Modulation of oligodendrocyte generation during a critical temporal window after NG2 cell division

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Oligodendrocytes in the mammalian brain are continuously generated from NG2 cells throughout postnatal life. However, it is unclear when the decision is made for NG2 cells to self-renew or differentiate into oligodendrocytes after cell division. Using a combination of in vivo and ex vivo imaging and fate analysis of proliferated NG2 cells in fixed tissue, we demonstrate that in the postnatal developing mouse brain, the majority of divided NG2 cells differentiate into oligodendrocytes during a critical age-specific temporal window of 3–8 d. Notably, within this time period, damage to myelin and oligodendrocytes accelerated oligodendrocyte differentiation from divided cells, and whisker removal decreased the survival of divided cells in the deprived somatosensory cortex. These findings indicate that during the critical temporal window of plasticity, the fate of divided NG2 cells is sensitive to modulation by external signals.

RESULTS
Stereotyped oligodendrocyte generation from divided NG2 cells

To determine the temporal dynamics of NG2 cell differentiation into oligodendrocytes after division in vivo, we first performed pulse-chase labeling with EdU in NG2creER:YFP mice that were double transgenic for tamoxifen-inducible NG2creER and the Cre reporter gt(ROSA)26Sor tm1(EYFP)Cos (YFP). We injected 4-hydroxytamoxifen (4OHT) from postnatal days 6 to 8 (P6–P8) to induce YFP expression in NG2 cells, followed by a single EdU injection at P8. We sacrificed animals 2 h (P8+0) or 1–4 d (P8+1, P8+2, P8+3 and P8+4) after EdU injection (Fig. 1). We determined the proportion of YFP+EdU+ cells that expressed the CC1 oligodendrocyte antigen at each time point in both the cortex and corpus callosum (Fig. 1b,e). The first emergence of YFP+EdU+CC1+ cells occurred at P8+2, suggesting that in vivo NG2 cells in both the cortex and corpus callosum take at least 48 h after DNA replication to differentiate into CC1+ oligodendrocytes. The percentage of YFP+EdU+ cells that expressed CC1 increased and reached a plateau over the next 2 d. More than 40% of the divided cells differentiated into the CC1+ oligodendrocyte stage within 3 d after division (Fig. 1e).

In addition to population analysis, we characterized the fate of single pairs of YFP+EdU+ cells in NG2creER:YFP mice. Three days of 4OHT injections from P6 to P8 yielded an efficiency of Cre induction that was low enough (25.7 ± 1.5% in the cortex and 24.8 ± 0.9% in the corpus callusom) to enable identification of isolated pairs of YFP+EdU+ cells. Daughter cell pairs were defined as two cells that were in the process of differentiating into oligodendrocytes during this critical temporal window.

Oligodendrocytes in the mammalian CNS are generated from NG2 cells (also known as polydendrocytes or oligodendrocyte precursor cells (OPCs)). NG2 cells in rodent telencephalon appear in late gestation and continue to expand through the first 2 weeks of postnatal life. Even after peak oligodendrocyte production during the third postnatal week, NG2 cells persist as a uniformly distributed resident glial cell population in the adult CNS and retain their proliferative ability throughout life. Recent genetic fate-mapping studies have revealed that NG2 cells continue to generate oligodendrocytes asynchronously and continue to express the NG2 proteoglycan for several days before one or both differentiate into oligodendrocytes. These observations suggested that the fate of divided NG2 cells might be determined by the nature and timing of the physiological signals that lead to the decision of divided NG2 cells to differentiate, self-renew or die.

We have previously shown that NG2 cells from early postnatal brain divide symmetrically to generate two daughter NG2 cells, which continue to express the NG2 proteoglycan for several days before one or both differentiate into oligodendrocytes. These observations suggested that the fate of divided NG2 cells might be determined by the microenvironment during this latency period. Here we have directly tested this hypothesis using a combination of slice cultures, in vivo 5-ethynyl-2′-deoxyuridine (EdU) pulse-chase labeling, and transcranial two-photon imaging of live mice carrying dual fluorescence reporter constructs. We demonstrate that there is a critical temporal window between NG2 cell division and differentiation, during which oligodendrocyte generation can be modulated by changes in the microenvironment.

The latency between NG2 cell division and oligodendrocyte differentiation is shortened by damage to myelin or oligodendrocytes. Moreover, sensory deprivation reduces the survival of divided NG2 cells that are in the process of differentiating into oligodendrocytes during this critical temporal window.

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Received 11 July; accepted 22 August; published online 28 September 2014; doi:10.1038/nn.3815
Figure 1 Temporal dynamics of oligodendrocyte differentiation after NG2 cell division in vivo. (a) Experimental protocol for EdU pulse-chase labeling in P8 and P21 NG2creER::YFP mice. (b) Labeling for YFP, EdU and CC1 at 1 and 4 d after EdU injection. Arrows, YFP+EdU+CC1− cells; arrowheads, YFP+EdU+CC1+ cells. (c) YFP+EdU+ cortical cell pairs immunostained for NG2 or CC1. Scale bars in b, c, 25 μm. Images in b and c represent data from 3 experiments. (d) Proportion of symmetric CC1− divisions (two CC1− cells), symmetric CC1+ divisions (two CC1+ cells) and asymmetric divisions (one CC1+ and one CC1− cell) divisions in the cortex (CTX) and corpus callosum (CC) 2, 3 and 4 d after EdU injection. (e,f) Percentage of YFP+EdU+ cells that were CC1+ on the indicated days after EdU injection at P8 (e) or P21 (f). Cortex 0–2, *P = 0.0002, t = 5.554; 0–3, *P < 0.0001, t = 12.92; 0–4, *P < 0.0001, t = 13.04; 1–2, *P = 0.0008, t = 4.946; 1–3, *P < 0.0001, t = 12.32; 1–4, *P < 0.0001, t = 12.43; 2–3, *P < 0.0001, t = 7.370; 2–4, *P < 0.0001, t = 7.483. Corpus callosum 0–2, *P < 0.0001, t = 9.639; 0–3, *P < 0.0001, t = 17.34; 0–4, *P < 0.0001, t = 17.73; 1–2, *P < 0.0001, t = 9.800; 1–3, *P < 0.0001, t = 17.50; 1–4, *P < 0.0001, t = 17.89; 2–3, *P < 0.0001, t = 7.701; 2–4, *P < 0.0001, t = 8.094. (f) Percentage of YFP+EdU+ cells that were CC1+ after EdU injection at P21. Cortex 1–4, *P = 0.0269, t = 3.427; 1–6, *P < 0.0001, t = 6.616; 1–8, *P < 0.0001, t = 7.277; 1–10, *P < 0.0001, t = 7.160; 2–4, *P = 0.0281, t = 3.410; 2–6, *P < 0.0001, t = 6.597; 2–8, *P < 0.0001, t = 7.261; 2–10, *P < 0.0001, t = 7.143; 4–8, *P = 0.0086, t = 3.850; 4–10, *P = 0.0119, t = 3.733. Corpus callosum 1–4, *P < 0.0001, t = 6.868; 1–6, *P < 0.0001, t = 11.77; 1–8, *P < 0.0001, t = 11.73; 1–10, *P < 0.0001, t = 13.09; 2–4, *P < 0.0001, t = 5.619; 2–6, *P < 0.0001, t = 10.33; 2–8, *P < 0.0001, t = 10.48; 2–10, *P < 0.0001, t = 11.84; 4–6, *P = 0.0088, t = 3.843; 4–8, *P = 0.0005, t = 4.862; 4–10, *P < 0.0001, t = 6.226; 6–10, *P = 0.0333, t = 3.346. n = 3 mice per condition and genotype. NS, not significant. Error bars, s.d. P values were obtained by two-way ANOVA, Bonferroni post-test.

YFP+EdU+ and were less than one cell-body diameter apart (Fig. 1c,d). At P8+3 and P8+4 we often observed YFP+EdU+ cell pairs with cell bodies very close to one another (for example, see Fig. 1c), and these cells often expressed CC1. Quantification revealed a greater proportion of cell pairs consisting of two CC1− cells (symmetric) in the corpus callosum than in the cortex (Fig. 1d). Furthermore, the percentage of cell pairs with asymmetric and/or symmetric CC1− differentiation outcomes increased from P8+2 to P8+3 but increased less steeply from P8+3 to P8+4 (Fig. 1d).

We performed a similar experiment in P21 mice. We injected 4OHT from P18–P21 to induce YFP expression in NG2 cells (Cre induction efficiency was 12–70% in the cortex and 13–45% in the corpus callosum), followed by a single EdU injection at P21 (Fig. 1a). We sacrificed animals at 1, 2, 4, 6, 8 and 10 d after EdU injection and determined the proportion of YFP+EdU+ cells that had differentiated into CC1+ oligodendrocytes in the corpus callosum and cortex (Fig. 1f). There was a steep rise in the proportion of YFP+EdU+ cells that were also CC1+ between 2 and 4 d after EdU injection, which was followed by a more modest increase. In the cortex, the proportion of YFP+EdU+ cells that were CC1+ reached a plateau on day P21+8 at 48%, and there was no significant increase from P21+8 to P21+10 (P > 0.999). In the corpus callosum, the rate of increase in the proportion of YFP+EdU+ cells that were CC1+ also slowed beyond P21+6. However, unlike in the cortex, the proportion of divided YFP+ cells that became oligodendrocytes continued to increase slightly (65% at P21+6, 73% at P21+8 and 80% at P21+10), although the
increase between P21+8 and P21+10 was not statistically significant ($P > 0.999$). This suggested that at both P8 and P21, there was a temporal window after NG2 cell division during which the majority of the differentiation events occurred, although the duration of this window was longer in P21 mice.

**In vivo imaging using dual reporter mice**

We generated mice that were triple transgenic for NG2cre, expressing constitutively active Cre in NG2 cells, the Cre reporter Z/EG, and PLPDsRed$^{16}$, which expresses DsRed under the control of the proteolipid protein (PLP) promoter. In P21 NG2cre:ZEG:PLPDsRed triple-heterozygous transgenic mice, GFP was detected in both NG2 cells (NG2$^+$PDGFRα$^+$) and oligodendrocytes (CC1$^+$), whereas DsRed was detected only in mature oligodendrocytes that expressed CC1, and not in NG2 cells expressing NG2 or PDGFRα (Fig. 2a–f). DsRed$^+$ cells first appeared in the corpus callosum at P6–P8 and their density steadily increased over the following weeks in both corpus callosum and deeper cortical layers (Fig. 2g), whereas in cortical layer I they did not appear until P21–P23. Although all PLPDsRed$^+$ cells were CC1$^+$, only a subpopulation of CC1$^+$ cells were DsRed$^+$ (Fig. 2h and Supplementary Fig. 1), suggesting that DsRed fluorescence is restricted mostly to myelinating oligodendrocytes$^{17}$.

To investigate NG2 cell division and differentiation dynamics of these cells in vivo, we performed repeated transcranial two-photon fluorescence imaging in NG2cre:ZEG:PLPDsRed mice (Fig. 3a and Supplementary Fig. 1). NG2cre:ZEG double-transgenic mice express GFP in cells throughout the oligodendrocyte lineage as well as in vascular pericytes$^{18,19}$. Pericytes were easily distinguished from oligodendroglial cells by their unique bipolar morphology and association with the vasculature, visualized via intravenous injection of Texas Red dextran; NG2 cells were identified by their distinct multilayered morphology, visualized via intravenous injection of Texas Red dextran; NG2 cells were identified by their distinct multilayered morphology, visualized via intravenous injection of Texas Red dextran; NG2 cells were identified by their distinct multilayered morphology. Using NG2cre:ZEG:PLPDsRed mice, we captured image stacks beginning at P20–P22 once per day for 10 d through layers I–III of the same region from the somatosensory cortex (Supplementary Movie 1). Excitation at 975-nm wavelength effectively elicited both GFP and DsRed fluorescence, providing an in vivo dual reporter system to identify both NG2 cells and mature oligodendrocytes (Fig. 3a and Supplementary Movie 1).

**NG2 cells divide and translocate with variable trajectories**

Analysis of the behavior of GFP$^+$ cells in NG2cre:ZEG mice from P21–P32 revealed that although pericyte location remained highly stable, many NG2 cells translocated their cell bodies from day to day in the $x$, $y$ and $z$ dimensions. Many NG2 cells divided during the 10- to 12-day imaging session, and some underwent multiple cell divisions (Fig. 3c and Supplementary Movie 2), consistent with previous reports of their proliferative properties$^{20,21}$. We also observed many GFP$^+$ cells disappearing or migrating out of the field of view over the imaging period.

**Figure 2** NG2cre:ZEG:PLPDsRed triple-transgenic mice identify cells at distinct stages of oligodendrocyte differentiation. Images in a–f represent data from three experiments. (a) Low-magnification images taken from the forebrain of fixed tissue from P15 NG2cre:ZEG:PLPDsRed transgenic mice. CC, corpus callosum; CTX, cortex. (b–d) High-magnification images taken from P15 corpus callosum immunostained for NG2 (b), PDGFRα (c) or CC1 (d), showing DsRed fluorescence in a subpopulation of CC1$^+$ cells (arrowheads) but not in NG2 cells that are NG2$^+$ or PDGFRα$^+$. (e) High-magnification image of a DsRed$^+$ oligodendrocyte in the corpus callosum showing typical myelinating oligodendrocyte morphology with multiple parallel processes. (f) Example of a cortical GFP$^+$DsRed$^+$ cell that is also CC1$^+$. (g) Low-magnification images of DsRed expression at P10, P15 and P30 in the cortex (layers I–VI) and corpus callosum (CC), showing the increase in the density of DsRed-expressing oligodendrocytes during the first month of postnatal development. Scale bars, 100 µm (a, left), 50 µm (a, right), 25 µm (b–d), 25 µm (e,f) and 250 µm (g). (h) Quantification in PLPDsRed transgenic mice demonstrating that DsRed$^+$ cells are a subset of CC1$^+$ cells in the cerebral cortex and corpus callosum. Error bars, s.d. $n = 3$ mice.
session (Fig. 3d and Supplementary Movie 3), which occurred either with or without a cell division. Quantification revealed similar numbers of dividing and disappearing cells per day in each of the four cortical depths. Of 82 cells that were imaged in vivo over 10 d (P22–P31), 9.9% disappeared without division, 10.4% disappeared after division, 15.4% divided and did not disappear, and 64.4% remained stable (Supplementary Fig. 1). Cells that disappeared after division disappeared between 2–6 d (average of 3.6 d) after division. Cells that disappeared without division disappeared between 2–6 d (average of 3.4 d) after the start of imaging. The total density of GFP+ cells over the imaging period remained remarkably stable over 10–12 d (Fig. 3e).

We next determined whether cell division and cell separation followed a stereotyped behavior by measuring the distance between individual divided cells 1 d after division had occurred. We found that separation was highly variable, ranging from 1.6 to 24.2 µm, with an average of 8.5 µm (Fig. 3f). The distance between individual divided cells over multiple days also varied considerably, ranging from 5 to 55 µm within 5 d after division (Fig. 3g).

Finally, we sought to determine whether NG2 cells divided in a specified division plane. Out of 86 cell divisions that were imaged, 21%, 29%, 29% and 21% divided at an angle of 45°, 90°, 135° and 180°, respectively, relative to the pial surface (Fig. 3h). According to these data, the plane of division appeared to be random and was highly variable between individual cell division events.

Protracted differentiation after NG2 cell division in vivo
On the first day of in vivo imaging (P20–P22), DsRed+ cells were rarely detected in NG2cre:ZEG:PLPDsRed mouse (Fig. 4a–c), consistent with fixed-tissue analysis of DsRed developmental expression and with reported developmental myelination of cortical layers I–III imaged in vivo17. On subsequent days (up to P32), in addition to finding many GFP+ cells dividing and migrating over variable distances, we saw multiple GFP+ cells gradually becoming DsRed+ (Fig. 4c and Supplementary Movie 4). Notably, over the 10- to 12-day imaging session, we never observed a GFP+ cell that divided and then became DsRed+ (869 GFP+ cells imaged over 5–12 d in four mice).
These data suggest that it takes more than 10 d for most of the divided NG2 cells to differentiate into oligodendrocytes in the neocortex of P21 mice. Quantification of total GFP+/DsRed+ cells showed a gradual increase over time, with the largest increase occurring in the uppermost region (Fig. 4d), consistent with myelination of layer I cortical axons.

To determine the region- and age-dependent temporal dynamics of oligodendrocyte maturation after NG2 cell division in vivo, we performed pulse-chase labeling with EdU in PLPDsRed mice after EdU injection at P8 (Supplementary Fig. 2a–c) or P21 (Supplementary Fig. 2d–f). EdU-injected mice were sacrificed 3, 4, 5, 6, 8 or 10 d after injection. In the P8 group, the first DsRed+EdU+ cells appeared at P8+4 in both the corpus callosum and cortex (Supplementary Fig. 2a–c), indicating that the onset of PLPDsRed fluorescence lags behind the onset of the expression of the CC1 antigen by approximately 2 d (compare with Fig. 2h). In the corpus callosum, the proportion of DsRed+ cells labeled with EdU at P8 continued to increase and reached a plateau of 15% at P8+8. By contrast, in the neocortex the proportion of EdU+DsRed+ cells remained below 1.5% (Supplementary Fig. 2c). In P21 mice, EdU+DsRed+ cells did not
Myelin damage accelerates differentiation after division

We next examined whether the time to oligodendrocyte differentiation after NG2 cell division could be altered by environmental conditions. NG2 cells are known to undergo enhanced proliferation and oligodendrocyte production in response to demyelination or developmental defects in myelin or oligodendrocyte production.22,23 To model myelin and oligodendrocyte injury, we exposed forebrain slice cultures from P8 NG2cre:ZEG:PLPDsRed mice to α-lysophosphatidylcholine (LPC), which causes an acute demyelinating injury in the cortex and corpus callosum of control slice cultures and were also GFP+ and CC1+ (Fig. 5a). LPC exposure resulted in reduced and disorganized myelin and degenerated appearance of oligodendrocyte processes, compared with control slices showing robust parallel oligodendrocyte processes extended along axons (Fig. 5b). In contrast, smaller cells with finer processes resembling NG2 cells appeared relatively unaffected by LPC.

After vehicle or LPC exposure, we performed time-lapse imaging on the slices to track the fate of dividing GFP+ cells as previously described.20 Of 16 divided pairs examined in the control slices, none of the daughter GFP+ cells became DsRed+ over the 78-h imaging session, and this daughter cell was also CC1+. Of 16 divided pairs examined in the control slices, none of the daughter GFP+ cells became DsRed+ over the 78-h imaging session, and this daughter cell was also CC1+. LPC exposure resulted in reduced and disorganized myelin and degenerated appearance of oligodendrocyte processes, compared with control slices showing robust parallel oligodendrocyte processes extended along axons (Fig. 5b). In contrast, smaller cells with finer processes resembling NG2 cells appeared relatively unaffected by LPC.

After vehicle or LPC exposure, we performed time-lapse imaging on the slices to track the fate of dividing GFP+ cells as previously described.20 Of 16 divided pairs examined in the control slices, none of the daughter GFP+ cells became DsRed+ over the 78-h imaging session, although we often observed GFP+ cells becoming DsRed+ without any division occurring (Fig. 5c, Supplementary Fig. 4 and Supplementary Movies 5 and 6). By contrast, after LPC treatment, 3 of 32 division events generated one daughter cell that became DsRed+ within the 78-h imaging session, and this daughter cell was also CC1+ after fixation (Fig. 5d and Supplementary Movie 7). Post hoc immunostaining revealed that in control slices, 15 of the 16 GFP+ cell divisions we imaged generated two GFP+CC1+ daughter cells, 1 generated one GFP+CC1+ daughter cell, and none of the division events generated...
two CC1+ daughter cells (Fig. 5e). By contrast, among 32 GFP+ cell divisions imaged after LPC exposure, 19 generated two GFP+CC1- daughter cells, 12 generated one GFP+CC1+ daughter cell, and 1 generated two GFP+CC1+ daughter cells (Fig. 5e). Statistical analysis revealed that LPC treatment significantly increased the percentage of cell divisions with asymmetric NG2 cell and oligodendrocyte fates (*P = 0.0168, unpaired Student’s t-test; Fig. 5e). These data suggest that the integrity or the density of myelin and oligodendrocytes regulated the timing of oligodendrocyte maturation after NG2 cell division.

To determine whether division orientation might be more strongly correlated with fate outcomes in slice cultures than in vivo, we analyzed the division plane in both control and LPC-treated slices (Supplementary Fig. 5). Of 56 cell divisions imaged in the cortex and corpus callosum, the proportions of vertical and horizontal divisions (relative to the tangent on the pial surface) were not substantially different between control and LPC-treated slices (Supplementary Fig. 5b). Furthermore, the proportion of divided cells that became CC1+ did not differ for vertical compared with horizontal divisions (Supplementary Fig. 5c), although there was a slight trend toward more horizontal divisions after LPC and a greater proportion of CC1+ progeny after horizontal division. Further experiments are necessary to determine more definitively whether the plane of division is a predictor of differentiation both in slice cultures and in vivo.

Sensory deprivation increases apoptosis of divided cells

We used the somatosensory barrel cortex to analyze whether sensory deprivation caused by whisker clipping could influence the production of oligodendrocytes from divided NG2 cells. We injected NG2creER: YFP mice with 4OHT from P6 to P8 and clipped all the whiskers on the right side every day from P6 until the day of sacrifice, 4 or 6 d later (Fig. 6a–h and Supplementary Fig. 6). This targeted a developmental stage when the critical period for barrel structural plasticity had ended and active cortical myelination was just beginning. The density of total CC1+ oligodendrocytes and YFP+CC1+ cells was significantly smaller in the deprived somatosensory cortex compared with the spared cortex at P6+4 and at P6+6 (statistical significance *P value threshold of *P < 0.05 was used for whisker sensory deprivation experiments; exact *P values can be found in figure legends; Fig. 6a–e). Furthermore, the proportion of YFP+ cells that had become CC1+ oligodendrocytes was significantly lower in the deprived somatosensory cortex at P6+4 and P6+6 (Fig. 6f). The effect of whisker clipping on oligodendrocyte density was specific to the somatosensory cortex. At both time points we did not detect significant differences in the density of oligodendrocytes or the proportion of YFP+CC1+ cells between the deprived and spared motor cortex in the same sections adjacent to the somatosensory cortex (Supplementary Fig. 7b–d). These data suggest that whisker sensory input influences NG2 cell differentiation into oligodendrocytes during the second postnatal week.

We next investigated the effect of whisker clipping on oligodendrocyte differentiation after NG2 cell division. A single injection of EdU was given to NG2creER:YFP mice at P8 that had whiskers clipped unilaterally starting at P6 and 4OHT injected from P6 to P8. Mice were perfused 2 and 4 d after EdU injections (P8+2 and P8+4), and the fate of YFP+EdU+ cells was determined in the spared and deprived somatosensory cortex. The density of CC1+EdU+ cells was significantly lower in deprived sides at P8+4, and the percentage of YFP+CC1+ EdU+ cells among all YFP+EdU+ cells was fivefold lower in the deprived somatosensory cortex at both P8+2 and P8+4 compared with the spared somatosensory cortex (Fig. 7a–h), but there was no difference in the motor cortex (Supplementary Fig. 7e,f). When whisker clipping was performed in PLPΔsRed mice, the density of DsRed+ cells was significantly lower in deprived sides 8 and 15 d after the start of clipping (Fig. 7j). Furthermore, the density and percentage of divided cells (EdU+) that became DsRed+ were significantly lower in the deprived compared with the spared somatosensory cortex at 6 d after EdU injection at P8 (Fig. 7j). These data indicate that sensory deprivation significantly reduces oligodendrocyte production from divided NG2 cells during the window of time after division when differentiation into CC1+ oligodendrocytes plateaus under normal conditions. A 2-h pulse labeling of EdU revealed that NG2 cell proliferation was slightly higher in the deprived compared with the spared somatosensory cortex at 4 d (18% higher) and 6 d (37% higher) after initiation of whisker clipping (Fig. 7a).

To determine whether the reduced oligodendrocyte production in the deprived cortex was caused by increased death of newly divided cells, we injected NG2creER:YFP mice with 4OHT from P6 to P8, clipped all the whiskers on the right side every day from P6 until P10 and performed a single injection of EdU at P8 (Fig. 7b–f and Supplementary Fig. 6). Staining for active caspase-3 in mice...
sacrificed at P8+2 revealed cells with typical apoptotic morphology (Fig. 7c–f). The densities of caspase-3\(^+\) cells, caspase-3\(^+\)YFP\(^+\) cells and caspase-3\(^+\)EdU\(^+\) cells were significantly higher in the deprived cortex relative to the spared cortex (Fig. 7b). Caspase-3\(^+\)CC1\(^+\) cells were present in both deprived and spared cortex, and there was no significant difference in their density (Fig. 7b,f). Although we did not detect caspase-3 in cells with typical NG2 cell morphology and strong PDGFR\(\alpha\) immunoreactivity, there were caspase-3\(^+\) round cells that had diffuse weak PDGFR\(\alpha\) immunoreactivity (Fig. 7d,e), some of which also had weak CC1 immunoreactivity. These findings suggest that sensory deprivation reduced the survival of divided NG2 cells that were in the process of differentiating into CC1\(^+\) oligodendrocytes.

**DISCUSSION**

Using a combination of *in vivo* and *ex vivo* imaging, cell fate mapping and EdU pulse-chase experiments, we demonstrated that during postnatal development, the fate of divided NG2 cells is determined within a critical temporal window between cell division and oligodendrocyte differentiation, the duration of which is region and age specific. At P8 the rate of oligodendrocyte differentiation from divided NG2 cells reached a plateau within 3–4 d. A temporal window between NG2 cell division and oligodendrocyte differentiation also existed in P21 mice but was protracted to 6–8 d. Interestingly, this plateau effect seemed to be more pronounced for cells in the cortex, whereas divided NG2 cells in the corpus callosum tended to continue to generate oligodendrocytes beyond the period of rapid oligodendrocyte production. Newly generated CC1\(^+\) oligodendrocytes subsequently matured and acquired PLP/\(\alpha\)DSRed fluorescence over the following days, and the length of time from division to oligodendrocyte maturation was longer in P21 animals and in the cortical gray matter compared to the corpus callosum. Most notably, we demonstrated that the temporal window between NG2 cell division and oligodendrocyte production is a period when the divided cells are sensitive to fate modulation by external signals. Specifically, damage to myelin or oligodendrocytes accelerated the differentiation after division, whereas a loss in sensory input compromised the survival of divided cells that were transitioning into oligodendrocytes.

Together, our observations from *in vivo* and slice-culture imaging and from EdU pulse-chase labeling followed by analysis of fixed brains suggest that NG2 cells initially undergo symmetrical division into two daughter NG2 cells, which remain as NG2 cells for a few days before one differentiates into an oligodendrocyte. This is consistent with an earlier 5-bromo-2’-deoxyuridine (BrdU) pulse-chase study in adult rats. By contrast, a recent study reported that the NG2 protein is asymmetrically distributed during cell division, suggesting that the differentiation process begins during division. Although the reasons for these contradictory findings are not clear, they could arise from differences in the age of the animals or the clonal preculture conditions.

**NG2cre:ZEG:PLPDsRed triple-transgenic mice allowed us to directly visualize the entire oligodendrocyte lineage and follow oligodendrocyte differentiation for the first time in a living mouse. During the 10 d of imaging, we did not detect DsRed fluorescence in the progeny of divided cells, even though DsRed fluorescence increased in intensity over time in oligodendrocyte lineage cells that had not divided during the imaging period. This was also evident with the EdU pulse-chase labeling of P21 PLPDsRed mice, which showed that fewer than 4% of the cortical EdU\(^+\) cells became DsRed\(^+\) even 10 d after EdU labeling. Unlike embryonic neural progenitor cells, the progeny of divided NG2 cells remain as two daughter NG2 cells for several days to weeks after division, depending on the age of the animal.**

This has also been noted through *in vivo* imaging of live adult NG2-mEGFP mice, although oligodendrocytes were not directly labeled. Use of constitutive NG2cre rather than inducible NG2creER mice was advantageous despite the presence of GFP throughout the lineage, as we were focused on the fate of dividing NG2 cells and this mouse line enabled us to sample a large number of cell division events. The findings from the NG2cre mouse line were consistent with those from the EdU pulse-chase experiments performed in the NG2creER line. Future studies could be conducted with additional new mouse lines to further define and investigate the temporal window.

A recent live imaging study in zebrafish reported that the number of myelin sheaths an oligodendrocyte makes is determined during a relatively short time frame after initial contact with unmyelinated axons. Our analyses revealed another critical temporal window that occurs earlier, between NG2 cell division and terminal oligodendrocyte differentiation, during which the fate of divided cells is influenced by their microenvironment. Our data suggest that cell-intrinsic changes, such as chromatin remodelling or oscillation of transcription factors such as Olig2 (ref. 35), may be occurring in the divided NG2 cells during this ‘sensitivity window’, which could alter the cells’ ability to respond to extracellular differentiation cues. Further investigation of the intracellular changes that occur in NG2 cells after division may shed light on whether all divided NG2 cells maintain the competence of differentiating into oligodendrocytes or whether some NG2 cells are programmed to permanently remain as NG2 cells.

The discovery of the narrow window of time between cell division and oligodendrocyte differentiation led us to investigate whether changes in external environment could modulate the fate of the divided cells. Using live imaging of slice cultures, we observed that a subset of divided NG2 cells in LPC-treated slices matured into DsRed\(^+\) oligodendrocytes faster than under control conditions. Thus, the time to oligodendrocyte differentiation after NG2 cell division is plastic and can be altered when oligodendrocyte production is necessary. This suggests a sensing mechanism that enables NG2 cells to detect changes in myelin or oligodendrocyte density or the presence of axons that would normally be myelinated. This hypothesis is supported by acute time-lapse imaging in zebrafish—which has shown that oligodendrocyte progenitors proliferate and replace laser-ablated oligodendrocytes—as well as by the increased rate of oligodendrocyte generation from proliferated NG2 cells in the hypomyelinating mutant *shiverer*.

We have found for the first time, to our knowledge, that deprivation of whisker sensory input reduces oligodendrocyte production after NG2 cell division specifically in the deprived somatosensory cortex and not in the neighboring motor cortex. We have previously reported that when whiskers are removed shortly after birth, before the end of the critical period for structural plasticity, there are no changes in the distribution of NG2 cells or the NG2 molecule in the somatosensory cortex, whereas another study has reported an increase in NG2 cell proliferation after whisker removal during the same developmental window. In the current study, we found a modest increase in NG2 cell proliferation after whisker clipping. However, immunolabeling for active caspase-3 revealed a 4.5-fold higher density of caspase-3\(^+\)YFP\(^+\) cells in the deprived cortex than in the spared cortex, suggesting that the increase in NG2 cell proliferation does not result in a sustained increase in oligodendrocyte-lineage cell density. The density of YFP\(^+\) caspase-3\(^+\) cells in the deprived cortex (240 per mm\(^3\)) accounts for only one-third of the difference in density of YFP\(^+\)CC1\(^+\) cells between the deprived and spared cortex (320 per mm\(^3\) in deprived and 1,164 per mm\(^3\) in spared). It is likely that caspase-3 immunolabeling detected only a subpopulation of all...
dying cells owing to the short duration of the cell-death process and rapid clearing of the dead cells. The density of caspase-3+CC1+ cells did not differ between deprived and spared cortex, which suggests that a certain fraction of differentiated oligodendrocytes die under normal conditions (the caspase-3+CC1+/CC1+ ratio was 0.55% at P10), although the fraction of caspase-3+ cells was considerably lower than the 50% rate reported for apoptotic cells in the optic nerve39. By contrast, the fraction of newly generated oligodendrocytes that were caspase-3+ (caspase-3+ YFP+/CC1+) was substantially higher at 3.53%. The effect of sensory deprivation was most pronounced in the newly divided cells. Caspase-3+ cells that were also EdU+ were found only in the deprived cortex, where caspase-3 was detected in cells with a round morphology and diffuse PDGFRα immunoreactivity or in YFP+ cells that were neither PDGFRα+ nor CC1+. These observations suggest that sensory deprivation specifically affects divided cells that are in the process of terminally differentiating into oligodendrocytes and have downregulated PDGFRα but not yet acquired CC1, consistent with reports for the normal CNS showing cell death of newly differentiated oligodendrocytes39,40.

Physiological neuronal activity could, by enhancing secretion of growth and trophic factors, influence the survival of divided NG2 cells that are in the process of differentiating into oligodendrocytes49. Brain-derived neurotrophic factor (BDNF) is known to be secreted by YFP+ cells that were neither PDGFRα+ nor CC1+. These observations are consistent with reports for the normal CNS showing cell death of newly differentiated oligodendrocytes and having a critical temporal window after NG2 cell division during which the cells are susceptible to environmental factors that affect their oligodendrocyte differentiation or survival. This is, to our knowledge, the first demonstration that the critical temporal window can be altered by changes in myelin or oligodendrocyte health, and that the survival of recently divided cells that are in the process of differentiating into oligodendrocytes can be modulated during the critical time window by alterations in physiological neuronal activity.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGMENTS**

This work was funded by grants from the US National Multiple Sclerosis Society (RG41579 to A.N.), the US National Institutes of Health (NIH R01NS073425 to A.N. and NIH R01AG27855 to J.G.) and the US National Science Foundation (A.N.). We thank F. Kirchhoff (University of Saarland) for providing PLP/Pi3-red transgenic mice, K. Ikenaka (National Institute for Physiological Science, Japan) for the DM20/PLP antibody, M. Nedergaard for initial training in vivo imaging experiments, and Y. Sun for assistance in maintaining the transgenic mouse colony.

**AUTHOR CONTRIBUTIONS**

R.A.H. designed, conducted and analyzed EdU pulse chase, live imaging and whisker ablation experiments, and wrote the manuscript. K.D.P. performed and analyzed EdU labeling and cell death experiments. C.M.G. performed a portion of EdU pulse chase labeling in P21 mice. J.G. supervised in vivo imaging and provided input on the manuscript. A.N. coordinated and supervised the experiments and edited the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Animals and induction of Cre mediated recombination. For differentiation experiments, we used both male and female mice that were triple transgenic for NG2creBAC54,18 (Jackson Labs #008533), the Cre reporter Z/EO19 (Jackson Labs #003920) and PLPDsRed48 (NG2creZEG:PLPDsRed). PLPDsRed mice were used in this study18 were generated using the same Plp promoter construct that had previously been used to generate PLP-GFP mice, in which 95% of the GFP+ cells were CC1+ oligodendrocytes50. Thus, unlike other PLP:EGFP mice generated with the 3′-untranslated region of the Plp gene in which EGFP is expressed in NG2 cells as well as in oligodendrocytes54, DS Red fluorescence in the PLPDsRed mice was seen mostly in myelinating oligodendrocytes51 and was not found in NG2 cells or immature oligodendrocytes (see Fig. 2). For EdU pulse-chase and whisker removal experiments, we used male and female inducible mice that were double heterozygous for NG2creER2 (Jackson Labs #008538) and gt(Rosa26)Sortm1(TEVP)Us (Jackson Labs #006148) (NG2creER: YFP). Cre was induced in P6–P8 mice by daily intraperitoneal injections of 0.2 mg 4-hydroxytamoxifen (4OHT, Sigma) as described previously6. To label cells in the S phase of the cell cycle, one intraperitoneal injection of 5 μg/g of 5-ethyl-2′-deoxyuridine (EdU, Life Technologies Cat. #C10338) was administered. Animals were housed in a 12/12 light/dark cycle, housed in litters of 4–8 before weaning age (P21) and housed at a density of 2–5 per cage after weaning. All animal procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Connecticut and Yale University.

In vivo two-photon imaging. For chronic transcranial time-lapse imaging, the thin skull procedure was used52. P20–P22 NG2creZEG and NG2creZEG: PLPDsRed mice were anesthetized by intraperitoneal injection of ketamine and xylazine, and the scalp was shaved and sterilized. A region of the skull approximately 1 mm in diameter was thinned with a high speed drill and a microsurgical blade to a thickness of 20–30 μm. Images were acquired using a two-photon microscope (Prairie Technologies) with a mode locked MaiTai tunable near infrared laser (Spectra Physics) and a 20x water immersion objective with a numerical aperture of 1.0 (Leica). In some cases 70,000 molecular weight Texas Red dextran (Life Technologies) was intravascularly injected to visualize the cortical vasculature. The following wavelengths were used for two-photon fluorescence excitation: 900 nm for GFP alone, 975 nm for GFP and DSRed, and 1040 nm for DSRed alone. Z stacks of the same cortical region over a depth of 200–320 μm with a 4 μm step size were captured through the thinned skull on consecutive days at intervals noted in the text. Images were analyzed using ImageJ.

Slice culture preparation and ex vivo time-lapse imaging. Forebrain slice cultures were prepared as described previously24,25. Briefly, 300 μm coronal forebrain sections from P8 NG2creZEG:PLPDsRed triple-transgenic mice were placed on 0.45 μm Millicell culture inserts (Millipore) and maintained in a 35 mm dish with a 10x objective using a Zeiss Axiovert 200 M inverted microscope equipped with an ORCA ER camera. The slices were maintained in the incubator intervals noted in the text. Images were acquired using a two-photon microscope (Prairie Technologies) with a mode locked MaiTai tunable near infrared laser (Spectra Physics) and a 20x water immersion objective with a numerical aperture of 1.0 (Leica). In some cases 70,000 molecular weight Texas Red dextran (Life Technologies) was intravascularly injected to visualize the cortical vasculature. The following wavelengths were used for two-photon fluorescence excitation: 900 nm for GFP alone, 975 nm for GFP and DSRed, and 1040 nm for DSRed alone. Z stacks of the same cortical region over a depth of 200–320 μm with a 4 μm step size were captured through the thinned skull on consecutive days at intervals noted in the text. Images were analyzed using ImageJ.

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LPC exposure in slice cultures. Cortical slice cultures from P8 NG2cre: ZEG:PLPDsRed triple-transgenic mice were exposed to 0.5 mg/ml α-hydroxybutyric acid (LPC, lsylocetin) after 3 d in vitro to cause an acute injury to myelin/oligodendrocyte membranes. After 17 h in LPC, the slices were returned to normal slice culture medium. Time-lapse imaging was started on 5 d in vitro, and images were taken manually every 4 h for a total of 78 h. Slices were then fixed with PFA-PLP and processed for immunohistochemistry using anti-GFP and CC1 antibodies (see below).

Animal perfusions and immunohistochemistry. Animals were anesthetized with isoflurane and transcardially perfused with PFA-PLP fix. Brains were post-fixed for 1 h in the same solution followed by 4 washes in 0.2M sodium phosphate buffer. Fifty-micrometer vibratome sections were cut and processed for immunohistochemistry. If antigen retrieval was necessary, sections were micro-waved for 5 min in pH 6.0 sodium citrate buffer, cooled to room temperature, and then washed in phosphate-buffered saline (PBS). After blocking and permeabilizing for 1 h in 5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS, slices were incubated at 4 °C overnight with primary antibodies diluted in PBS containing 5% NGS. Primary antibodies used were rabbit anti-NG2 antibody24 (Chemicon Cat. #AB5320, 1:500), chicken anti-GFP antibody24 (Aves Labs Cat. #GFP-1020, 1:500), mouse antibody to adenomatous polyposis coli antibody49 (Abcam Cat. #AB13847, 1:200; http://www.abcam.com/active–proliferation–antibody-ab13847.html) and AA3 rat monoclonal antibody that recognizes both DM20 and PLP splice variants of the Plp gene (obtained from Dr. Kaz Ikenaka, Okazaki, Japan). After PBS wash, slices were incubated in secondary antibodies at room temperature for 1 h in PBS containing 5% NGS. Secondary antibodies used were Alexa 488-conjugated antibodies (Molecular Probes, 1:500), Cy3-conjugated antibodies (Jackson ImmunoResearch, 1:500), and Dylight 649-conjugated antibodies (Jackson ImmunoResearch 1:200). Detection of EdU was performed following PBS wash according to the manufacturer’s protocol. Slices were then mounted on glass slides in Vectashield mounting medium (Vector Laboratories) containing 4-6-diamidino-2-phenylindole (DAPI). Fixed tissue imaging was performed using either a Zeiss Axiovert 200 M microscope with an ORCA ER camera (Hamamatsu) and Apotome grid confocal system or Leica SP2 and SP5 (Yale) confocal microscopes.

Whisker trimming and analysis of NG2 cell differentiation. The right whiskers of NG2creER:YFP or PLPDsRed animals were first trimmed at postnatal day 6 (P6) and 4OHT was injected into NG2creER:YFP mice from P6–P8. In some mice a single injection of EdU was also administered at P8. Whiskers were trimmed every day until the date of perfusion between P10 and P21. Sections from NG2creER:YFP mice were labeled for EdU, GFP, and CC1 or NG2, and the phenotype of YFP+ cells or YFP+EdU+ cells was analyzed. Sections from PLPDsRed mice were labeled for EdU only, and the EdU+ cells were scored for DSRed fluorescence.

Quantification and statistics. For EdU pulse-chase experiments with NG2creER:YFP and PLPDsRed transgenic mice, fluorescence-reporter-positive, EdU+ and NG2 or CC1+ cells were quantified in both the cortex and corpus callosum by randomly selecting regions with a defined area grid. Littermates were randomly assigned to each age group. At least four separate fields were quantified for each brain region in each section, and at least three sections were quantified for each mouse. For slice culture imaging, slices were prepared from 3 separate mice; 5 slices were analyzed for control conditions and 8–9 slices were analyzed for LPC. For in vivo imaging experiments, image stacks were compiled into time-lapse videos and the cell density, division, division orientation, migration, disappearance and differentiation was recorded in specified 80-μm z-projections indicated in the text. For whisker manipulation experiments, cell quantification was blindly performed in layer IV of the contralateral and ipsilateral somatosensory and motor cortices. For all experiments data was compiled and analyzed from at least 3 animals for each age, genotype, time point and condition. No animals were excluded from this study. For each brain area to be quantified, unbiased quantification was performed by randomly sampling 4 fields of view based on DAPI staining and then viewing the other fluorescence channels for quantification. Two-way ANOVA analyses with Bonferroni post hoc analyses were performed on data acquired from age- and region-dependent EdU pulse-chase experiments (Fig. 1 and Supplementary Fig. 2). Unpaired or paired two-tailed Student’s t-test analyses were performed for comparison of control and LPC-treated slice cultures (Fig. 5 and Supplementary Fig. 5) and spared and deprived somatosensory and motor cortices (Figs. 6 and 7 and Supplementary Fig. 7), assuming equal variance between groups. Using Minitab, the sample sizes used in the experiments were calculated to give a power of analysis of 80% or greater based on the differences and s.d., assuming an
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A Supplementary Methods Checklist is available.

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