Loss of functional Dicer in mouse radial glia cell-autonomously prolongs cortical neurogenesis

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Radial glia of the mouse cerebral cortex emerge from neuroepithelial stem cells around embryonic day 11 and produce excitatory cortical neurons until a few days before birth. The molecular mechanisms that regulate the end of cortical neurogenesis remain largely unknown. Here we investigated if the Dicer-dependent microRNA (miRNA) pathway is involved. By electroporating a cre-recombinase expression vector into the cortex of E13.5 embryos carrying a conditional allele of Dicer1, we induced mosaic recombination causing Dicer1 deletion and reporter activation in a subset of radial glia. We analysed the long-term fates of their progeny. We found that mutant radial glia produced abnormally large numbers of Cux1-positive neurons, many of which populated the superficial cortical layers. Injections of the S-phase marker bromodeoxyuridine between postnatal days 3 and 14 showed that much of this population was generated postnatally. Our findings suggest a role for Dicer-dependent processes in limiting the timespan of cortical neurogenesis.

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Introduction

Radial glia are a class of undifferentiated cerebral cortical progenitor cells that give rise to excitatory neurons and astrocytes (Miyata et al., 2001, 2010; Noctor et al., 2001; Okano and Temple, 2009; Voigt, 1989). Cortical neurogenesis in the mouse normally occurs between E11 and E17, after which radial glia generate mainly astrocytes (Caviness, 1982; Gillies and Price, 1993; Levers et al., 2001; Takahashi et al., 1995). Prolongation of the neurogenic period is believed to have coincided with neocortical expansion in mammals (Kriegstein et al., 2006). It is likely that modifications to the molecular mechanisms controlling the timing of the switch from radial glial neurogenesis to gliogenesis were instrumental in neocortical evolution. While the switching mechanism is of great interest, it still remains relatively obscure (Miyata et al., 2010; Okano and Temple, 2009).

MicroRNAs (miRNAs) are short non-coding RNA molecules that fine-tune gene expression by inhibiting the translation of target mRNAs. Most primary miRNA gene transcripts (pri-miRNAs) are generated by RNA polymerase II. Hairpins containing mature miRNAs (pre-miRNAs) are released from pri-miRNAs by endoribonuclease cleavage by Drosha in the nucleus (Lee et al., 2003). Pre-miRNA hairpins are exported into the cytoplasm and cleaved by Dicer (Bernstein et al., 2003; Nicholson and Nicholson, 2002) into mature miRNA and loaded into the RNA-induced silencing complex (Hutvagner and Zamore, 2002; Mourelatos et al., 2002), where they interact with target sequences in the mRNAs and induce post-transcriptional gene silencing. While most studies identifying miRNA–mRNA target interactions in mammalian systems report only moderate effects of miRNA action on protein levels (Baek et al., 2008), genetic ablation of the miRNA biosynthesis pathway causes severe abnormalities early in embryonic development (Bernstein et al., 2003). Several studies investigated the roles of Dicer-dependent processes in cortical development using conditional genetic removal of the RNase IIIb domain of Dicer in the forebrain with Cre-recombinase driven from Foxg1, Nes, Nex and Camk2 promoters (Davis et al., 2008; De Pietri Tonelli et al., 2008; Kawase-Koga et al., 2009, 2010; Li et al., 2011; Makeyev et al., 2007; Nowakowski et al., 2011) as well as the Hes5 regulatory elements (Knuckles et al., 2012). In all of these in vivo studies, removal of functional Dicer was found to result in an onset of apoptotic cell death, defects in radial glia specification and survival, defects in differentiation, migration and maturation of cortical neurons. Reduced viability of Dicer-deficient cortical progenitors was recently proposed to be associated with the loss of trophic factor support (Andersson et al., 2010). Consistent with this finding, studies inducing Dicer deletion in a small proportion of radial glial or neuronal cells in vivo found that apoptotic cell death is not always an inevitable outcome of Dicer loss.

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consequence of Dicer loss and that Dicer-deficient cells can survive when surrounded by wild-type cells (Cuellar et al., 2008; Nowakowski et al., 2013), offering the opportunity to examine other defects.

Here we extended our analysis of the cell autonomous functions of miRNAs in cortical progenitors. Lineage analysis revealed that radial glia lacking functional Dicer produced abnormally large numbers of Dicer\(^{-/-}\) neurons in the postnatal cortex. Further investigation of this surprising finding revealed that many of these Dicer\(^{-/-}\) neurons were generated after the normal cortical neurogenesis period.

Materials and methods

The licence authorising this work was approved by the University of Edinburgh Ethical Review Committee of 22nd September 2008 (application number PL35-08) and by the Home Office on 6th November 2008. Animal husbandry was in accordance with the UK Animals (Scientific Procedures) Act 1986 regulations. Mice homozygous for Dicer\(^{fl}\) (Harle et al., 2005) were crossed to mice carrying the Rosa26R:YFP (R26RYFP) transgene (Srinivas et al., 2001). Experimental embryos were generated and electroplanted in utero as described before (Nowakowski et al., 2013; Saito, 2006). Bromodeoxyuridine (BrdU) (Sigma) was administered intraperitoneally (50 \(\mu\)g/g body weight). The cre-recombinase construct was kindly provided by Anjen Chenn, Northwestern University. Embryonic brains were fixed in 4\% paraformaldehyde (PFA). Postnatal animals were perfused with oxygenated aCSF containing picrotoxin (50 \(\mu\)M) and varied between embryos of the two genotypes. By P14, control and Dicer-deficient radial glial progenitors were identified by immunohistochemistry using an antibody against both EGFP and YFP (Fig. 1 Ba n d C, Srinivas et al., 2001). The progeny of Dicer-deficient glial progenitors were identified by immunohistochemistry using an antibody against both EGFP and YFP (Fig. 1 Ba n d C, Srinivas et al., 2001). Immunohistochemistry was applied in PBS at 1:10000. Slides were mounted in Vectashield hard-set (Vector) or Prolong gold (Invitrogen). Primary antibodies included in this study were against: BrdU (rat, 1:50, Abcam), Dcx (rabbit, 1:1000, Abcam), Cux1 (rabbit, 1:50, Santa Cruz), GFP/YFP (goat, 1:400, Abcam), NeuN (mouse, 1:500, Chemicon), Sox9 (rabbit, 1:1500, Millipore), and Br2 (rabbit, 1:100, Abcam). Binding was revealed using an appropriate fluorophore-conjugated secondary AlexaFluor antibody raised in Donkey (1:400, Invitrogen). Nuclear counterstain DAPI (Vector) was applied in PBS at 1:10000. Slides were mounted in Vectashield hard-set (Vector) or Prolong gold (Invitrogen).

Electrophysiology

Brains of P14 pups were dissected in ice-cold buffer containing 86 mM NaCl, 1.2 mM Na\(_2\)HPO\(_4\), 25 mM KCl, 25 mM NaHCO\(_3\), 20 mM glucose, 75 mM sucrose, 0.5 mM CaCl\(_2\), and 300 mM coronal slices were prepared using a Leica VT1200S vibratome. Slices were visualised using infra-red DIC/epi fluorescence and betamethylene (NaHCO\(_3\), 20 mM MgCl\(_2\), and 1 mM MMgCl\(_2\)). Borders were defined using Sigmastat (SYSTAT Software). In all figures: *--p < 0.05, **--p < 0.01, and ***--p < 0.001.

Results

Dicer deficient progenitors generate more cortical neurons

We tested the effects on postnatal day 14 (P14) lateral cortex of electroplanted cre-recombinase expression vector (CAG-cre-IRES-EGFP) into E13.5 Dicer\(^{fl}\), Rosa26R:YFP\(^{fl}\) or Dicer1\(^{ft}\) mice and Rosa26R:YFP\(^{fl}\) (control) embryos (Fig. 1 A–C). The progeny of Dicer-deficient radial glial progenitors were identified by immunohistochemistry using an antibody against both EGFP and YFP (Fig. 1 B and C). Dicer-deficient radial glial progenitors were identified by immunohistochemistry using an antibody against both EGFP and YFP (Fig. 1 B and C). Immunohistochemistry was applied in PBS at 1:10000. Slides were mounted in Vectashield hard-set (Vector) or Prolong gold (Invitrogen).

Immunofluorescent sections were imaged using either a Leica microscope connected to a Leica DFC 360 FX digital camera or a Zeiss LSM 150 confocal system. Image analysis including image intensity, area, cortical thickness or distance measurements were performed either in ImageJ or in MatLabR2009a (Mathworks). For embryonic sections, optical sections through the dorso-lateral telencephalon containing electroporated cells were acquired at constant separation. Counting ladders consisting of 200 \(\mu\)m (wide) \(\times\) 40 \(\mu\)m (deep) boxes were positioned in Adobe Photoshop with the base along the white matter. For postnatal sections, brains of all surviving mice were sectioned and analysed for GFP expression. Only brains with GFP\(^{+}\) cells in the dorso-lateral cortex were considered. Counting ladders consisting of 500 \(\mu\)m \(\times\) 100 \(\mu\)m boxes were positioned with the base along the pial edge. For quantification of Doublecortin (Dcx) positive cells, single 400 \(\mu\)m wide box spanning the cortical wall was used. Borders between cortical layers were established based on nuclear counterstaining with DAPI. Student's t-tests were performed using Microsoft Office Excel; Tukey's tests and three-way ANOVA were performed using SigmaStat (SYSTAT Software). In all figures: *--p < 0.05, **--p < 0.01, and ***--p < 0.001.

Immunostaining with antibodies against the neuronal marker NeuN (mouse, 1:500, Chemicon), Sox9 (rabbit, 1:1500, Millipore), and Br2 (rabbit, 1:100, Abcam). Binding was revealed using an appropriate fluorophore-conjugated secondary AlexaFluor antibody raised in Donkey (1:400, Invitrogen). Nuclear counterstain DAPI (Vector) was applied in PBS at 1:10000. Slides were mounted in Vectashield hard-set (Vector) or Prolong gold (Invitrogen).

Electrophysiology

Brains of P14 pups were dissected in ice-cold buffer containing 86 mM NaCl, 1.2 mM Na\(_2\)HPO\(_4\), 25 mM KCl, 25 mM NaHCO\(_3\), 20 mM glucose, 75 mM sucrose, 0.5 mM CaCl\(_2\), 7 mM MgCl\(_2\), and 300 \(\mu\)m coronal slices were prepared using a Leica VT1200S vibratome. Slices were recovered for 30 min at 35 °C in artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 1.2 mM Na\(_2\)HPO\(_4\), 25 mM KCl, 25 mM NaHCO\(_3\), 20 mM glucose, 2 mM CaCl\(_2\), and 1 mM MgCl\(_2\). For recording, slices were visualised using infra-red DIC/epifluorescence optics using an Olympus BX51WI upright microscope and continuously perfused with oxygenated aCSF containing picrotoxin (50 \(\mu\)M) at 33–35°C. Whole-cell recordings were obtained using an Axon Multiclamp 700B amplifier and a custom National Instruments data acquisition/analysis system. Patch-pipettes (resistance 3–5 MΩm) were filled with an internal recording solution containing 130 mM K-methyisulphate, 10 mM KCl, 10 mM HEPES, 0.1 mM EGTA, 10 mM glucose, 10 mM Na-phosphocreatine, 4 mM Mg-ATP, 0.5 mM Mg-GTP, and 5 mM Alexa555 disodium salt, (pH 7.3 with KOH; 290–300 mOsM). Neuron-like YFP-positive cells in layer II/III of the cortex were visually identified and once patched their identity was confirmed by dialysis with Alexa555. Series resistance was monitored throughout each experiment; only experiments where series resistance was < 25 MOhm and varied < 15% were included for analysis.

Imaging and quantification

Immunofluorescent sections were imaged using either a Leica microscope connected to a Leica DFC 360 FX digital camera or a Zeiss LSM 150 confocal system. Image analysis including image intensity, area, cortical thickness or distance measurements were performed either in ImageJ or in MatLabR2009a (Mathworks). For embryonic sections, optical sections through the dorso-lateral telencephalon containing electroporated cells were acquired at constant separation. Counting ladders consisting of 200 \(\mu\)m (wide) \(\times\) 40 \(\mu\)m (deep) boxes were positioned in Adobe Photoshop with the base along the white matter. For postnatal sections, brains of all surviving mice were sectioned and analysed for GFP expression. Only brains with GFP\(^{+}\) cells in the dorso-lateral cortex were considered. Counting ladders consisting of 500 \(\mu\)m \(\times\) 100 \(\mu\)m boxes were positioned with the base along the pial edge. For quantification of Doublecortin (Dcx) positive cells, single 400 \(\mu\)m wide box spanning the cortical wall was used. Borders between cortical layers were established based on nuclear counterstaining with DAPI. Student's t-tests were performed using Microsoft Office Excel; Tukey's tests and three-way ANOVA were performed using SigmaStat (SYSTAT Software). In all figures: *--p < 0.05, **--p < 0.01, and ***--p < 0.001.
Loss of functional Dicer1 causes overproduction of cortical neurons by P14. (A) The experimental design: Dicer1\textsuperscript{fl/fl} and control Dicer1\textsuperscript{fl/+} embryos carrying the R26RYFP reporter allele were electroporated with cre-expression vector at E13.5 and developed until E15.5 or P14. Cells derived from electroporated radial glia were found reproducibly in the lateral cortex (red box). (B) At E15.5, they contributed to the ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ) and the cortical plate (CP), with high densities migrating in the IZ. (C) At P14, they contributed throughout the cortical wall (scale bars in C and D are 50 μm). (D) At P14, average densities of GFP\textsuperscript{+} cells in the developing cortex did not differ between genotypes ($p=0.64, \text{student's } t\text{-test}, n=8$ animals, from three surgeries, 2 sections counted per brain). (E) At P14, average densities of GFP\textsuperscript{+} cells were significantly higher ($p=0.041, \text{student's } t\text{-test}, n=6$ animals, 5 sections counted per brain) in Dicer1\textsuperscript{fl/fl} cortices. Data in D represent means ± s.e.m. $n=9$ animals from 3 surgeries with 1 section analysed per animal. Data in E represent means ± s.e.m. $n=6$ animals from 3 surgeries with 5 sections analysed per animal. (F) At P14 the contribution of GFP\textsuperscript{+} cells to cortical layers I, II/III, V and VI was higher in Dicer1\textsuperscript{fl/fl} than in Dicer1\textsuperscript{fl/+} mice. (G) Average thicknesses of cortical layers were not different between genotypes. (H) Average densities of cell nuclei were higher in layers I, II/III and V of cortices containing Dicer1\textsuperscript{fl/fl} cells. (I) Average densities of GFP\textsuperscript{+}/C0 cells were not different between genotypes. Data in F–I represent means ± s.e.m. $n=6$ animals from 3 surgeries with 5 sections analysed per animal; borders between cortical layers were identified with DAPI nuclear counterstain. (J) Cells generated from Dicer1\textsuperscript{fl/fl} and (K) Dicer1\textsuperscript{fl/+} radial glia were able to adopt neuronal morphologies (arrows). Scale bar, 25 μm. (L–O) To validate that Dicer1\textsuperscript{fl/fl} GFP\textsuperscript{+} cells can develop into functional neurons, whole-cell patch clamp recordings were obtained from layer II/III GFP\textsuperscript{+} cells at P14 ($n=6$ cells from 4 animals from separate surgeries). (L) Wide-field image showing the position of recording electrode. (L) A layer II/III GFP/YFP-expressing Dicer1\textsuperscript{fl/fl} cell filled with AlexaFluor dye following the recording. Scale bar, 100 μm. (M) Depolarisation of a patched layer II/III Dicer1\textsuperscript{fl/fl} neuron causes the neuron to fire action potentials. (N) Whole cell patch-clamp recording from a representative layer II/III Dicer1\textsuperscript{fl/fl} neuron receiving synaptic input. (O) Postsynaptic currents were sensitive to CNQX (5 μM, $n=3$ cells examined).
P14 $\text{Dicer}^{1/-}$ cells fired fast, regenerating action potentials upon depolarisation (Fig. 1L, L', and M), had passive electrical properties normal for fast-spiking excitatory cortical neurons (Fig. 1N) and exhibited spontaneous excitatory postsynaptic currents that were sensitive to CNQX (Fig. 1O). These observations indicate that $\text{Dicer}^{1/-}$ cells can differentiate to form functional neurons capable of responding to synaptic input.

$\text{Dicer}^{1/-}$ cortical progenitors continue to generate increased numbers of cortical neurons postnatally

Comparison of the embryonic migration of $\text{Dicer}^{1/-}$ and control postmitotic neurons using pulses of bromodeoxyuridine (BrdU) at E13.5 or E16.5 (Fig. 2A) revealed no differences in their distributions by E18.5 (Fig. 2B–G). This result suggested that the

**Fig. 2.** Loss of mature miRNAs does not affect the migration of embryonically-generated postmitotic neurons. (A) Experimental design: E13.5 $\text{Dicer}^{100/100}$ or $\text{Dicer}^{100/+}$ embryos were electroporated with cre-expression vector and exposed to a pulse of BrdU on E13.5 or E16.5 and sacrificed on E18.5. (B and C) Representative images of coronal sections through the cortical plate of (B) $\text{Dicer}^{100/100}$ and (C) $\text{Dicer}^{100/+}$ embryos pulse-labelled with BrdU on E13.5. A counting ladder composed of 11 counting boxes was used to quantify the relative distribution of GFP$^+$ cells that were labelled with BrdU given at E13.5 shows no difference between genotypes (data represent means ± s.e.m., p-value calculated using Tukey's test for 10 control and 12 experimental embryos from 3 surgeries, 5 sections analysed per brain). (D) Relative distribution at E18.5 of GFP$^+$ cells that were labelled with BrdU given at E13.5 shows no difference between genotypes (data represent means ± s.e.m., p-value calculated using Tukey's test for 10 control and 12 experimental embryos from 3 surgeries, 5 sections analysed per brain).
explanation for the large postnatal increase in Dicer1−/− neurons might be found after birth. We tested whether increased neurogenesis persists postnatally among Dicer1−/− cortical cells.

We administered a daily pulse of BrdU to electroporated Dicerfl/fl and control Dicerfl/+ animals between P3 and P14 (Fig. 3A). Proportions of GFP+ cells that were BrdU+/ were higher in Dicerfl/fl cortices than in control cortices, with significant increases of up to six-fold particularly in layer I and also in layers II/III, V and VI (Fig. 3B and C), corresponding with the increased contribution of Dicer1−/− GFP+ cells to these layers shown in Fig. 1F. The average densities of GFP−BrdU+ cells were not different between genotypes (not shown). In Dicerfl/fl cortices (n=3), many BrdU+/GFP+ double-positive cells were positive for the neuronal marker NeuN (Fig. 3E, arrows), whereas we never observed such triple-labelled cells in control cortices (n=3) (Fig. 3D–D′). To distinguish astroglial and neuronal populations, cells were immunolabelled for neuronal marker Cux1 and astrocyte marker Sox9. In controls, almost all BrdU+/GFP+ cells in layer I expressed Sox9 but very few expressed Cux1 [Fig. 3F, G–G′, I, J, K–K′, and M], whereas about a third of Dicer1−/− BrdU+/GFP+ cells in layer I expressed Cux1 and about 40% expressed Sox9 (Fig. 3F, G–G′, H–H′, I, J, L–L′, and M). It is likely that the reduction in the proportions of Dicer1−/− GFP/BrdU double-positive cells that expressed Sox9 (Fig. 3M) occurred because a greater proportion of these double-positive cells were neuronal, rather than there being an absolute reduction in the generation of astroglia from Dicer−/− cells. To test this, we compared the average fractions of the Sox9+ cell populations that expressed GFP in the electroporated regions of Dicerfl/fl and Dicer1+/+ cortices. The vast majority, about 90%, of
Sox9 cells were non-electroporated (i.e., GFP−) and there was no evidence that their densities were affected by intermingled electroporated (GFP+) cells. The average contributions (about 10%) of Dicer+/fl and Dicer+/m animals to the glial populations did not differ significantly between genotypes, neither specifically in layer I (Fig. 3N), which has the highest BrdU+ GFP+ cell contribution, nor overall in the cortex (Fig. 3O). These results suggest that 3 surgeries.

In both cases, the requirement for miRNAs was cell autonomous as there was no difference in the densities of surrounding Tbr2+/GFP− cells (Fig. 4E) or Dcx+/GFP− cells (Fig. 4I) in the cortex.

These data indicate that loss of Dicer significantly enhanced the production of neurons postnatally. They suggest that miRNAs normally cell-autonomously restrict the production and persistence of Tbr2-expressing intermediate cortical progenitor cells and hence limit the neuronal output of the cerebral cortex.

Discussion

In this study, we analysed a model of mosaic deletion of Dicer in a subset of radial glia and found an abnormally large population of cortical neurons generated outside the normal time-window of cortical neurogenesis. Furthermore, we observed abnormal maintenance of Tbr2-expressing intermediate progenitors in the SVZ of P14 cortex. Since intermediate progenitors are believed to produce neurons directly (Kowaclzycz et al., 2009), it is likely that postnatal persistence of abnormally large numbers of intermediate progenitors accounts for the extended time-span of mouse cortical neurogenesis. Interestingly, a recent study reported increased numbers of proliferating and apoptotic cells in the dentate gyrus, and reduced numbers of mature hippocampal neurons (Li et al., 2011). This phenotype suggested that long-term loss of functional Dicer may extend the maintenance of undifferentiated neuronal progenitors, or prevent their entry into quiescence, in the hippocampal dentate gyrus, one of the regions where neuronal progenitors are maintained throughout life. Cortical neurogenesis takes place between E11 and E18 and to the best of our knowledge, no manipulation prolonging the maintenance of intermediate cortical progenitors or cortical neurogenesis beyond that period has so far been reported.
While canonical Drosha-Dgcr8-dependent pre-miRNAs remain the best studied substrates for Dicer, recent reports demonstrated that Dicer is also responsible for the cleavage of retrotransposon transcripts (Kaneko et al., 2011) as well as for the maturation of numerous non-canonical miRNAs, endogenous small interfering RNAs and short-hairpin RNAs (Babiarz and Blelloch, 2008; Babiarz et al., 2008; Castellano and Stebbing, 2013). Conditional removal of Dgcr8 from the developing cortex results in a less severe range of phenotypes compared to the loss of Dicer (Babiarz et al., 2011). It remains to be investigated whether loss of particular Dicer-dependent but Dgcr8-independent short RNAs contributes to Dicer knockout-specific phenotypes in the developing cortex.

An intriguing feature of our results was that many of the overproduced Dicer$^{-/-}$ neurons were found in layer I by P14. This could be explained by the loss of miRNAs having a cell autonomous effect, for example by affecting the transcript levels of genes that promote neuronal migration or inhibit terminal differentiation. In line with this hypothesis, miR-134 was recently proposed to regulate migration and differentiation of cortical neurons (Gaughwin et al., 2011). A further contributing factor could be that Cajal–Retzius neurons, which safeguard layer I from invasion by new neurons during normal period of cortical neurogenesis (Hashimoto-Torii et al., 2008), disappear early in postnatal development (Chowdhury et al., 2010) and may therefore not be able to prevent the migration into layer I of Dicer$^{-/-}$ neurons born postnatally. Further studies will be needed to investigate the impact of cellular autonomous loss of Dicer on terminal differentiation of cortical neurons by allowing some of the animals to develop to adulthood and looking at neurite outgrowth complexity and spine development. Several miRNAs have been implicated in these processes, including miR-124, miR-34a and miR-132 (Agostini et al., 2011; Clovis et al., 2012; Franke et al., 2012; Vo et al., 2005; Yu et al., 2008) and it will be interesting to see if loss of Dicer partially or completely recapitulates the defects seen following the specific loss of these miRNAs.

For two aspects of development, the formation of functional neuronal properties and embryonic migration, we did not detect abnormalities among the progeny of the electroporated Dicer$^{-/-}$ radial glia. The latter result contrasts with previous findings from studies using cre-recombinase expressing mice, which reported severe defects in neuronal migration (De Pietri Tonelli et al., 2008; Kawase-Koga et al., 2009; Nowakowski et al., 2011). A possible explanation is that in our mosaics the presence of numerous non-electroporated radial glial fibres may provide sufficient support to rescue many neuronal migration defects observed in models with more widespread deletion throughout the radial glial population. Another possibility is that residual Dicer1 and/or miRNAs persisting after deletion in our model might be sufficient for neuronal migration to occur over the following days. This would be hard to exclude.

Many molecular mechanisms have been implicated in the regulation of the switch from neuro- to gliogenesis of the radial glia, in particular the Notch and BMP signalling pathways and epigenetic mechanisms (Miyata et al., 2010). Various miRNAs have been shown to directly regulate the expression of genes involved in most of these diverse pathways (Fineberg et al., 2012; Gaughwin et al., 2011). While it is likely that the effects we describe here are caused by a loss of many miRNAs deregulating multiple pathways, loss of miRNAs regulating cell autonomous mechanisms are likely to be particularly important. Interestingly, it was suggested recently that miRNAs control the developmental ‘clock’ of radial glia neurogenesis, since early cortical ablation of Dicer1 induced using Emx1$^{*}$ results in a sustained generation of deep instead of superficial cortical layer neurons (Saurat et al., 2013). This study also showed that early loss of functional Dicer in radial glia did not abolish or delay gliogenesis (Saurat et al., 2013), which is in agreement with our findings that Dicer-deficient radial glia contribute normally to the glial population by P14.

It is a strong possibility that at least some of the postnatally-born Dicer$^{-/-}$ neurons are generated from Dicer$^{-/-}$, Tbr2$^+$ intermediate progenitors that are present postnatally in abnormally large numbers. This is suggested by our observation of increased numbers of Tbr2$^+$, Dicer$^{-/-}$ cells postnatally. In a previous study, we observed that mosaic loss of Dicer caused an increased production of Tbr2$^+$, cells prenatally (Nowakowski et al., 2013). It is possible that an expanded population of Dicer$^{-/-}$, Tbr2$^+$ cells present at birth persists postnatally because their cell cycle times become very long, or a combination of mechanisms may be responsible. It is even conceivable that, following the loss of Dicer, Tbr2 becomes re-expressed in either a quiescent cortical progenitor population or in postmitotic cells leading to their re-entry into the cell cycle. Further studies will be needed to investigate these interesting possible mechanisms. Recent studies implicated miR-92a/b in the regulation of intermediate progenitor cell specification in the mouse SVZ by regulating the expression of Tbr2 transcription factor (Bian et al., 2013; Nowakowski et al., 2013). It is possible that over-expression of Tbr2 due to the loss of miR-92a/b could to some extent account for the persistence of abnormally large numbers of progenitors that we observed postnatally. To the best of our knowledge, a study of the long-term postnatal effects of earlier Tbr2 gain-of-function has not been performed, but such a study would help to determine whether an early increase in the production of intermediate progenitor cells would extend the time-span of mouse cortical neurogenesis postnatally due to delayed postnatal depletion of that population.

Based on studies analysing periods of cortical neurogenesis in mouse, ferret and macaque it has been proposed that expansion of the SVZ and prolongation of cortical neurogenesis contributed to the evolutionary expansion of the neocortex (Kriegstein et al., 2006). In agreement with this hypothesis, time windows of cortical neurogenesis in ferret and macaque are, respectively, threefold and eight-fold longer than in mouse (Kornack and Rakic, 1998; Noctor et al., 1997), allowing for additional neurogenic divisions of cortical progenitors. Recent analysis of miRNA expression in developing human, chimp and macaque brains showed rapid changes in developmental expression profiles of a number of conserved miRNAs, including miR-92, in the primate lineage and proposed that miRNAs contributed to the brain’s evolutionary expansion (Somel et al., 2011). It remains to be investigated what developmental processes might have changed due to altered miRNA expression in the developing primate brain. Our current analysis of the genetic ablation of miRNA production in the mouse radial glia demonstrates that miRNAs regulate the timespan of cortical neurogenesis and suggest a mechanism by which changes in miRNA maturation and/or in miRNA expression levels could contribute to the evolution of the cerebral cortex.

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References


