer cko brains, and in the dentate migratory stream in the Emx1-Dicer cko hippocampus (Fig. 7A). We next examined expression of Prox1, a DG-specific neuronal marker. While a great reduction of Prox1+ cells was observed in the Nestin-Dicer cko hippocampus, no Prox1+ cells were detected in the Emx1-Dicer cko hippocampus (Fig. 7C, D).

Interestingly, more early-born neurons labeled by Tbr1 were observed in the E18.5 Nestin-Dicer cko hippocampus compared to controls (Fig. 7E, F). In particular, Tbr1+ cells were significantly increased in the CA1 and DG regions of the Nestin-Dicer cko hippocampus. However, the number of mature neurons labeled by NeuN was greatly reduced in the CA1, CA3 and DG regions, while the number of NeuN+/Tbr1+ neurons was increased (Fig. 7G-J). Tbr1 and NeuN expression was undetectable in the E18.5 Emx1-Dicer cko hippocampus due to underdeveloped hippocampal region (Fig. 7E, G). Ectopic Tbr2 expressing progenitors may contribute to increased Tbr1+ neurons in the hippocampus. However, maturation of hippocampal neurons, labeled by Prox1 and NeuN, is reduced. Therefore, our results indicate that while blocking miRNA biogenesis causes early neuronal differentiation, it has a negative effect on maturation of hippocampal neurons.

We next examined hippocampal neurogenesis in P22 Nex-Dicer cko brains, in which miRNA biogenesis was blocked at a later developmental stage. Similar to the Nestin-Dicer cko hippocampus, the number of NeuN+ cells was greatly reduced in the CA1, CA3 and DG regions, in particular, the CA3 region was more severely affected than other regions (Fig. 8A, B). Furthermore, a higher frequency of mispositioned NeuN+ cells was observed along the CA1 and CA3 regions of the Nestin-Dicer cko hippocampus. Our results indicate that different timings of Dicer deletion affect neurogenesis in distinct regions of the hippocampus, with the CA1 and DG more sensitive to an early loss of miRNAs and the CA3 more sensitive to a late loss of miRNAs.

Interestingly, further examination of progenitor cells in the P22 hippocampus of Nex-Dicer cko reveals an increase of Ki67+ cell numbers in the CA3 and DG regions, and an increase of PH3+ cell numbers in the DG region compared to controls (Fig. 8A, C).
Moreover, a great increase of apoptotic cells were detected in the CA3 and DG regions in the P22 Nestin-Dicer cko hippocampus (Fig. 8A, D). Our results suggest that a late loss of miRNAs selectively affects progenitor proliferation and apoptosis in the CA3 and DG regions of the postnatal hippocampus.

**Discussion**

Using mouse genetic tools, miRNA biogenesis was blocked in the developing hippocampus at different embryonic time points. We have found that early miRNA function is required for proper hippocampal morphogenesis, and that it is essential for continuous expansion of hippocampal neuroepithelial and dentate granular progenitors. Interestingly, we have found that the CA1 and dentate gyrus (DG) regions are more sensitive to an early loss of miRNAs, and the CA3 region is more sensitive to a late loss of miRNAs (Fig. 9). Our studies imply that miRNAs may target distinct genes at different time points to ensure proper embryonic and postnatal hippocampal development and functions.

**Early requirement of Dicer for hippocampal formation**

The role of miRNAs has proven to be essential in the development and function of the CNS by ablating Dicer using different Cre lines [23–25,28–30]. In particular, late embryonic deletion of Dicer has caused dendritic branching defects of hippocampal neurons and neurodegeneration [28,29]. These studies indicate the importance of miRNAs, processed by Dicer, in hippocampal formation and functions.

We here have revealed the requirement of miRNA functions in early hippocampal morphogenesis and progenitor expansion. Previous work has demonstrated that patterning molecules, in particular from the cortical hem, play a critical role in early
We have shown that mice with early ablation of Dicer using the Emx1-Cre line by E9.5 fail to develop detectable CA1, CA3 and DG regions. Moreover, there are altered expressions of early hippocampal markers and ectopic Reelin-expressing Cajal-Retzius cells in the cortex. These suggest that miRNAs may regulate proper expression levels and domains of hippocampal patterning molecules such as Wnt and BMPs and their downstream genes, and participate in the process of early hippocampal morphogenesis and the development of Cajal-Retzius cells derived from the cortical hem.

In addition, our studies have shown reduced numbers of hippocampal neuroepithelial cells and dentate granular progenitors when early Dicer expression is deleted by the Emx1-Cre and Nestin-Cre lines (Fig. 9). Reduced Lhx2 expression in progenitors may contribute to smaller hippocampus in the Emx1-Dicer cko and Nestin-Dicer cko hippocampus. Consistent with the role of Dicer in neural stem cell and neural progenitor development, miRNA functions are generally required for expansion of the neural progenitor pool in the cortex and the hippocampus [26,27].

Although increased cell death was detected in the Dicer deficient hippocampus, the reduced hippocampal size was most likely caused by a pronounced proliferation defect of hippocampal progenitors.

The hippocampal defects are more profound in the Emx1-Dicer cko brain than the Nestin-Dicer cko brain, which is consistent with phenotypes observed in the cerebral cortex [24]. We think the following reasons may explain the difference: 1) the timing difference. The hippocampal development is timing sensitive. Earlier deletion of miRNAs by the Emx1-Cre line causes more severe defects in the hippocampus than the Nestin-Cre line; 2) the strength of the Cre activity. The Emx1-Cre may have stronger excision effects of Dicer than the Nestin-Cre and causes a rapid ablation of miRNAs.

Early and late loss of Dicer affects distinct hippocampal regions

Early deletion of Dicer using the Nestin-Cre line reduces progenitor numbers in all hippocampal regions. However, we...
have found that apoptotic cells are mostly localized in the CA1 and DG regions. Moreover, the increase in early differentiated hippocampal neurons is more prominent in the CA1 and DG regions than the CA3 region when miRNA biogenesis is blocked by E10.5. These results suggest that early miRNA functions have a higher impact on neurogenesis in the CA1 and DG regions of the hippocampus (Fig. 9). Interestingly, when Dicer ablation occurs by E13.5 using the Nex-Cre line, the overall hippocampal morphogenesis appears normal. However, we have found that the CA3 region is more severely affected than other regions. Our results are consistent with a previous report showing more severely reduced mature neurons in the CA3 regions when Dicer is ablated by the CaMKII-Cre line [29].

Our results and others indicate the timing requirement of miRNA functions for the proper development of distinct hippocampal regions. Some miRNAs that are highly expressed in the hippocampus at an early stage, for example by E10.5, perhaps play an important role in the formation of the CA1 and DG regions, while some miRNAs highly expressed by E13.5 may be essential for CA3 region development. These miRNAs may regulate different target genes and ensure proper hippocampal morphogenesis. Moreover, our results also imply that the developmental timings of distinct hippocampal regions are different: the commencement of the CA1 and DG regions is perhaps earlier than the CA3 region.

Moreover, surprisingly, we have found an increase in proliferating and apoptotic cells in the CA3 and DG regions of the postnatal hippocampus in P22 Nex-Dicer cko mice, suggesting an active turnover of progenitors in the CA3 and DG regions. Loss of miRNAs at a later stage may release their repression of some target genes that normally promote proliferation and apoptosis, and in turn selectively increases progenitor numbers in the CA3 and DG regions. Interestingly, a recent study has reported that mice with Dicer ablation using inducible CaMKII-Cre line at adult stages display enhanced learning and memory [30]. NSCs in the adult DG region have the potential to continuously generate new neurons, which is important for learning and memory functions [3,4]. Concomitantly, late ablation of Dicer in adult mice may
selectively promote expansion or turnover of NSCs and progenitors in the DG region and contribute to enhanced learning and memory performance [30]. Due to the postnatal lethality of Nex-Dicer cko mice by P23 (Hong and Sun, unpublished data), we could not further examine whether more proliferating cells in the CA3 and DG regions are also detectable in the adult Dicer knockout hippocampus.

Our findings of time-based functions of miRNAs for hippocampal development indicate the complexity of the miRNA regulation in proliferation, survival and differentiation of hippocampal progenitors (Fig. 9). There is perhaps a group of miRNAs that are highly expressed in the hippocampus at early stages. These miRNAs may play a critical role in patterning the specific domains for the hippocampus and the cortical hem. These miRNAs also function in maintaining proliferation and survival status of hippocampal progenitors. Moreover, a group of miRNAs may be highly expressed in the postnatal hippocampus and play a role in controlling differentiation and maturation of neurons in distinct hippocampal regions such as the CA1 and CA3 region. Identifying specific miRNAs will help reveal miRNA-target networks that are essential for embryonic and postnatal development of the hippocampus. These studies will further shed light on noncoding miRNA functions in cognitive behaviors such as learning and memory.

Materials and Methods

Mouse lines and genotyping

The floxed Dicer transgenic mice (Dicer^flox/flox^) (C57/BL6 x 129 background, provided by the Greg Hannon’s lab at the Cold Spring Harbor Laboratory) [36] were bred with three Cre lines: Nestin-Cre (Stock Number: 003771, C57/BL6 background), Emx1-Cre mice (Stock Number: 005628, C57/BL6 background) (The Jackson Laboratory) and Nex-Cre (C57/BL6 background, provided by Drs. M. Schwab and K. Nave at Max-Planck-Institute of Experimental Medicine, Goettingen, Germany). Subsequently generated Dicer conditional knockout (cko) mice were named Emx1-Dicer cko (Dicer^flox/flox^; Emx1-Cre), Nestin-Dicer cko (Dicer^flox/flox^; Nestin-Cre) and Nex-Dicer cko (Dicer^flox/flox^; Nex-Cre).

For staging of embryos, midday of vaginal plug formation was considered embryonic day 0.5 (E0.5), and the first 24 hours after birth was defined as postnatal day 0 (P0).

All animal procedures reported herein were conducted under IACUC protocol (#0807-770A) approved by Weill Cornell Medical College.

Genotyping of Dicer conditional knockout mice

Mouse tail-tip biopsies were used for genotyping by PCR reactions using the following primer pairs: for Cre, 5'-TAAAGATATCTCTCAGTGACTGACGTTG-3’ and 5'-TCTCTTGACCA-GAGTCATCCCTTAGC-3’ (product size: 330 bp); for Dicer, 5'-ATTGTACACGCCCTTAGAATTCC-3’ and 5'-GTACGTCTGACAATTGCTATG-3’ (product sizes: 767 bp from the Dicer^flox^ allele and 560 bp from the wild-type Dicer gene).

BrdU Incorporation

To assess proliferation of neural progenitors in the developing hippocampus, one dose of BrdU (50 μg/g body weight) was administrated by intraperitoneal injection to mice half an hour before sacrifice.

Tissue preparation and immunohistochemistry

Mouse brains were collected at different ages for Emx1-Dicer cko, Nestin-Dicer cko and Nex-Dicer cko mice, and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4°C overnight, followed by incubating in 30% sucrose in PBS. Brain tissues were embedded for OCT and stored at −80°C until use. Brains were sectioned coronally (10 μm/section for immunohistochemistry; 16 μm/section for in situ hybridization) with a Leica cryostat (Leica, CM3050 S).

For immunohistochemistry, sections were incubated in heated solution (1 mM EDTA, 5 mM Tris, pH 8.0; 95–100°C) for 15–20 min for antigen recovery, and cooled down for 20-30 min. Before applying antibodies, sections were blocked in 10% normal goat serum (NGS) in PBS (PBS with 0.1% Tween-20) for 1 hour. Sections were incubated with primary antibodies at 4°C overnight and visualized using goat anti-rabbit IgG-Alexa-Fluor-488 and/or goat anti-mouse IgG-Alexa-Fluor-594 (1:550, Molecular Probes) for 1 hour at room temperature. Images were captured using a Leica digital camera under a fluorescent microscope (Leica DMi6000B). Primary antibodies against the following antigens were used: bromodeoxyuridine (BrdU) (1:50, DSHB), phospho-histone H3 (PH 3) (1:1000, Upstate), Ki67 (1:500, Abcam), Tbr2

Figure 9. Summary of miRNA functions at different time points of hippocampal development. Dicer ablation using the Emx1-Cre, Nestin-Cre and Nex-Cre lines at different embryonic stages in mice affects progenitor proliferation, cell death and differentiation in the hippocampus (HP) and in different regions in the HP such as CA1, CA3 and dentate gyrus (DG). The up and down arrows point out an increased or decreased effect of miRNAs, respectively.

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(1:500, Abcam), Proxl (1:1000, Chemicon), Tbr1 (1:500, Abcam) and NeuN (1:300, Chemicon).

Nissl staining

Brain sections (10 μm) were processed through incubation in the following solutions in order: ethanol/chloroform (1:1, overnight), 100% ethanol (30 sec), 95% ethanol (30 sec), distilled water (30 sec, twice), cresyl violet (3–5 min), distilled water (2 min, three times), 50% ethanol (2 min), 95% ethanol (5–30 min), 100% ethanol (5 min, twice), xylene (3 min, twice), and then mounted with a coverslip.

In situ hybridization

Digoxigenin (DIG)-labeled sense and antisense mRNA probes were produced by in situ transcription. The in situ hybridization on sections was performed as described [37]. Briefly, the sections were hybridized at 65°C overnight and washed. After blocking for 2 hours, sections were labeled with anti-DIG antibody (1:1,500, Roche) at 4°C overnight, sections were labeled with anti-DIG antibody (1:1,500, Roche) at 4°C overnight and washed, stained with BM purple (Roche) at room temperature until ideal intensity. The images of in situ hybridization were collected using a Leica digital camera under a dissection scope (Leica, MZ16F).

In situ hybridization for microRNA expression was performed according to previously published methods with modifications using locked nucleic acid (LNA) probes [38]. Briefly, after fixation with 4% PFA, acetylation with acetylation buffer (1.33% acetic anhydride, 0.25% Triethanolamine, 0.25% Acetic anhydride, 20 mM HCl), sections were hybridized with DIG-labeled LNA probes at 45–55°C overnight and washed, stained with BM purple (Roche) at 4°C overnight. Brain sections were washed with 1xMABT and staining buffer (0.1 M NaCl, 5 mM EDTA, pH 7.5), brain sections were hybridized with DIG-labeled LNA probes at 45–55°C overnight and washed, stained with BM purple (Roche) at room temperature until ideal intensity. The microRNA LNA probes (Exiqon) were 3’ end labeled with DIG–diUTP with terminal transferase using the DIG–3’ end labeling kit (Roche).

TUNEL Assay

To identify apoptotic cells in the hippocampus, a TUNEL assay was performed using the Apop Tag Fluorescein in situ Apoptosis detection kit (Chemicon) on 10 μm frozen sections according to the manufacturer’s instructions.

Cell counting and statistical analysis

Coronal sections were collected in the medial hippocampal region. At least four sections from each brain were chosen for antibody labeling and TUNEL assay. Positive cells were counted in a field, a randomly selected view in the hippocampus. For instance, the hippocampal neuroepithelium (HN) and dentate gyrus (DG) in the E15.5 hippocampus, and the CA1, CA3 and DG regions in the E18.5 and P22 hippocampus were analyzed. At least 5 fields were selected in each hippocampal region on each section. Positive cells and percentage of positive cells for each marker in distinct hippocampal regions were presented.

At least three Dicer conditional knockout and three control littermate animals were used for all statistical analyses. Data were shown as mean ± standard error of the mean (S.E.M). Statistical comparison was made by an analysis of variance (unpaired Student’s t-Test).

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Author Contributions

Conceived and designed the experiments: QL TS. Performed the experiments: QL SB YKK JH EZ. Analyzed the data: QL SB YZ LY. Wrote the paper: TS.
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