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Age-dependent fate and lineage restriction of single NG2 cells

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SUMMARY

NG2-expressing glia (NG2 cells, polydendrocytes) appear in the embryonic brain, expand perinatally, and persist widely throughout the gray and white matter of the mature central nervous system. We have previously reported that NG2 cells generate oligodendrocytes in both gray and white matter and a subset of protoplasmic astrocytes in the gray matter of the ventral forebrain and spinal cord. To investigate the temporal changes in NG2 cell fate, we generated NG2creER^{TM}BAC transgenic mice, in which tamoxifen-inducible Cre is expressed in NG2 cells. Cre induction at embryonic day 16.5, postnatal day (P) 2, P30 and P60 in mice that were double transgenic for NG2creER^{TM}BAC and the Cre reporter revealed that NG2 cells in the postnatal brain generate only NG2 cells or oligodendrocytes, whereas NG2 cells in the embryonic brain generate protoplasmic astrocytes in the gray matter of the ventral forebrain in addition to oligodendrocytes and NG2 cells. Analysis of cell clusters from single NG2 cells revealed that more than 80% of the NG2 cells in the P2 brain give rise to clusters consisting exclusively of oligodendrocytes, whereas the majority of the NG2 cells in the P60 brain generate clusters that contain only NG2 cells or a mixture of oligodendrocytes and NG2 cells. Furthermore, live cell imaging of single NG2 cells from early postnatal brain slices revealed that NG2 cells initially divide symmetrically to produce two daughter NG2 cells and that differentiation into oligodendrocytes occurred after 2-3 days.

KEY WORDS: NG2 (Cspg4), Oligodendrocyte, Astrocyte, Cre, Glia, Tamoxifen, Mouse

INTRODUCTION

Cells that express the NG2 (Cspg4 – Mouse Genome Informatics) proteoglycan in the central nervous system (CNS) (NG2 cells or polydendrocytes) constitute 2-9% of total cells and represent the largest pool of dividing cells in the adult brain (Dawson et al., 2003). In the mouse forebrain, NG2 cells start to appear around embryonic day (E) 14.5, become uniformly distributed after birth, and persist in the adult CNS (Nishiyama et al., 1996; Nishiyama et al., 2009). It has been highly debated whether NG2 cells are committed oligodendrocyte progenitor cells or generate other cell types (reviewed by Nishiyama et al., 2009).

In a genetic fate-mapping study using NG2creBAC transgenic mice, which express constitutively active Cre in NG2 cells, we have provided direct in vivo evidence that NG2 cells generate oligodendrocytes throughout the brain and spinal cord and a subset of protoplasmic astrocytes in the gray matter of the ventral forebrain and spinal cord (Zhu et al., 2008a; Zhu et al., 2008b). Subsequently, other mouse lines were used to follow the fate of NG2 cells in adult and perinatal brain (Guo et al., 2009). Rivers et al. (Rivers et al., 2008) used a transgenic mouse line that expresses tamoxifen-inducible Cre (CreER^{T2}) under the regulation of the platelet-derived growth factor alpha (*Pdgfra*) gene and reported that NG2 cells in the adult brain generate oligodendrocytes and a small number of neurons in the piriform cortex, but not astrocytes. Dimou et al. (Dimou et al., 2008) used Olig2-CreER^{TM} mice (Takebayashi et al., 2002) to show that NG2 cells in the adult brain generate oligodendrocytes and a small number of protoplasmic astrocytes, but not neurons. In PLP-CreER^{T} mice, NG2 cells were shown to generate astrocytes in the ventral gray matter and some neurons in the piriform cortex in addition to oligodendrocytes (Guo et al., 2009; Guo et al., 2010).

In order to determine the time course of NG2 cell differentiation into oligodendrocytes and astrocytes, we generated NG2creER^{TM}BAC transgenic mice in which tamoxifen-inducible Cre (CreER^{TM}) (Hayashi and McMahon, 2002; Danielian et al., 1993; Takebayashi et al., 2002) is specifically expressed in NG2 cells. By crossing NG2creER^{TM}BAC transgenic mice with the Cre reporter mouse lines and inducing Cre-mediated recombination at different developmental stages, we have found that NG2 cells in the postnatal brain generate oligodendrocytes, whereas those in the embryonic brain generate a subset of astrocytes in the ventral forebrain in addition to oligodendrocytes. We have also performed cluster analysis and live cell imaging to follow the fate of single NG2 cells.

MATERIALS AND METHODS

Cre reporter mice

Z/EG Cre reporter mice were obtained from Dr Caiying Guo (University of Connecticut Health Center, currently at HHMI Janelia Farm). GtROSA26R, gtROSA26-EYFP (YFP) and gtROSA26-mTmG (mTmG) (Muzumdar et al., 2007) mice were obtained from the Jackson Laboratory. The reporter lines were maintained as homozygotes.

Generation of NG2creER^{TM}BAC transgenic mice

NG2CreER^{TM}BAC transgenic mice were generated using the same bacterial artificial chromosome (BAC) modification approach that had been taken to generate the NG2DsRed BAC transgenic mice, as described previously (Zhu et al., 2008a) (Jackson Laboratory stock number 008241), except that DsRed cDNA was replaced by CreER^{TM} cDNA (Danielian et

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al., 1993; Littlewood et al., 1995) (obtained from Dr Takebayashi, Kumamoto, Japan). Linearized NG2creER^{TM}BAC DNA was microinjected into fertilized C57Bl/6 or C57Bl6/SJLF1 oocytes in the transgenic facility at the University of Connecticut Health Center and at Johns Hopkins Medical School, Baltimore, MD, USA. Founder mice were identified by PCR using forward (5′-CCGTACACCAAAATTTGCC-3′) and reverse (5′-ATCGCGAACATCTTCAGG-3′) primers from the Cre cDNA.

Three founder lines were obtained that expressed different levels of Cre activity. When crossed with the Z/EG (Novak et al., 2000) or the gtROSA26R reporter line (Soriano, 1999), one line (NG2CreERA) exhibited leaky reporter expression in the absence of tamoxifen. Another line (NG2CreERC) expressed little detectable reporter after tamoxifen injection. The third line (NG2creERB) had no background reporter expression in the absence of tamoxifen, and the reporter was detected specifically in NG2-expressing cells after tamoxifen injection. Therefore, all the studies described here were performed with the NG2CreERB line, which we will refer to as the NG2creER^{TM}BAC line (Jackson Laboratory stock number 008538). NG2creER^{TM}BAC mice were maintained as homozygotes, and double-heterozygous transgenic mice were used for the experiments.

Induction of Cre-mediated recombination with 4-hydroxytamoxifen

Cre activity in postnatal NG2creER^{TM}BAC:reporter double-transgenic mice was induced by intraperitoneal injection of 4-hydroxytamoxifen (4-OHT, Sigma). A 10 mg/ml stock solution was prepared by dissolving and sonicating 4-OHT in 19:1 autoclaved vegetable oil:ethanol. Postnatal day (P) 30 and P60 mice were injected with 1 mg 4-OHT intraperitoneally twice a day for five consecutive days. P2 mice were injected with 0.2 mg 4-OHT intraperitoneally once a day for four consecutive days. Control animals were injected with the same volume of vehicle. Cre induction efficiency in the forebrain of NG2creER^{TM}BAC:ZEG mice at P2, P30 and P60 was 1.5%, 1.7% and 1.2%, respectively. Cre induction efficiency in NG2creER^{TM}BAC:YFP mice at P2 and P60 was 45.3% and 42.6%, respectively.

Cre activity was induced in embryonic NG2creER^{TM}BAC:ZEG double-transgenic mice by administering 8 mg of tamoxifen to pregnant mothers at E16.5 via gastric gavage using 18-gauge curved stainless steel feeding needles with round tips. Tamoxifen (Sigma) was dissolved in autoclaved corn oil at 20 mg/ml. At E19.5, the pups were delivered by caesarean section. Some pups were sacrificed and processed for analysis. The other pups were fostered until P14, when they were sacrificed and processed for analysis.

Tissue processing

Mice were fixed and processed for immunohistochemistry as previously described (Zhu et al., 2008a). Vibratome sections (50 μm) were prepared for postnatal tissues and cryostat sections (20 μm) were prepared for embryonic tissues.

Antibodies

Primary antibodies were obtained from the following sources. Guinea pig anti-rat NG2 antibody was a gift from Dr William Stallcup (Burnham Institute, La Jolla, CA, USA). Monoclonal antibody to the adenomatous polyposis coli antigen (APC; clone CC1) (Bhat et al., 1996) was purchased from Calbiochem (La Jolla, CA, USA). Mouse antibody to S100β was obtained from Sigma. Rabbit anti-NG2 antibodies, mouse and rabbit anti-GFP antibodies and monoclonal antibody to NeuN were obtained from Chemicon (Temecula, CA, USA). Mouse antibody to aldehyde dehydrogenase 1 L1 (Aldh1L1) was obtained from NeuroMab (Davis, CA, USA).

Cy3- and Cy5-conjugated (Jackson ImmunoResearch, West Grove, PA, USA) and Alexa-conjugated (Molecular Probes, Eugene, OR, USA) secondary antibodies were used.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Zhu et al., 2008a). Dilutions of primary antibodies were as follows: 1:500 for rabbit and mouse GFP and rabbit NG2; 1:200 for guinea pig NG2, for CC1 and NeuN; 1:2000 for S100β; and 1:1000 for Aldh1L1. Dilutions of secondary antibodies were: 1:500 for Cy3-conjugated; 1:50 for Cy5-conjugated; and 1:1000 for Alexa 488-conjugated antibodies. Labeled sections were mounted in Vectashield containing DAPI (Vector Laboratories) and examined using a Zeiss Axiovert 200M microscope equipped with an ORCA ER camera (Hamamatsu) and Apotome or a Leica TCS SP2 confocal microscope.

Cell counts

Cre induction efficiency was obtained 1 day after the last 4-OHT injection (1 dpi) by dividing the number of EGFP$^+$ or YFP$^+$ NG2$^+$ cells by the total number of NG2$^+$ cells in randomly selected fields of defined area in coronal sections through the cerebral hemispheres at the level of the anterior commissure or dorsal hippocampus.

To determine the phenotype of EGFP$^+$ cells in NG2creER^{TM}BAC:ZEG mice, the total number of EGFP$^+$ cells, NG2$^+$ EGFP$^+$ cells or CC1$^+$ EGFP$^+$ cells was obtained from the entirety of each coronal section, and the area of the section was obtained using IPLab software (Scanalytics) on a Leica DMR epifluorescence upright microscope equipped with an ORCA digital camera. To determine the phenotype of YFP$^+$ cells in NG2creER^{TM}BAC:YFP mice, the number of YFP$^+$ cells that were NG2$^+$ or CC1$^+$ was counted in randomly selected defined areas.

To determine the phenotype of EGFP$^+$ cells in EGFP$^+$ cell clusters, clusters with at least two EGFP$^+$ cells that were separated from other clusters by more than 50 μm were scanned throughout one hemisphere. Each cell in the clusters was scored for the expression of NG2 or CC1, and their location (cortex or corpus callosum) was noted.

Three mice from each group were used for all the analyses. All the values are expressed as averages ± s.d.

Time-lapse analysis

To follow the fate of NG2 cells as they divided, slice cultures from P4 or P8 mice that were double transgenic for NG2creBAC (Jackson Laboratory stock 008533) and Z/EG were prepared as described (Bahr et al., 1995) using MilliCell culture inserts (0.45 μm pore size, Millipore). Several fields in each slice were marked, and the same fields were imaged with a 10× objective at 4- to 10-hour intervals. The slices were maintained in the incubator between imaging. At the end of the incubation, slices were fixed in 4% paraformaldehyde for 30 minutes, and the slices were processed for immunohistochemistry using anti-NG2 and CC1 antibodies.

RESULTS

Generation of transgenic mice with inducible CreER^{TM} under the *Cspg4* promoter

Using NG2creBAC:ZEG double-transgenic mice, in which constitutively active Cre in NG2-expressing cells permanently activates EGFP expression in all the progeny of NG2 cells, we previously demonstrated that NG2 cells generate oligodendrocytes throughout the brain and a subset of protoplasmic astrocytes in the gray matter of the ventral forebrain (Zhu et al., 2008a). To examine the ability of NG2 cells to differentiate into oligodendrocytes and astrocytes at different developmental ages, we generated NG2creER^{TM}BAC transgenic mice that express tamoxifen-inducible CreER^{TM} specifically in NG2-expressing cells and crossed them with Z/EG Cre reporter mice. Cre was induced in NG2creER^{TM}BAC:ZEG double-transgenic mice at P2, P30 and P60, and brains were analyzed at 1, 4, 10, 20 and 60 days after the last 4-OHT injection (dpi). At 1 dpi, at all ages, EGFP$^+$ cells in the neocortex and corpus callosum expressed either NG2 or the oligodendrocyte antigen CC1, and the majority of the EGFP$^+$ cells were NG2$^+$ (see Fig. S1A-F in the supplementary material). A small number of EGFP$^+$ cells were CC1$^+$ oligodendrocytes (not shown).

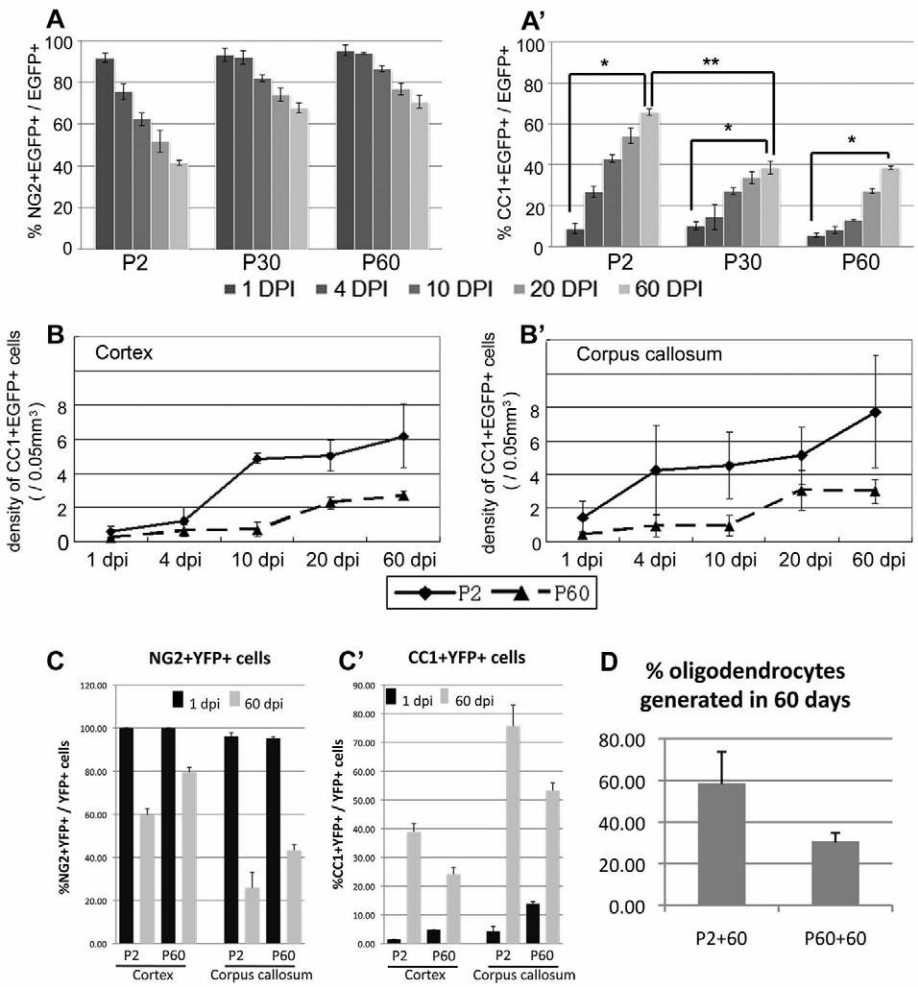

Fig. 1. Oligodendrocyte differentiation from NG2 cells in P2, P30 and P60 mouse brain. (**A**,**A′**) Changes in the composition of EGFP$^+$ cells in the cerebrum over time after Cre induction in NG2creER^{TM}BAC:ZEG mice. (A) The percentage of EGFP$^+$ cells that were NG2$^+$ at 1, 4, 10, 20 and 60 days after Cre induction at P2, P30 and P60. (A′) The percentage of EGFP$^+$ cells that were CC1$^+$ oligodendrocytes at the same time points as in A. The proportion of oligodendrocytes among the EGFP$^+$ cells is significantly higher at 60 days after 4-OHT injection (dpi) than at 1 dpi in all three age groups (*, P<0.05; One-way ANOVA, Tukey's test). The proportion of oligodendrocytes among the EGFP$^+$ cells at 60 dpi in P2-induced mice is significantly higher than that in mice induced at P30 or P60 (**, P<0.05; Student's t-test). Similarly, the proportion of oligodendrocytes among the EGFP$^+$ cells at 4, 10 and 20 dpi is significantly higher in mice induced at P2 than the proportion at the corresponding time points in mice induced at P30 or P60. There is no significant difference between P30 and P60 in the proportion of oligodendrocytes among the EGFP$^+$ cells at any of the survival time points (one-way ANOVA, Tukey's test). (**B**,**B′**) Changes in the density of EGFP$^+$ CC1$^+$ oligodendrocytes in the neocortex (B) and corpus callosum (B′) from 1 to 60 dpi after Cre induction at P2 (solid lines) and P60 (dashed lines). (**C**,**C′**) The percentage of YFP$^+$ cells that were NG2$^+$ (C) or CC1$^+$ (C′) in the neocortex or corpus callosum at 1 or 60 days after Cre induction at P2 or P60 NG2creER^{TM}BAC:YFP mice. (**D**) The fraction of oligodendrocytes in the corpus callosum of NG2creER^{TM}BAC:YFP mice that was newly generated from NG2 cells during a 60-day period starting at P2 or P60. Error bars indicate s.d.

Oligodendrocyte differentiation from NG2 cells continues to occur in the adult but declines with age in NG2creER^{TM}BAC:ZEG mice

To compare the ability of NG2 cells to generate oligodendrocytes at P2, P30 and P60, the proportion of EGFP$^+$ cells that were CC1$^+$ oligodendrocytes at 1, 4, 10, 20 and 60 dpi was determined in NG2creER^{TM}BAC:ZEG double-transgenic mice. All the EGFP$^+$ cells in both the gray and white matter of the cerebral hemispheres were included in the quantification.

The proportion of NG2 cells among EGFP$^+$ cells decreased from 1 dpi to 60 dpi (Fig. 1A), whereas the proportion of oligodendrocytes among EGFP$^+$ cells increased (Fig. 1A′), suggesting that NG2 cells continuously generate oligodendrocytes, even in the mature brain. At 1 dpi, more than 90% of the EGFP$^+$ cells were NG2$^+$, and fewer than 10% were CC1$^+$ in all three age groups. After survival times of 4 days or longer, the percentage of EGFP$^+$ cells that were CC1$^+$ oligodendrocytes was significantly higher in mice induced at P2 than in mice induced at P30 and P60 (Fig. 1A′; P<0.05, one-way ANOVA, Tukey's test). There was no significant difference between the P30 and P60 groups in the percentage of oligodendrocytes among the induced cells. At 60 dpi in P2 mice, 66% of the EGFP$^+$ cells were CC1$^+$ oligodendrocytes. By contrast, only 39% of the EGFP$^+$ cells were CC1$^+$ oligodendrocytes 60 days after 4-OHT injection in P30 or P60 mice. These findings indicate that more oligodendrocytes were generated from perinatal NG2 cells than from NG2 cells in the adult brain.

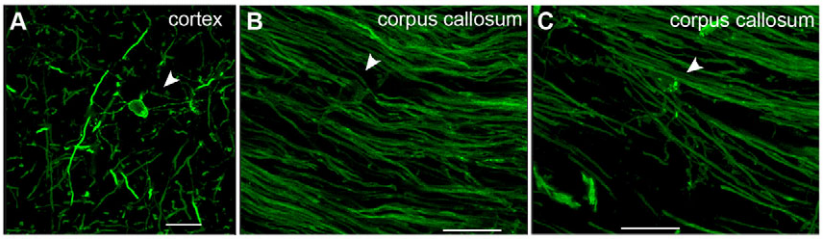

Fig. 2. Myelination by NG2 cell-derived oligodendrocytes. Membrane-bound EGFP expression detected in (**A**) the cortex or (**B**,**C**) the corpus callosum of NG2creER^{TM}BAC:mTmG mice sacrificed 150 days after Cre induction at P60. Green is EGFP fluorescence. Dorsal is top. Arrowhead indicates oligodendrocyte cell body. Scale bar: 15 μm.

The timing of generation of oligodendrocytes from NG2 cells differed at different ages. In the neocortex, there was a steep increase in the density of CC1$^+$ EGFP$^+$ cells between 4 and 10 dpi when Cre was induced at P2, whereas the steepest rise in oligodendrocyte generation occurred between 10 and 20 dpi when Cre was induced at P60 (Fig. 1B). This difference was more pronounced in the corpus callosum, where the steepest rise in the density of EGFP$^+$ oligodendrocytes occurred between 1 and 4 dpi after Cre induction at P2 (Fig. 1B′). Thus, oligodendrocyte differentiation from NG2 cells occurred faster in the perinatal than mature brain.

Oligodendrocyte differentiation in postnatal neocortex and corpus callosum in NG2creER^{TM}BAC:YFP and NG2CreER^{TM}BAC:mTmG mice

When we crossed NG2creER^{TM} mice into YFP reporter mice, we found that the Cre induction efficiency was almost 30-fold higher (45%) than that in NG2creER^{TM}BAC:ZEG mice, and all the YFP$^+$ cells were either NG2$^+$ or CC1$^+$, as in NG2creER^{TM}:ZEG mice (see Fig. S2 in the supplementary material). The higher recombination efficiency of the YFP reporter allowed us to detect a sufficient number of YFP$^+$ cells per section to separately quantify NG2 cell-derived oligodendrocytes in the neocortex and corpus callosum. Fig. 1 shows the percentage of YFP$^+$ cells that were NG2$^+$ (Fig. 1C) or CC1$^+$ (Fig. 1C′) at 1 or 60 days after 4-OHT injection at P2 or P60. The proportion of the YFP$^+$ cells in the corpus callosum that differentiated into oligodendrocytes (76% at P2 + 60 dpi and 53% at P60 + 60 dpi) was 2-fold greater than that in the cortex (39% at P2 + 60 dpi and 24% at P60 + 60 dpi).

To determine whether oligodendrocytes that differentiated from NG2 cells made myelin sheaths, we crossed NG2creER^{TM}BAC mice to mTmG reporter mice that express membrane-anchored EGFP after recombination (Muzumdar et al., 2007). When Cre was induced in the double-transgenic mice at P60 and the brain was analyzed at 150 dpi, EGFP$^+$ myelinated fibers were found in both gray and white matter throughout the brain and spinal cord. In the neocortex, there were isolated oligodendrocytes with multiple myelinating processes (Fig. 2A). In the corpus callosum, numerous parallel EGFP$^+$ myelinated fibers were seen with oligodendrocyte processes connected to them (Fig. 2B,C). We also noted occasional oligodendrocytes with delicate processes connected to thin myelin-like structures, which is suggestive of a cell that is beginning to myelinate axons (Fig. 2C).

The proportion of oligodendrocytes in the corpus callosum that are newly generated from NG2 cells declines with age

We used NG2creER^{TM}BAC:YFP double-transgenic mice to compare the proportion of oligodendrocytes in the corpus callosum that were generated from NG2 cells during a 60-day period after Cre induction at P2 and P60. The proportion of oligodendrocytes that were generated in 60 days was obtained by dividing the density of newly generated CC1$^+$ cells by total CC1$^+$ cells (both YFP$^+$ and YFP$^-$) at P2 + 60 dpi and at P60 + 60 dpi. The density of newly generated CC1$^+$ cells was estimated by dividing the density of YFP$^+$ CC1$^+$ cells by the Cre induction efficiency in the corpus callosum, which was 45% and 38% at P2 and P60, respectively. At P2 + 60 dpi, 58% of the oligodendrocytes/mm^3 of the corpus callosum had been generated during the 60-day period following Cre induction, whereas at P60 + 60 dpi this value was 30% (Fig. 1D). This suggests that the fraction of oligodendrocytes in the corpus callosum that are newly generated from NG2 cells declines with age, but even in the mature corpus callosum approximately one-third of the oligodendrocytes in a given volume are generated from NG2 cells during a 60-day period.

Fate analysis of isolated EGFP$^+$ cell clusters

The low Cre induction efficiency in NG2creER^{TM}BAC:ZEG double-transgenic mice allowed us to perform fate analyses of isolated clusters of EGFP$^+$ cells. One day after Cre induction, 80-130 EGFP$^+$ cells were detected in single coronal sections through the cerebral hemisphere. The EGFP$^+$ cells were scattered throughout the cerebral hemispheres, and the majority were NG2$^+$ (Fig. 3A,D). At 4 dpi, most of the EGFP$^+$ cell clusters consisted of two or three cells. A cell cluster was defined as a group of at least two EGFP$^+$ cells that were separated from other EGFP$^+$ cells by at least 50 μm. Some EGFP$^+$ cell clusters contained only NG2 cells (Fig. 3B′,C′,E′,F′) or only oligodendrocytes (Fig. 3B‴,C‴), whereas others contained both NG2 cells and oligodendrocytes (Fig. 3B″,C″,E″,F″).

The phenotype of the cells in each of the EGFP$^+$ cell clusters was analyzed at 4, 20 and 60 days after Cre induction at P2, P30 and P60 (Fig. 3G). At 4 dpi, more than half of the clusters consisted exclusively of NG2 cells (54%, 65% and 80% of the clusters in mice induced at P2, P30 and P60, respectively), and the majority of the remaining clusters comprised a mixture of NG2 cells and oligodendrocytes. In addition, at P2 + 4 dpi, a small proportion of the EGFP$^+$ clusters (7%) consisted exclusively of oligodendrocytes.

After longer survival times, the proportion of NG2 cell-only clusters declined in all three age groups, as the proportion of oligodendrocyte-only and mixed clusters increased. There was a significant difference in the cellular composition of the EGFP$^+$ clusters derived from perinatal or adult NG2 cells. At P2 + 60 dpi, 48% of the clusters consisted exclusively of oligodendrocytes and only 15.3% of the clusters comprised exclusively NG2 cells. By contrast, when Cre was induced at P30 or P60, the percentage of oligodendrocyte-only clusters at 60 dpi was much lower (14% and 11%, respectively). At P60 + 60 dpi, 36% of the clusters comprised only NG2 cells and 53% of the clusters contained both NG2 cells and oligodendrocytes. These observations suggest that perinatal NG2 cells are more likely to generate daughter cells with equivalent oligodendrogliogenic fate.

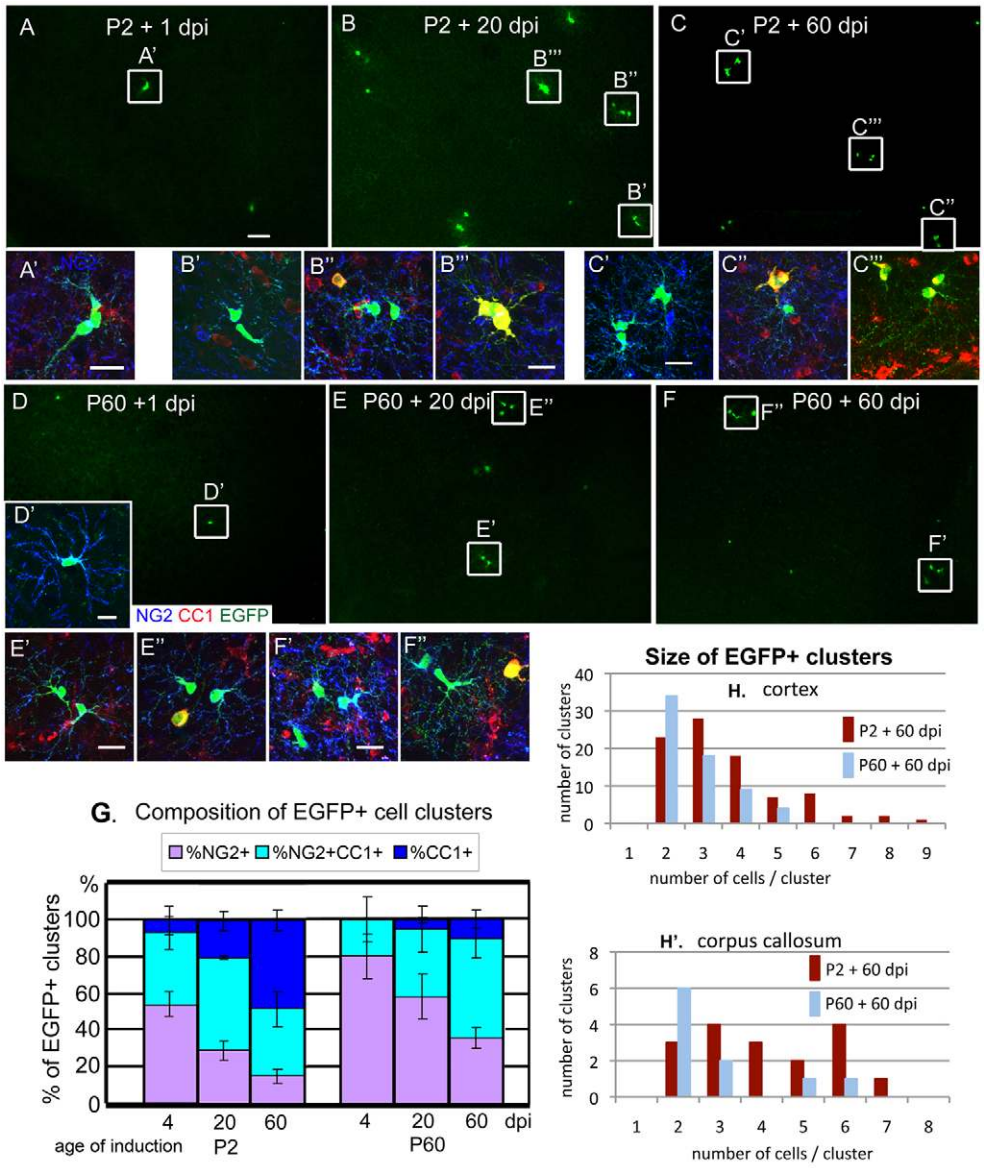

Fig. 3. The composition of EGFP$^+$ cell clusters generated from single NG2 cells in NG2creER^{TM}BAC:ZEG mice. (**A-F″**) Coronal sections through the cerebral hemispheres immunolabeled with rabbit anti-NG2 antibody (Cy5, blue) and mouse CC1 antibody (Cy3, red) 1 (A,D), 20 (B,E) and 60 (C,F) days after Cre induction at P2 (A-C) and P60 (D-F). Green represents EGFP fluorescence. Insets are higher magnifications of the boxed areas in the corresponding panels showing NG2 or CC1 expression of EGFP$^+$ cells in the cluster. Scale bars: 100 μm in A for A-F; 20 μm in A′-F″. (**G**) The composition of EGFP$^+$ cell clusters in the cerebral hemispheres at 4, 20 and 60 dpi in mice induced at P2 and P60. Error bars indicate s.d. (**H**,**H′**) The size of EGFP$^+$ cell clusters in the cortex (H) and corpus callosum (H′) 60 days after Cre induction at P2 (red bars) or P60 (blue bars).

There were some differences in the phenotype of clusters in the cortex and corpus callosum. The data shown in Fig. 3G were compiled from clusters in both the cortex and corpus callosum, with 85% of the clusters in the cortex. The proportion of oligodendrocyte-only clusters in the cortex and corpus callosum at P2 + 60 dpi was 48% and 81%, respectively. A more pronounced regional difference was seen in mice at P60 + 60 dpi, when 11% and 50% of the clusters in the cortex and corpus callosum, respectively, comprised oligodendrocytes only. These results suggest a greater tendency of NG2 cells in the white matter to differentiate symmetrically into oligodendrocytes and are consistent with the results from the analysis of total EGFP$^+$ cells shown in Fig. 1C′ and the findings of Dimou et al. (Dimou et al., 2008).

We also determined the size of the clusters in the cortex and corpus callosum at P2 + 60 dpi and P60 + 60 dpi (Fig. 3H). At P2 + 60 dpi, the cluster size ranged from two to nine cells in the cortex and from two to seven cells in the corpus callosum. Occasionally, there were extremely large clusters consisting of more than 20 cells in the corpus callosum, but these were excluded from the analysis because we could not be certain that they had originated from single NG2 cells. By contrast, at P60 + 60 dpi the majority of the clusters consisted of only two or three cells in both the cortex and corpus callosum.

As a way of assessing whether there was any intermingling of cells from neighboring clusters, we first compared the distance between the clusters at P2 + 4 dpi and P2 + 60 dpi. The average distance between the clusters did not change significantly between 4 and 60 dpi (283 μm in the cortex and 449 μm in the corpus callosum at P2 + 4 dpi; 239 μm in the cortex and 403 μm in the corpus callosum at P2 + 60 dpi). This suggests that the clusters remained separated over the 60-day period that we examined.

As another way to verify the observations made on the clusters, we performed live cell imaging of slices of cerebral hemispheres prepared from P4 or P8 NG2creBAC:ZEG double-transgenic mice (Fig. 4). Out of 56 EGFP$^+$ cell divisions that were imaged, 82% generated two NG2$^+$ daughter cells, 7% generated two CC1$^+$ cells, and 11% generated one NG2$^+$ cell and one CC1$^+$ oligodendrocyte. Thus, all three modes of division that had been observed in the cluster analysis in vivo were seen in live cells. However, it was interesting to note that all the pairs from P8 cultures that contained

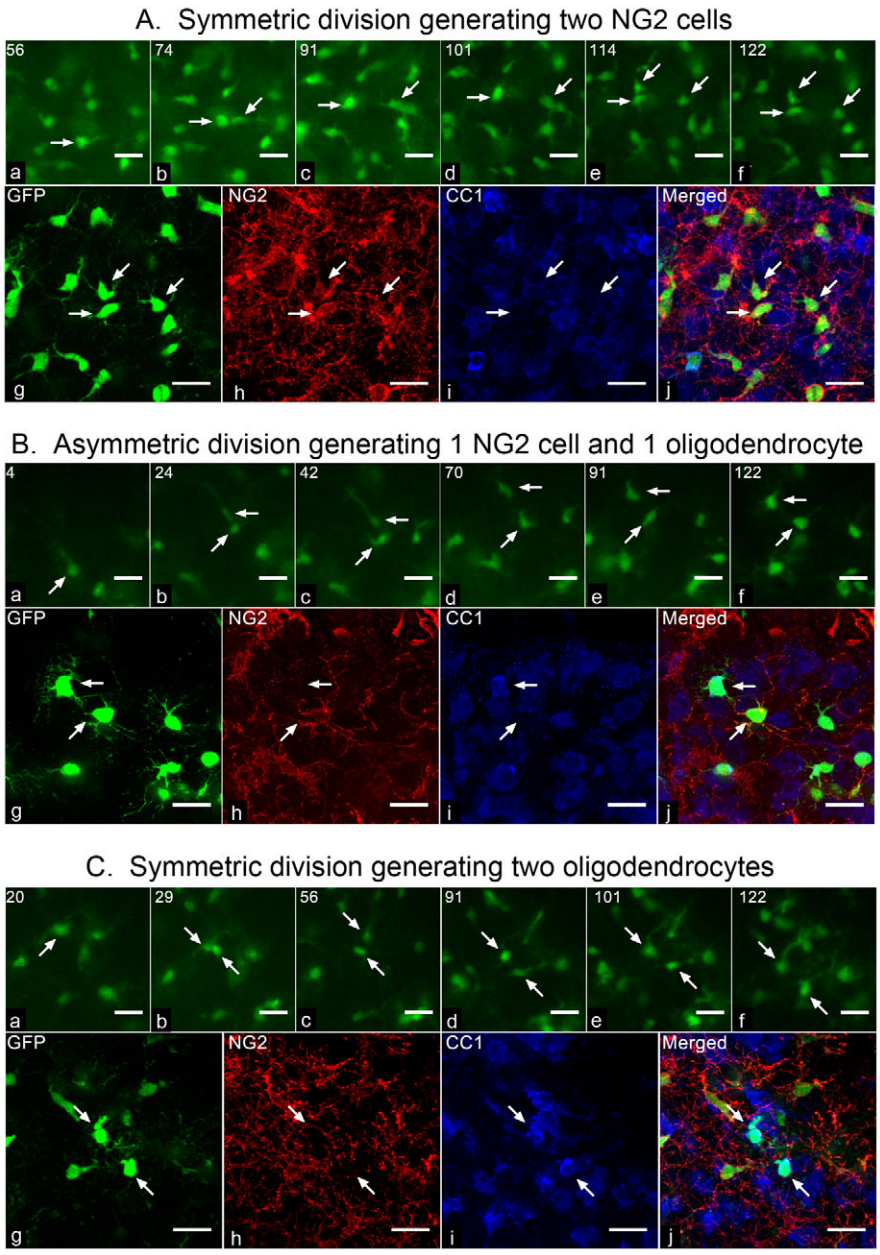

Fig. 4. Time-lapse imaging of the fate of NG2 cell progeny in slice cultures. (**A-C**) Three sets of time-lapse analyses representing: (Aa-j) symmetric division that generated two NG2 cells; (Ba-j) asymmetric division that generated one NG2 cell and one oligodendrocyte; and (Ca-j) symmetric division that generated two oligodendrocytes. Slices from P4 (A,C) or P8 (B) NG2CreBAC:ZEG mice were imaged at the indicated hours (top rows). Hour 0 is designated as 2 hours after slice preparation. At the end of the live slice imaging (122 hours), the slices were fixed and immunolabeled with anti-NG2 (red) and CC1 (blue) antibodies (bottom rows). Arrows indicate the cell that divided during the time-lapse and its daughter cells that were immunolabeled after fixation. Scale bars: 25 μm.

at least one oligodendrocyte had been fixed and stained at least 66 hours after the cell division, whereas many of the NG2 cell-only pairs were found in slices that had been fixed sooner after the division. This suggests that NG2 cells first divide into two daughter NG2 cells, and that differentiation of one or both daughter cells into oligodendrocytes occurs gradually over at least 2-3 days.

NG2 cells in the embryonic but not postnatal brain generate astrocytes

In NG2CreBAC:ZEG double-transgenic mice, which express constitutively active Cre in NG2 cells, some EGFP$^+$ protoplasmic astrocytes were found in the gray matter of the ventral forebrain, suggesting that they are derived from NG2 cells (Zhu et al., 2008a). However, we did not find any EGFP$^+$ protoplasmic astrocytes in NG2creER^{TM}BAC:ZEG double-transgenic mice when Cre was induced postnatally at P2, P30 or P60, even at 60 dpi. Nor did we find any YFP$^+$ protoplasmic astrocytes in NG2creER^{TM}BAC:YFP double-transgenic mice at P2 + 60 dpi or at P60 + 60 dpi. Some EGFP$^+$ cells were S100β$^+$, but these cells also co-expressed NG2, as previously shown (Hachem et al., 2005), exhibited the morphology of typical NG2 cells, and were morphologically distinct from bushy protoplasmic astrocytes.

We previously observed cells that appeared to be in transition from NG2 cells to astrocytes in the embryonic brains of NG2creBAC:ZEG double-transgenic mice (Zhu et al., 2008a). To test whether NG2 cells in the embryonic brain give rise to astrocytes, Cre was induced at E16.5 in NG2creER^{TM}BAC:ZEG double-transgenic mice and the brains analyzed at E19.5 or P14. Only a small number of EGFP$^+$ cells were detected at 3 dpi in the brains of E19.5 double-transgenic embryos, all of which were NG2 cells. By P14, EGFP$^+$ cells were more numerous and existed in clusters. A subpopulation of EGFP$^+$ cell clusters in the hippocampus and in the ventral forebrain at P14 had the characteristic bushy morphology of protoplasmic astrocytes

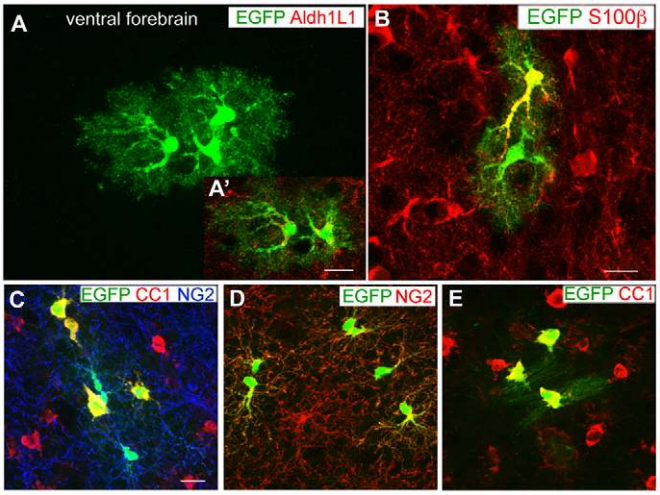

Fig. 5. Generation of protoplasmic astrocytes from embryonic NG2 cells. Immunolabeling of EGFP$^+$ cells in P14 NG2creER^{TM}:ZEG double-transgenic mice after Cre induction at E16.5. (**A-B**) Coronal sections through the ventral forebrain immunolabeled with mouse anti-Aldh1L1 (A; red) or anti-S100β (B; red) antibody showing EGFP$^+$ astrocyte clusters. Inset (A′) shows a merged image of EGFP fluorescence and Aldh1L1 immunoreactivity. (**C-E**) Coronal sections through the dorsal forebrain double immunolabeled with mouse CC1 (C; Cy3, red) and rabbit anti-NG2 (C; Cy5, blue) antibodies or single labeled with rabbit anti-NG2 antibody (D; Cy3, red) or mouse CC1 antibody (E; Cy3, red) showing EGFP$^+$ cell clusters consisting of NG2 cells and/or oligodendrocytes. Green represents EGFP fluorescence. Scale bar: 20 μm.

(Fig. 5). They were immunoreactive for the astrocytic antigens aldehyde dehydrogenase 1 L1 (Aldh1L1) (Cahoy et al., 2008) and S100β (Fig. 5A,B). All the EGFP$^+$ clusters that contained astrocytes were homogeneous and consisted only of astrocytes. None of the clusters contained both astrocytes and NG2 cells or astrocytes and oligodendrocytes. The phenotype of the embryonically induced EGFP$^+$ cell clusters that did not contain astrocytes was similar to that of postnatally induced clusters and was heterogeneous: some contained only oligodendrocytes, some contained only NG2 cells, and others contained both oligodendrocytes and NG2 cells (Fig. 5C-E). Our data suggest that NG2 cells in the late embryonic brain, but not in the postnatal brain, generate astrocytes. Astrocytes appear to be generated from a distinct subset of embryonic NG2 cells and not from bipotential NG2 cells that generate both astrocytes and oligodendrocytes.

NG2 cells do not generate neurons

When Cre was induced at P30 or P60 in NG2creER^{TM}BAC:ZEG mice, we detected at 1 and 4 dpi rare EGFP$^+$ cells that exhibited the morphology of pyramidal neurons and expressed NeuN (Neuna60 – Mouse Genome Informatics) in the neocortex. Only one or two such NeuN$^+$ EGFP$^+$ cells were found in a series of coronal sections at these time points, and EGFP$^+$ neurons were not found in mice in which Cre had been induced at P2 or E16.5. EGFP$^+$ neurons were not found at any of the other survival times examined (Fig. 6). Similarly, we did not find any NeuN$^+$ YFP$^+$ neurons in NG2creER^{TM}BAC:YFP mice at 60 dpi when Cre was induced at P2 or P60. Many YFP$^+$ cells were directly apposed to NeuN$^+$ neurons, but the DAPI channel indicated that the satellite YFP$^+$ cells were distinct from the YFP$^-$ NeuN$^+$ neurons. These

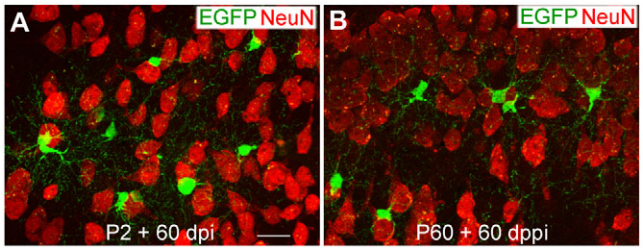

Fig. 6. No neuronal differentiation from NG2 cells. Coronal sections through the neocortex of NG2creER^{TM}BAC:ZEG double-transgenic mice immunolabeled with mouse anti-NeuN antibody (Cy3, red) 60 days after Cre induction at (**A**) P2 or (**B**) P60. Green represents EGFP fluorescence. Scale bar: 20 μm.

observations suggest that NG2 cells do not generate neurons. We attribute the rare NeuN expression in the EGFP$^+$ cells to sporadic neuronal expression of Cre, and possibly NG2, in older mice, as observed elsewhere (X.Z., R.S. and A.N., unpublished observations).

DISCUSSION

Oligodendrocyte generation from NG2 cells continues in the mature brain but declines with age

We used tamoxifen-inducible NG2creER^{TM} transgenic mice crossed with Z/EG reporter mice to study the fate of NG2 cells at three developmental stages: P2, P30 and P60. Immediately after Cre induction at 1 dpi, we found that the majority of the EGFP$^+$ cells were NG2$^+$ polydendrocytes, and a small fraction of EGFP$^+$ cells were oligodendrocytes, confirming the specificity of transgene expression. The presence of a small number of reporter-positive oligodendrocytes detected as early as 1 dpi could be due to the differentiation of NG2 cells into oligodendrocytes during the time between the first 4-OHT injection and sacrifice.

Our observation that NG2 cells continue to generate oligodendrocytes in the adult brain is consistent with the results of recent fate-mapping studies performed in PDGFRαCreER^{T2} (Rivers et al., 2008) and Olig2CreER^{TM} (Dimou et al., 2008) transgenic mouse lines. It is also consistent with earlier ultrastructural studies following [^{3}H]thymidine pulse labeling which showed that new oligodendrocytes continue to be generated from proliferating precursors and persist in the mature brain for at least 6 months (McCarthy and Leblond, 1988).

Our results demonstrate that NG2 cells in the perinatal brain generate more oligodendrocytes in 60 days than do NG2 cells in the more mature brain. This is consistent with previous studies that showed that oligodendrocyte and myelin production peak during the second postnatal week and decline thereafter (Skoff et al., 1976; Sturrock, 1980). The majority of NG2 cells marked at P2 became oligodendrocytes within 10 days, whereas those marked at P60 took 20 days or longer to differentiate into oligodendrocytes. Thus, NG2 cells in the perinatal brain not only generated more oligodendrocytes than those in the mature brain but they also generated oligodendrocytes more quickly than those in the mature brain. This could be due to a combination of intrinsic and extrinsic mechanisms. Although perinatal and adult NG2 cells share the expression of common progenitor antigens such as NG2 and PDGFRα and are morphologically similar, some of their properties related to their ability to proliferate and differentiate change over time as the animal matures, as described over 20 years ago for

oligodendrocyte progenitor cells in culture (Wolswijk and Noble, 1989). The precise mechanism underlying this change is not clear, but epigenetic mechanisms might play a role (Shen et al., 2008). Extrinsic factors, such as the availability of axons to be myelinated, might also influence oligodendrocyte differentiation from NG2 cells.

The extent to which new oligodendrocytes are added to, or replace, existing oligodendrocytes in the young and mature CNS has remained unclear. Using NG2creER^{TM}BAC:YFP double-transgenic mice, we estimated that 30% of the oligodendrocytes in the corpus callosum of P120 mice are generated from NG2 cells between P60 and P120. Cre induction at P60 in NG2creER^{TM}BAC:mTmG mice revealed that a substantial amount of myelin is generated after P60, especially in the corpus callosum. It remains unclear whether the newly formed myelin is added to, or replaces, the existing myelin. The proportion of newly formed oligodendrocytes calculated here was higher than the 17% rate reported by Rivers et al. using PDGFRαCreER^{T2} transgenic mice for a period of 90 days starting at P45 (Rivers et al., 2008). This disparity might result from differences in the oligodendrocyte identification methods used, Cre induction efficiencies, or the survival rates of the induced cells owing to other experimental factors such as BrdU incorporation. It is also possible that Cre induction continued to occur after the last 4-OHT injection, as recently described (Guo et al., 2010), which could lead to an underestimation of Cre induction efficiency and an overestimation of oligodendrocyte production.

EGFP$^+$ cell clusters derived from single NG2 cells are heterogeneous

Analysis of the phenotype of the progeny of NG2$^+$ EGFP$^+$ cells in isolated clusters in NG2creER^{TM}BAC:ZEG mice revealed that the proportion of oligodendrocyte-only EGFP$^+$ clusters increased over time after Cre induction in all age groups. At P2 + 60 dpi, more than 50% of the clusters contained only oligodendrocytes, whereas only 11% of the clusters in P60 + 60 dpi brains consisted only of oligodendrocytes. These findings are strikingly similar to those obtained from clonal analysis in vitro (Wren et al., 1992). The tendency to generate oligodendrocyte-only clusters was not only higher in perinatal brains but was also more pronounced in the corpus callosum, where 81% of the clusters consisted exclusively of oligodendrocytes at P2 + 60 dpi, and none of the clusters contained exclusively NG2 cells. By contrast, at P60 + 60 dpi, 90% and 50% of the clusters in the cortex and corpus callosum, respectively, contained NG2 cells.

Cluster size analysis revealed that the majority of the clusters at P60 + 60 dpi had two or three cells, whereas the size of P2 + 60 dpi clusters spanned two to nine cells. The cluster size was smaller than expected based on reported cell cycle times of less than 2 days at P6 and 9 days at P60 (Psachoulia et al., 2010) and the large size of clones observed in culture (Temple and Raff, 1986). This could be due to the death of newly differentiated cells (Barres et al., 1992) or the stringent criteria that we used for defining the clusters to avoid ‘lumping errors’.

Our findings suggest that NG2 cells are heterogeneous in their ability to self-renew and generate oligodendrocytes. This was verified by time-lapse imaging of slice cultures from perinatal brains, which also revealed cell divisions that generated two NG2 cells, two oligodendrocytes, or one NG2 cell and one oligodendrocyte. However, unlike neocortical neuroblasts, whose cell fate is determined immediately after division through the unequal distribution of molecules in the two daughter cells (Zhong and Chia, 2008), the majority of NG2 cells appeared to initially divide symmetrically, producing two daughter cells that continued to express NG2 for at least several hours or days, after which one or both cells gradually became oligodendrocytes. The symmetric nature of the initial NG2 cell division is consistent with published observations (Kukley et al., 2008; Dawson et al., 2003).

A subpopulation of NG2 cells in the embryonic ventral forebrain generates astrocytes

When Cre was induced in postnatal mice, we did not find any EGFP$^+$ astrocytes even after 60 dpi. However, when Cre was induced at E16.5, we observed clusters of EGFP$^+$ astrocytes in the ventral forebrain and hippocampus at P14, similar to the distribution of NG2 cell-derived astrocytes observed in NG2creBAC:ZEG mice (Zhu et al., 2008a). Based on our previous observation that NG2 and EGFP are initially expressed ventrally away from the germinal zones in NG2creBAC:ZEG embryos (Zhu et al., 2008a), and the specific expression of EGFP in NG2$^+$ cells 3 days after Cre induction in NG2creER^{TM}:ZEG embryos, it is unlikely that the expression of EGFP detected in astrocytes had been caused by ectopic expression of Cre in the ventral germinal zones. Our observation that NG2 cells in the adult brain do not generate astrocytes is consistent with the results of NG2 cell fate-mapping in PDGFRαCreER^{T2} mice (Rivers et al., 2008), but differs from the results of Dimou et al., who showed that 5-8% of the induced cells in adult Olig2-CreER^{TM} transgenic mice generate protoplasmic astrocytes in the gray but not white matter (Dimou et al., 2008). It is possible that Olig2 is not restricted to NG2 cells. For example, Olig2 has been detected in a subpopulation of type B and C cells in the adult subventricular zone (Menn et al., 2006), which are likely to be NG2$^-$ (Komitova et al., 2009; Platel et al., 2009; Cesetti et al., 2009). Furthermore, loss of one allele of *Olig2* in the Olig2-CreER^{TM} mice might have a subtle effect on cell fate. In a recent study using PLP-CreER^T mice, it was shown that 20% of the *Plp* (*Plp1* – Mouse Genome Informatics) promoter-positive cells at P8, most of which were NG2$^+$, generated protoplasmic astrocytes in the ventral forebrain (Guo et al., 2009). The variance of this result from our observations might be attributed to distinct cell types that were targeted by the different promoters used and to possible differences in the kinetics of Cre activation of the different tamoxifen-inducible mutants of the estrogen receptor.

The embryonic generation of astrocytes from NG2 cells is consistent with our observation in NG2creBAC:ZEG mice that cells that appeared to be in transition from NG2 cells to astrocytes were readily detected at E18.5, but were more difficult to find in the postnatal brain. This is also consistent with earlier studies which demonstrated that astrocyte development occurs in late embryonic stages and is completed by the end of the first postnatal week, prior to the peak of oligodendrocyte generation (Skoff et al., 1990; Gomez et al., 2003).

In mice in which Cre was induced at E16.5, we did not detect any EGFP$^+$ clusters at P14 that contained both astrocytes and oligodendrocytes. Therefore, NG2 cells in the embryonic brain that generate astrocytes are unlikely to be bipotential cells that generate both astrocytes and oligodendrocytes (Raff et al., 1983; Rao et al., 1997). Instead, there might be heterogeneity among NG2 cells in the embryonic brain. It is likely that embryonic NG2 cells that exclusively generate astrocytes become depleted, and postnatal NG2 cells predominantly give rise to oligodendrocytes or remain NG2$^+$.

NG2 cells do not generate neurons

We have not obtained any evidence that NG2 cells generate neurons in NG2creBAC (Zhu et al., 2008a; Komitova et al., 2009) or NG2creER^{TM} mice. We detected a very small number of scattered EGFP$^+$ NeuN$^+$ cells at 1 and 4 days after Cre induction in the mature brain, but such cells were not observed at longer survival times. The sporadic occurrence of EGFP$^+$ NeuN$^+$ cells in the NG2creER^{TM}:ZEG brain is likely to be caused by Cre expression in NeuN$^+$ neurons in the mature brain (X.Z., R.S. and A.N., unpublished observations). We did not detect any YFP$^+$ neurons in the piriform cortex or elsewhere at P2 + 60 dpi or P60 + 60 dpi in NG2creER^{TM}BAC:YFP mice with higher Cre induction efficiency. Our findings are consistent with those of Dimou et al. (Dimou et al., 2008), who did not detect neuronal differentiation from Olig2$^+$ cells in the adult brain, but differ from the results of fate mapping in PDGFRα-CreER^{T2} or PLP-CreER^T mice (Rivers et al., 2008; Guo et al., 2009; Guo et al., 2010), in which a small number of neurons were generated from *Pdgfra* or *Plp* promoter-positive cells. Further studies on the specificity of Cre expression and additional mouse lines are needed to resolve these differences.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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