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Cell cycle dynamics of NG2 cells in the postnatal and ageing mouse brain

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Summary

Oligodendrocyte precursors (OLPs or "NG2 cells") are abundant in the adult mouse brain, where they continue to proliferate and generate new myelinating oligodendrocytes. By cumulative BrdU labelling we estimated the cell cycle time T_C and the proportion of NG2 cells that is actively cycling (the growth fraction) at ~postnatal day 6 (P6), P60, P240 and P540. In the corpus callosum, T_C increased from <2 days at P6 to ~9 days at P60 to ~70 days at P240 and P540. In the cortex, T_C increased from ~2 days to >150 days over the same period. The growth fraction was ~50% at all ages in both corpus callosum and cortex – that is, similar numbers of mitotically active and inactive NG2 cells co-exist at all ages. Our data imply that a stable population of quiescent NG2 cells appears before the end of the first postnatal week and persists throughout life. The mitotically active population acts as a source of new oligodendrocytes during adulthood, while the biological significance of the quiescent population remains to be determined. We found that the mitotic status of adult NG2 cells is unrelated to their developmental site of origin in the ventral or dorsal telencephalon. We also report that new oligodendrocytes continue to be formed at a slow rate from NG2 cells even after P240 (eight months of age).

Keywords

NG2 cell; cell cycle; oligodendrocyte; corpus callosum; cerebral cortex; ageing; adult brain; PDGFRA; Cre recombinase; transgenic mice

Introduction

Adult cells with the antigenic phenotype of oligodendrocyte precursors (OLPs) were first identified in the rat optic nerve and later in other parts of the adult mammalian central nervous system (CNS) (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989; Engel and Wolswijk, 1996; Reynolds and Hardy, 1997; Chang et al., 2000; Horner et al, 2000;

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Statement of Interest
"Cycling and non-cycling populations of NG2 cells in the postnatal mouse brain" by Psachoulia, K., Jamen, F., Young, K.M., and Richardson, W.D.
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Reynolds et al. 2002). Like their perinatal counterparts, adult OLPs can generate either oligodendrocytes or GFAP+ "type-2 astrocytes" in culture, depending on the composition of the culture medium. Also like perinatal OLPs, adult OLPs express the NG2 proteoglycan and the platelet-derived growth factor receptor (alpha subtype, PDGFRA) and can divide and migrate in vitro in response to PDGF, although their migration rate and cell cycle are both slower than perinatal OLPs (Wolswijk and Noble, 1989; Shi *et al.*, 1998). Because of their obvious similarities to perinatal OLPs, the adult cells were generally presumed to be glial precursors that fulfil a homeostatic role in the adult CNS, replacing oligodendrocytes and possibly astrocytes that might die as a result of injury or disease or through normal wear-and-tear. PDGFRA/ NG2-expressing OLPs are distributed more-or-less uniformly throughout the adult brain and spinal cord. They are relatively abundant, comprising ~5% of all cells in the mature CNS (Pringle *et al.*, 1992; Dawson *et al.*, 2003) (for review see Nishiyama *et al.*, 2009).

Study of adult OLPs started to take off with the development of antibodies against NG2 and so they came to be known as "NG2 cells" or NG2 glia". The morphology of NG2 cells in vivo revealed that they are complex, ramified cells that contact neurons at synapses and nodes of Ranvier – not the simple morphology one might expect of immature progenitor cells (Butt *et al.*, 1999; Ong and Levine, 1999; Chang *et al.*, 2000; Hamilton *et al.*, 2009). These observations, together with the ubiquitous distribution of NG2 cells in both grey and white matter, have raised questions about the function of NG2 cells in the adult. Are they really glial precursors, or differentiated cells that perform some essential physiological role in their own right? The latter view has been encouraged by the finding that postnatal NG2 cells express ligand- and voltage-gated ion channels and receive synaptic input from neurons (Gallo *et al.*, 1996; Bergles *et al.*, 2000; Lin and Bergles, 2002; Lin and Bergles, 2004; Lin *et al.*, 2005; Karadottir *et al.*, 2005; Salter and Fern, 2005). NG2 cells also form "en-passant" glutamatergic synapses with unmyelinated axons in white matter tracts (Kukley *et al.*, 2007; Ziskin *et al.*, 2007). It has also been reported that some NG2 cells can fire action potentials in response to an initial depolarizing trigger (Chittajallu *et al.*, 2004; Karadottir *et al.*, 2008). While not all studies support this conclusion (eg. Lin and Bergles, 2002), these findings raise the possibility that NG2 cells might participate in neural processing, by sensing neuronal activity and reporting this activity to neighbouring neurons or glia through vesicular release.

Some NG2 cells continue to divide and incorporate bromo-deoxyuridine (BrdU) during adulthood (Levison *et al.*, 1999; Horner *et al.*, 2000; Dawson *et al.*, 2003; Lasiene *et al.*, 2009). Since their number and distribution does not change much throughout life (Rivers *et al.*, 2008), this implies that half of the daughters of each division, on average, must either differentiate (losing NG2 and PDGFRA immunoreactivity) or else die. Recently, several groups have followed the fates of dividing NG2 cells by "Cre-lox*"* technology in adult transgenic mice. This approach relies on expressing a tamoxifen-inducible version of Cre recombinase (CreER) under transcriptional control of regulatory sequences associated with genes that are expressed specifically or preferentially in NG2 cells. When a Cre-conditional reporter transgene such as *Rosa26R-YFP* is also present, brief administration of tamoxifen induces Cre recombination, activating the *yellow fluorescent protein (YFP)* reporter irreversibly in NG2 cells and all of their descendents. Using *Pdgfra-CreER : Rosa26R-YFP* transgenic mice our own laboratory showed that PDGFRA/ NG2 cells generate many new

myelin-forming oligodendrocytes in the adult corpus callosum and other white matter tracts (Rivers *et al.*, 2008). Many new differentiated cells (PDGFRA- and NG2-negative) were also produced in the cortical grey matter. Although most of the latter did not make identifiable myelin sheaths they all expressed the oligodendrocyte lineage marker SOX10 and many expressed the myelin protein 2',3'-cyclic nucleotide phosphodiesterase (CNP), suggesting that they might be bona fide oligodendrocytes that make relatively few myelin sheaths compared to their counterparts in white matter. Alternatively, they might be a new type of non-myelinating oligodendrocyte lineage cell. An independent study using *Olig2-CreER* transgenic mice (in which transgene activity marked NG2 cells but not differentiated oligodendrocytes) came to similar conclusions (Dimou *et al.*, 2008). There were some differences between the studies – for example, Rivers *et al.* (2008) found evidence that small numbers of piriform projection neurons were produced during adulthood in addition to oligodendrocytes, whereas Dimou *et al.* (2008) found no evidence for neurogenesis. Nevertheless, both studies agreed that a major function of adult NG2 cells, like their perinatal counterparts, is to generate new myelinating oligodendrocytes in the white matter. This does not preclude the possibility that NG2 cells might perform other more "physiological" roles besides.

The fact that glutamate can influence the proliferation and differentiation of perinatal OLPs in culture suggests that their synaptic communication with unmyelinated axons in vivo might control the postnatal development of NG2 cells. Perhaps NG2 cells are "listening in" to electrical activity, which at some threshold might trigger their myelination program. This could ensure that only active circuits are myelinated and might even contribute to circuit plasticity during adulthood (Fields, 2008). Only around 30% of axons are normally myelinated in the corpus callosum of eight month-old mice, for example, so there is plenty of scope for de novo myelination in the adult CNS (Sturrock, 1980).

The idea that adult myelinogenesis might contribute to neural plasticity in humans is gaining ground. For example, it has been reported that extensive piano practise or juggling can cause long-term changes to the structure of white matter tracts, including parts of the corpus callosum, as revealed by magnetic resonance imaging (MRI) (Bengtsson *et al.*, 2005). There is also an accumulating body of evidence that some measures of general cognitive ability correlate with white matter volume. For example, cognitive ability and white matter increase in parallel into the fourth decade of life and both decline thereafter (Bartzokis *et al.*, 2001; Mabbott *et al.*, 2006; Hasan *et al.*, 2008; Ullen *et al.*, 2008; Bartzokis et al., 2009; Zahr *et al.*, 2009). The reasons behind these age-related changes are unknown but they could conceivably be related to changes in the ability of NG2 cells to proliferate and generate new oligodendrocytes as the brain matures and ages.

NG2 cells are also thought to be crucial for remyelination following demyelinating injury or disease. For example, during cytotoxin-induced focal demyelination and subsequent re-myelination in mouse spinal cord, the dynamic behaviour of NG2 cells in and around the lesion suggests that they are the source of remyelinating oligodendrocytes (Keirstead *et al.*, 1998; Reynolds et al., 2002; Watanabe *et al.*, 2002; Dawson *et al.*, 2003). The efficiency of remyelination following experimental demyelination decreases with age, which might be at least partly due to an age-related decline in the regenerative properties of NG2 cells (Sim *et*

al., 2002). Therefore, NG2 cell function is probably crucial not only during normal healthy adulthood but also for repair following demyelinating insults to the CNS. It is therefore important to understand how and why the behaviour of NG2 cells changes with age. To this end we have investigated the cell cycle dynamics of NG2 cells and how this relates to the production of new oligodendrocytes in the mouse brain throughout life.

By cumulative BrdU labelling we estimated cell cycle time (T_C) and the fraction of all NG2 cells that is actively engaged in the cell cycle (growth fraction) in the corpus callosum and cerebral cortex at ages ranging from P6 to P540 (~18 months). T_C increased dramatically from ~2 days at P6 to ~70 days at P240 and older. Unexpectedly, the growth fraction in both grey and white matter was approximately 50% at all ages examined; that is, from soon after birth there appear to be separate populations of dividing and non-dividing NG2 cells. We had previously shown that oligodendrocyte lineage cells (SOX10+) cells in the postnatal corpus callosum and cortex develop both from inwardly migrating (*Gsh2*-expressing) precursors that arise in the ventral telencephalon and local (*Emx1*-expressing) precursors in the cortical ventricular zone, in roughly equal proportions. We therefore asked whether the dividing and non-dividing subpopulations of NG2 cells have different developmental origins, by BrdU labelling in *Gsh2-iCre : Rosa26R-GFP* and *Emx1-iCre : Rosa26R-GFP* mice. We found that both dividing and non-dividing NG2 cells were equally likely to be derived from the ventral or dorsal telencephalon. Thus, the mechanism that subdivides the NG2 population remains obscure. It will be interesting in future to determine whether the dividing and non-dividing subpopulations fulfil different roles in the postnatal CNS.

We also investigated the rate at which NG2/ PDGFRA cells in *Pdgfra-CreER^{T2} : Rosa26R-YFP* mice produced differentiated (YFP+, PDGFRA-negative) progeny. This rate decreased dramatically in the early postnatal period, as expected, and continued to decline thereafter. Thus, NG2 cell differentiation roughly parallels their rate of cell division. Nevertheless, they continue to divide slowly and generate small numbers of new oligodendrocytes even after eight months of age.

Materials and Methods

Transgenic mice

Homozygous *Pdgfra-CreER^{T2}* BAC transgenic mice (Rivers *et al.*, 2008) were crossed with homozygous *Rosa26R-YFP* Cre-conditional reporters (Srinivas *et al.*, 2001) to generate double heterozygous offspring for analysis. Genotyping was by PCR as previously described (Rivers *et al.*, 2008).

Heterozygous *Gsh2-iCre* and *Emx1-iCre* BAC transgenic mice (Kessaris *et al.*, 2006) were separately crossed with homozygous *Rosa26R-YFP* or *Rosa26R-GFP* (Mao *et al.*, 2001). Double-heterozygous offspring were identified by PCR using primers designed to detect iCre (forward 5'-GAG GGA CTA CCT CCT GTA CC, reverse 5'-TGC CCA GAG TCA TCC TTG GC), giving a 630 bp product, and the modified *Rosa* locus (forward 5'-GCG AAG AGT TTG TCC TCA ACC, reverse 5'-GGA GCG GGA GAA ATG GAT ATG), giving either a 250 bp or a 1,100bp product for *Rosa26R-YFP* or *Rosa26R-GFP*, respectively.

Tamoxifen administration

Tamoxifen (Sigma) was dissolved at 40 mg/ml in corn oil by sonicating at 30ºC for 1 hour. It was administered to *Pdgfra-CreER*^{T2} : *Rosa26R-YFP* double heterozygous mice by oral gavage on four consecutive days (one dose of 300 mg tamoxifen/ Kg body weight per day).

BrdU cumulative label

For cumulative labelling, BrdU was administered via the drinking water at a concentration of 1 mg/ml. Alternatively, for early postnatal animals (P6; pups were aged between P4 and P6 at the beginning of the time-course), BrdU was dissolved in phosphate-buffered saline (PBS) at 20 mg/ml and 30 µl was injected subcutaneously every 3.5 hours.

Tissue preparation and immunolabelling

Mice were perfusion-fixed with 4% (w/v) paraformaldehyde (PFA) in PBS. Cryosections were collected and immunolabelled as described previously (Young *et al.*, 2007). Briefly, 30 µm coronal brain sections were labelled as floating sections with the following primary antibodies: rat monoclonal anti-PDGFRA (1:400; BD Pharmingen™), rabbit anti-GFP serum (1:6000; AbCam), mouse monoclonal anti-BrdU (1:10; American Type Culture Collection, Manassas, VA), mouse monoclonal anti-CNPase (1:2000; Chemicon International). Secondary antibodies were goat anti-rat, donkey anti-rabbit or goat anti-mouse conjugated with Alexa Fluor-488, -568 or -647 (1:1000; Invitrogen). Cell nuclei were visualized by post-staining with Hoescht 33258 (1:1000; Sigma).

Microscopy and cell counts

All images were collected on an Ultraview confocal microscope (Perkin Elmer) as Z stacks with 1 µm spacing, using standard excitation and emission filters for DAPI, FITC (Alexa Fluor 488), TRITC (Alexa Fluor 568) and Far Red (Alexa Fluor 647). Orthogonal views were produced using Volocity software (Perkin Elmer). Low magnification (20x) non-overlapping fields were counted along the length of the corpus callosum (8 fields) and throughout all layers of the medial (motor) cortex (12 fields). At least two sections from each of three mice were analyzed for each time point.

Estimation of cell cycle parameters

For a homogeneous population of cycling cells, the fraction of cells that label with BrdU ("labelling index") is expected to increase linearly with the duration of BrdU exposure until all dividing cells are labelled (phase 1). After this the labelling index can not increase further and a plateau is reached (phase 2). The rate at which cells incorporate BrdU is given by the slope (m) of phase 1. The maximum labelling index (phase 2 plateau value) is known as the "growth fraction" ("GF"), which we express as a percentage of the total cell population. From these data we can calculate the length of the cell cycle Tc = GF/m (Nowakowski *et al.*, 1989; see Fig. 3). Since T_C depends on the reciprocal of m, the standard error ($s.e._{Tc}$) is not symmetrical about the mean. However, for simplicity $s.e._{Tc}$ is shown as ± half of the full range, calculated as follows:

$$s.e._{Tc} = \left[\left\{\left(GF+s.e._{GF}\right)/\left(m-s.e._{m}\right)\right\} - \left\{\left(GF-s.e._{GF}\right)/\left(m+s.e._{m}\right)\right\}\right]/2$$

We subdivided our cumulative BrdU labelling plots into rising (phase 1) and plateau (phase 2) regions by an iterative process. We fitted the first three data points to a straight line by the method of least squares, then the first four data points and so on. The line with the smallest R value was selected as the “best-fit” for phase 1 and the remaining data points were assigned to the phase 2 plateau. Where three or more data points fell in the plateau region they were analyzed by analysis of variance (ANOVA). In no case were these significantly different from one another ($P > 0.05$). Comparisons were made between data sets using ANOVA and were considered significantly different at $P \leq 0.05$.

Results

The NG2 cell cycle slows dramatically with age

We administered BrdU to wild type mice continuously for up to 100 days starting on P6, P60, P240 (eight months) and P540 (eighteen months). After various BrdU labelling times we double-immunolabelled coronal brain sections for PDGFRA and BrdU (Fig. 1) to visualize OLPs/ NG2 cells that had undergone DNA replication during the labelling period. [We previously showed that >99% of NG2+ non-vascular cells in the postnatal corpus callosum and cerebral cortex co-express PDGFRA (Rivers *et al.*, 2008).] We counted cells in defined regions of the corpus callosum (Fig. 1a-d, Fig. 2) and medial cortex (Fig. 1e-h, Fig. 2) in confocal micrographs (see Methods) and plotted BrdU labelling index versus labelling period. From these data we determined the cell cycle time (T_C) and fraction of OLPs that was cycling (growth fraction, GF) as a function of age (see Methods and Fig. 3).

In the corpus callosum, T_C increased from <2 days at P6 to ~70 days at P240 and older (Fig. 3b, c). In the cortex, T_C also increased steadily from ~2 days at P6 to >100 days at P540. We cannot determine T_C in the P540 cortex with confidence because we do not know the GF; even at P540 + 100 days of BrdU labelling the labelling index still appeared to be on the increase (Fig. 1e). If we assume that GF ≅ 0.4 at P540, as it is at P240, then T_C (P540) ≅ 170 days. It is likely that most of the variation in T_C results from how long cells remain resting in early G1.

When we examined the relationship between age and cell cycle length in the cortex we found it to be linear (Fig. 3c). With every extra day after birth, the cell cycle increases by around one third of a day. This relationship allows us to estimate T_C for any given postnatal age. In the corpus callosum there was not a simple linear relationship between age and T_C (Fig. 3c); T_C reached its maximum around P240 and did not increase significantly after that.

Dividing and non-dividing NG2 sub-populations in the postnatal brain

In contrast to T_C, GF was relatively invariant with age, ~50% at all ages in both callosal white matter and in the cortical grey (Fig. 1a, e; Fig 3b). This implies that in the postnatal forebrain only around half of all NG2 cells are actively engaged in the cell cycle at any age,

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the other half being long-term quiescent. These separate dividing and quiescent populations are already present in the brain shortly after birth at P4-P7.

Oligodendrocyte production declines in parallel with the NG2 cell cycle

We previously showed that many new myelinating oligodendrocytes are formed in the corpus callosum during young adulthood (Rivers *et al.*, 2008). In that study we treated *Pdgfra-CreER^{T2} : Rosa26R-YFP* mice with tamoxifen at P45 and followed the subsequent differentiation of labelled NG2 cells (YFP+, PDGFRA+) into oligodendrocytes (YFP+, SOX10+, PDGFRA-negative) over the following months. By this means we found that ~29% of the myelin-forming oligodendrocytes present at P240 were formed after P45 (Rivers *et al.*, 2008). To ask whether oligodendrocyte production continues after P240 we have now administered tamoxifen to P240 *Pdgfra-CreER^{T2} : Rosa26R-YFP* mice and have followed the subsequent appearance of differentiated YFP+ progeny for up to 100 days post-tamoxifen (P240+100). We first determined that ~48% of all PDGFRA+ cells became YFP-labelled following tamoxifen administration (Fig. 4a-c) – very similar to the fraction that became labelled after tamoxifen administration at P45 (Rivers *et al.*, 2008). We detected no difference in the proportion of YFP-labelled cells in the corpus callosum versus the cortex. Maximal YFP-labelling of NG2 cells was achieved by 10 days after the first dose of tamoxifen (6 days after the final dose) (Fig. 4a). We previously found that maximal labelling in P45 mice took ~8 days (Rivers *et al.*, 2008), which might indicate that tamoxifen is metabolized to the biologically active form (4-hydroxy tamoxifen) more rapidly in younger mice or that accumulation of YFP takes longer in older cells. Nevertheless, at early times (10 days) post-tamoxifen the great majority of YFP-labelled cells in both white and grey matter were also PDGFRA+, as predicted (Fig. 4b, c).

The great majority of YFP+ cells continued to co-label for PDGFRA at all time points examined post-tamoxifen (Fig. 4d, e). However, there was a slow but steady accumulation of YFP+, PDGFRA-negative cells in the grey and white matter so that by P240+100 approximately 18% of YFP+ cells in both corpus callosum and cortex were PDGFRA-negative, differentiated cells Fig. 4e-g). The YFP+, PDGFRA-negative cells in the corpus callosum had the appearance of oligodendrocytes (Fig. 4f) and they co-immunolabelled for CNPase, a marker of differentiated oligodendrocytes (Fig. 4h). This confirmed that new oligodendrocytes continue to be generated from NG2 cells even after eight months of age.

Mitotic status of NG2 cells is unrelated to their developmental site of origin

We previously traced the embryonic origins of oligodendrocyte lineage cells (SOX10+) in the forebrain by Cre *lox* fate mapping and found that they have multiple developmental origins in the ventricular zone (VZ) of the ventral and dorsal telencephalon (Kessaris *et al.*, 2006). The first OLPs were generated in the ventral telencephalon around embryonic day 12.5 (E12.5), from the VZ of the medial ganglionic eminence (MGE). Subsequently, they arose more dorsally in the VZ of the lateral ganglionic eminence (LGE) and ultimately (after birth) from the cortical VZ (Kessaris *et al.*, 2006). The MGE- and LGE-derived OLPs migrated widely throughout the developing forebrain including the cerebral cortex, whereas those derived from the cortical VZ remained within the cortex. After birth, the MGE-derived population declined rapidly within the cortex, for unknown reasons, so that the postnatal

cortex and corpus callosum become populated by a roughly equal mixture of LGE - and cortex-derived oligodendrocyte lineage cells. It seemed possible that these ventrally- and dorsally-derived populations might correspond in some way to the dividing and quiescent NG2 cell populations identified in the present study. We therefore set out to determine whether there was a correlation between the developmental origin of NG2 cells and their ability to incorporate BrdU in the adult.

As in our previous study (Kessaris *et al.*, 2006), we visualized LGE- or cortex-derived cells in *Gsh2-iCre : Rosa26R-GFP* or *Emx1-iCre : Rosa26R-GFP* mice, respectively. At P60 we immunolabelled forebrain sections for PDGFRA and GFP (Fig. 5a, b) to identify OLPs/NG2 cells that had originated from the LGE or cortex. We confirmed that PDGFRA+ cells from each region were intermingled and were present in similar proportions (Fig. 5a-e). In both corpus callosum and cortex there was a modest fraction of NG2 cells (10-20%) that appeared not to be derived from either ventral or dorsal forebrain, consistent with our previous data. We think these might be derived from the diencephalon (Kessaris *et al.*, 2006).

We administered BrdU to P60 mice via their drinking water for 6 or 35 days, then immunolabelled brain sections for GFP, PDGFRA and BrdU. We counted triple-labelled cells in the corpus callosum and cortex and expressed these as a percentage of the total number of (GFP+, PDGFRA+) cells in the same region (Fig. 5f-i). The fraction of (GFP+, PDGFRA+) cells that incorporated BrdU was similar in both *Gsh2-iCre* : *Rosa26R-GFP* and *Emx1-iCre : Rosa26R-GFP* animals and each was representative of the PDGFRA+ cell population as a whole (Fig 5f-i). We conclude that developmental origin has no influence on the proliferative behaviour of NG2 cells.

Discussion

We have studied the cell cycle dynamics of NG2 cells in the postnatal forebrain and reach the following conclusions: 1) Cell cycle time increases dramatically from ~2 days to >70 days in the corpus callosum (>150 days in cortex) between ~P6 to P240; 2) Oligodendrocyte differentiation continues throughout adulthood but the rate of oligodendrocyte production declines in parallel with the rate of precursor cell division; 3) There are two distinct populations of NG2 cells, a mitotically active population and a separate quiescent population; 4) The subdivision into dividing and non-dividing NG2 cell populations is not related to their developmental origins in either the ventral or dorsal telencephalon.

A recent study of surgical tissue has provided evidence that, at any one time, a substantial fraction of cycling adult human NG2 cells is in the early G1 phase of the cycle (Geha *et al.*, 2009). This study exploited the fact that the cell cycle-related antigen Ki-67 (Mib-1) is detectable in cells from late G1 through to M-phase but not in early G1, whereas the mini-chromosome maintenance protein-2 (Mcm-2) is expressed at all stages of the cycle. They found that cycling cells identified by either Ki-67 or Mcm-2 in the cortical grey or white matter all co-expressed NG2 and OLIG2. However, Mcm-2+ cells outnumbered Ki-67+ cells ~3-fold, consistent with a long cell cycle and an extended G1. We ourselves found that ~29% of PDGFRA/NG2 cells in the P6 mouse corpus callosum were Ki-67+, dropping to

~15% at P60 (three sections from one animal in each case). Thus, the Ki-67 labelling index does not match the growth fraction estimated by cumulative BrdU labelling (~50%) but it does decline in parallel with the increase in the length of the cell cycle - as expected if the cell cycle slows down because the duration of G1 increases.

The mitotically active NG2 cells function as bona fide oligodendrocyte precursors but what is the function of the quiescent NG2 cells? Two categories of NG2 cells in the P7 cerebellar white matter have been identified on the basis of their electrical properties; one class expresses voltage-gated sodium and potassium channels and fires action potentials in response to a depolarizing stimulus whereas the other class does not express voltage-gated channels and displays a linear voltage-current relationship (Karadottir *et al.*, 2008). These two types of NG2 cell were also identified in the corpus callosum (Karadottir *et al.*, 2008). Intriguingly, the two electrophysiological subtypes of NG2 cell were present in approximately equal proportions, as are our dividing and non-dividing subtypes. One possibility is that, during development, all newly-generated NG2 cells attach to unmyelinated axons, some of which fire action potentials and deliver a mitogenic signal to their associated NG2 cells. These NG2 cells consequently divide, renewing themselves and producing myelinating oligodendrocytes. The other NG2 cells are associated with axons that never fire, or do not fire above a sufficient threshold, so these cells are destined to remain mitotically inactive (Fig. 6). They might also lose their expression of voltage-gated ion channels, rendering them electrically passive. These might contribute in some other way to the neuronal circuitry – for example they might perform some essential homeostatic function at nodes of Ranvier or at neuron-neuron synapses (Butt *et al.*, 2002). This model implies that the electrically active sub-class of NG2 cells might correspond to our mitotically active fraction. Against this, Kukley *et al.* (2008) found that some NG2 cells had synapses and sodium channels, whether or not they expressed PCNA, implying that both cycling and non-cycling cells are electrically active.

Are the non-dividing NG2 cells permanently post-mitotic, or can they ever re-enter the cell cycle? It seems possible that they do not divide under normal conditions because, once the BrdU labelling index reached plateau, there was no sign of any further increase even after a very long labelling period. For example, at P60 the BrdU labelling index in the corpus callosum reached 40-50% after ~8 days (~20 days in cortex) but did not increase further than that even after 100 days continuous BrdU exposure (until P160). At earlier (P6) and later (P240) ages the steady-state labelling index was very similar (~50%) so it seems that a stable non-dividing subpopulation forms during late embryogenesis or during the first few postnatal days and persists for many months.

An alternative scenario that might be consistent with our data is that there is a continuous flow of cells: non-dividing NG2 cell → dividing NG2 cell → differentiated oligodendrocyte. However, to maintain the NG2 cell population this would require continuous replacement of non-dividing NG2 cells from some other source. Where would they come from? A potential source might be the adult subventricular zone (SVZ), which has been shown to generate some glial precursors and oligodendrocytes during adulthood (Levison *et al.*, 1999; Menn *et al.*, 2006). However, we previously showed by Cre-lox fate mapping that only a tiny proportion of oligodendrocyte lineage cells in the adult corpus

callosum are SVZ-derived, the main source being the pre-existing population of adult NG2 cells (Rivers *et al.*, 2008). Furthermore, SVZ precursors are themselves a mitotically active population so, under the conditions of our cumulative labelling experiments, SVZ-derived NG2 cells would still be BrdU+. It therefore seems most likely that the quiescent NG2 cell population is stable long-term. The dividing population of NG2 cells also seems to be stable because although the cells divide more slowly with age they do not exit the cell cycle - i.e. the growth fraction does not decline with age. Our growth fraction measurements are in keeping with a previous report that the growth fraction (defined by PCNA immunolabelling) of P7-P12 mouse hippocampal NG2 cells is also close to 50% (Kukley *et al.*, 2008). Moreover, Keirstead *et al.* (1998) found that ~50% of NG2 cells in the adult rat spinal cord were resistant to killing by X-irradiation, suggesting that they were dividing very slowly or not at all.

Whether the non-dividing NG2 cells are intrinsically incapable of division, or else are inhibited from doing so by their local environmental, is another question. It will be interesting, for example, to see if the growth fraction can be increased by culturing dissociated cells in saturating concentrations of mitogens (e.g. PDGF), which would be expected to override environmental regulation. It will also be informative to discover whether the cell cycle of older NG2 cells can be accelerated by culturing in PDGF or other mitogens. We showed previously that the cell cycle time of OLPs in the embryonic mouse spinal cord increases from ~30 hours to 70-100 hours between E13 and E17, but that both E13 and E17 cells can accelerate their cycle to ~20 hours when cultured in medium containing saturating PDGF-AA. This demonstrated that slowing of the OLP cell cycle during late embryogenesis results from a change in their mitogenic environment, not a shift in the intrinsic properties of the cells (van Heyningen *et al.*, 2001). Transgenic mice engineered to over-express PDGF-A in the CNS have an increased number of NG2 cells during adulthood (Woodruff *et al.*, 2004) as well as in the embryo (van Heyningen *et al.*, 2001), suggesting that PDGF-AA is a critical mitogen for OLPs/ NG2 cells throughout life. It is likely that PDGF acts in concert with other mitogenic stimuli including electrical stimulation; for example, there is evidence that proliferation of OLPs in the perinatal rat optic nerve depends on electrical activity of retinal ganglion cell axons, one effect of which might be to stimulate release of PDGF from astrocytes within the nerve (Barres and Raff, 1993). The fact that NG2 cells receive synaptic input from axons, together with previous evidence that the neurotransmitter glutamate can inhibit proliferation and differentiation of OLPs/NG2 cells in culture, suggests that neurotransmitter release at axon-glial synapses might directly or indirectly influence the NG2 cell cycle and the myelination program.

Dividing NG2 cells in white matter tracts generate new myelinating oligodendrocytes. Since the number of NG2 cells stays relatively constant during the first year of life (Rivers *et al.*, 2008) it follows that half of the daughters of cell divisions must either differentiate or die. For example, at P45 the cell cycle time in the corpus callosum is ~7.5 days (extrapolating from the data in Fig. 3), so it will take ~15 days (two cell cycles) for that half of the NG2 cells that is dividing to generate a number of differentiated cells equal to the starting population of NG2 cells. Put another way, the fraction of YFP+ cells that is differentiated (therefore PDGFRA-negative) 15 days after administering tamoxifen to P45 *Pdgfra-CreER^{T2} : Rosa26R-YFP* mice is predicted to be 50%. At 14 days post-tamoxifen the

predicted fraction is ~48%, which is close to experiment (~45%), so it appears that there is rather little death of newly differentiated cells in the 14 days after P45. A similar calculation for P240 (T_C = 73 ± 12 days) predicts that at 100 days post-tamoxifen 41 ± 4 % of YFP+ cells should be PDGFRA-negative. The observed value in this case was only ~18%, suggesting that less than half of the differentiated progeny of NG2 cells survive long-term between P240 and P340. Nevertheless, the rate of oligodendrocyte production in the corpus callosum roughly follows the rate of NG2 cell division, as expected; the cell cycle slows down ~10-fold between P45 and P240 and the rate of oligodendrocyte production slows ~20-fold in the same period. The same principle applies in the cortex (data not shown), although both cell division and production of YFP+, PDGFRA-negative cells are slower in the cortex than in the corpus callosum at most ages. Note that our estimate of T_C in 8 month old mice (~73 days) is in line with long term retroviral tracing experiments that indicate an oligodendroglial cell doubling time in the 3-8 month old rat cortex of around three months (Levison *et al.*, 1999). An age-related increase in the cell cycle of OLPs in the mouse spinal cord was also noted by Lasiene *et al.* (2009). These authors also observed that the NG2 cell cycle started to speed up again in aged mice (21 months of age) but we did not observe this phenomenon in the forebrain of 18 month old animals in this study.

Following experimental demyelination in rodents, the number of NG2 cells in and around lesions and the local BrdU labelling index both increase, indicating that the NG2 cell cycle speeds up in response to demyelination (Kierstead *et al.*, 1998; Redwine & Armstrong, 1998; Levine and Reynolds, 1999; Watanabe *et al.*, 2002). What causes the cell cycle to speed up? It is possible, for example, that mitogenic factors are released from naked axons or that loss of myelin somehow triggers mitogen release from nearby cells such as astrocytes. Redwine & Armstrong (1998) found increased PDGF A immuno-reactivity in reactive astrocytes following MHV-induced demyelination. Also, remyelination was enhanced in the corpus callosum of cuprizone-treated transgenic mice engineered to over-express PDGF-A in astrocytes (Vana *et al.*, 2007). These observations suggest that astrocyte-derived PDGF might be involved in the mitogenic response of NG2 cells to demyelination. Fibroblast growth factor (FGF) might also be involved because NG2 cells express FGF receptors and up-regulate them following experimental demyelination (Redwine & Armstrong, 1998; Fortin et *al.*, 2005; Redwine *et al.*, 2007). Adult NG2 cell division in culture can be accelerated by a combination of PDGF and Glial Growth Factor (GGF2, a soluble Neuregulin-1 isoform) (Shi *et al.*, 1998). Thus, manipulating a range of growth factor signalling pathways might be useful in the context of enhancing remyelination by endogenous NG2 cells in demyelinating diseases like multiple sclerosis, as well as in other forms of injury with myelin involvement (McTigue & Tripathi, 2009). Note that even in demyelinated lesions the distinction between the cycling and non-cycling NG2 cell populations seems to be maintained and only the cycling population contributes to repair (Kierstead *et al.*, 1998).

The age-related reduction in the proliferative rate of NG2 cells and the corresponding reduction in the rate of production of new oligodendrocytes could play an important role in the normal ageing process. For example, it is becoming clear that white matter volume starts to decline in humans after the fourth decade of life and that this white matter loss correlates with general deterioration of cognitive and motor ability. It could be that myelinating

oligodendrocytes have a finite lifetime and, beyond this, the rate of new oligodendrocyte production cannot keep pace with accelerating oligodendrocyte loss. If so, finding ways to maintain NG2 cells in a more proliferation-competent state might help maintain white matter integrity and slow down age-related mental decline.

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Addendum

While this paper was in press we learned of another cumulative BrdU study (Hiroko Nakatani and Carlos Parras, Hôpital de la Pitié-Salpêtrière, Paris) similar to our own, which found consistently higher growth fractions than we did: ~90% in the corpus callosum, ~60% in the cortex at P80+21 days BrdU exposure in their study, compared to ~50% in the corpus callosum and ~40% in the cortex at P60+21 in our own study. We explored possible reasons for the discrepancy, including different tissue fixation and BrdU immunolabelling procedures. For example, the Parras group perfusion-fixed their mice with cold 2% (w/v) paraformaldehyde (PF) and post-fixed their sections with 4% PF for 5 minutes just prior to immunolabelling for PDGFRA, whereas we perfused with 4% PF followed by 45 minutes immersion fixation in 4% PF, before cutting sections, with no further fixation. In addition, the Parras group pre-treated their sections with 2M HCl for 30 minutes at 37°C prior to BrdU immunolabelling, whereas we used 6M HCl for 15 minutes at 20-25°C (Young et al., 2007). We therefore performed one new cumulative BrdU labeling experiment (P21+80) using the Parras fixation and BrdU labelling procedures. From that experiment we estimated the growth fraction of NG2 cells to be ~45% in both corpus callosum and cortex – roughly in line with our previous estimates (Figure 1). In parallel, we immunolabelled slides from the Parras lab (P80+21) and found the growth fraction in the cortex to be ~75% in that material. From this it appears that the difference might reside in the tissue (e.g the mice colonies), rather than the preparation procedures. This requires further investigation but at present we stand by the growth fraction estimates presented in our present paper. Our conclusion that the NG2 cell cycle lengthens dramatically with age is unaffected by these issues, although actual cell cycle lengths at all ages might be longer than we estimated. We thank Drs Parras and Nakatani for drawing our attention to this discrepancy and for helping us try to resolve it.

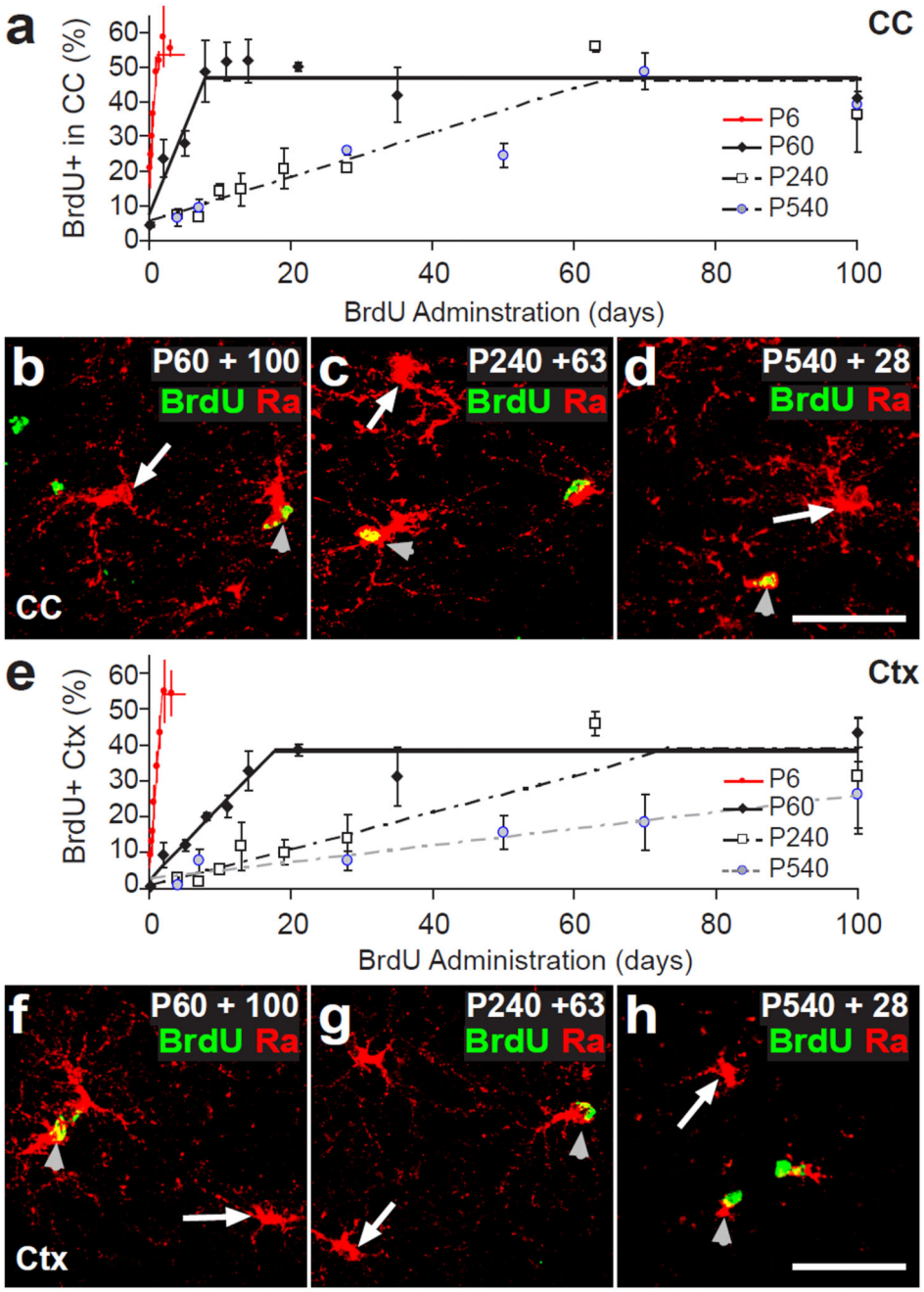

Figure 1.

NG2 cells continue to proliferate throughout postnatal life in the corpus callosum and cortex. BrdU was administered to mice by subcutaneous injection or via their drinking water (see Methods) for up to 100 days starting on ~P6, P60 (2 months), P240 (8 months) or P540 (18 months). At various times after the start of BrdU administration, the number of BrdU+, PDGFRA+ cells was counted in the corpus callosum and cerebral cortex and expressed as a percentage of the total number of PDGFRA+ cells (**a, e**). BrdU+ (Alexa 568, shown green), PDGFRA+ (Alexa 647, shown red) NG2 cells could be detected readily in the grey (**b-d**)

and white (**f-h**) matter at all ages. The point at which a plateau is reached indicates the fraction of the NG2 cell population that is actively cycling. Grey arrowheads indicate BrdU +, cycling NG2 cells and white arrowheads indicate non-cycling cells. CC, corpus callosum; Ctx, cortex. Scale bars: **b-d** and **f-h**, 20 μm

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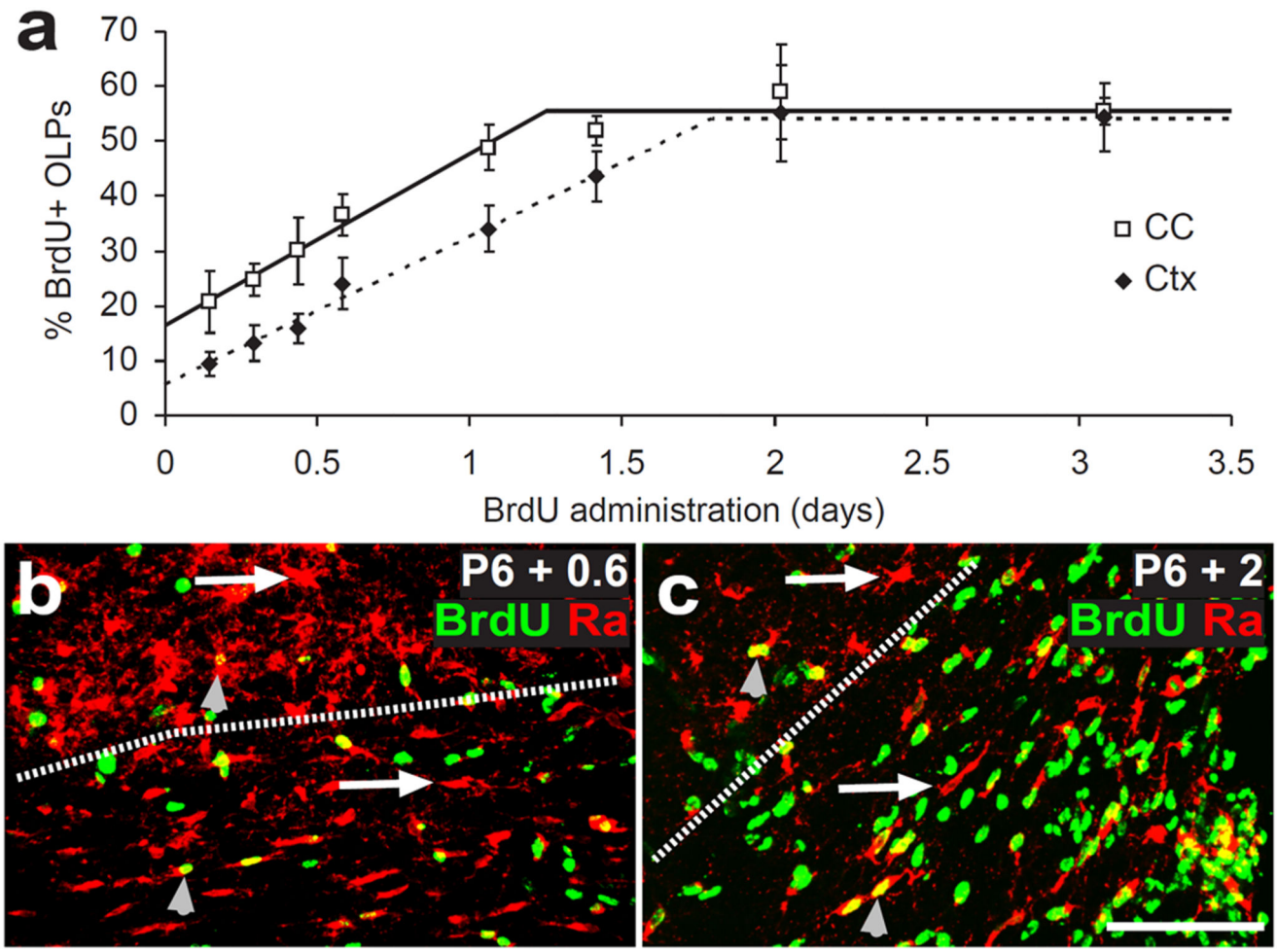
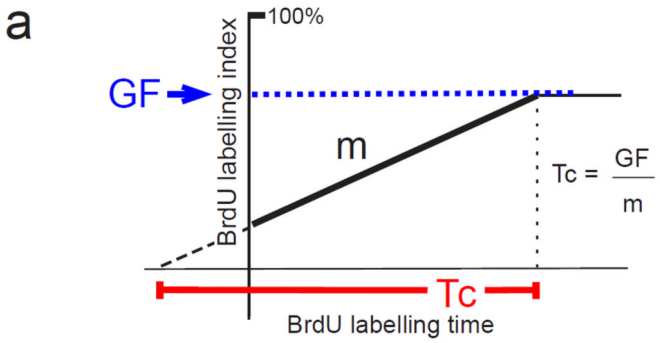


Figure 2.

Quiescent NG2 cells appear before the end of the first postnatal week. BrdU was administered for ~3 days starting on P6 by repeated subcutaneous injections. (These data are the same as those shown in red in Fig 1a, e). At various times after the start of BrdU administration, BrdU+, PDGFRA+ cells were counted in the corpus callosum and cortex and expressed as a percentage of the total number of PDGFRA+ cells (**a**). BrdU+ (green), PDGFRA+ (red) NG2 cells could be detected readily in the grey and white matter at all BrdU labelling periods. Grey arrowheads indicate BrdU+ cycling NG2 cells and white arrowheads non-cycling cells (**b-c**). The white dashed line indicates the border between corpus callosum and cortical grey matter. CC, corpus callosum; Ctx, cortex. Scale bars: **a-b**, 60 μm



b

		Postnatal Age (days)			
		6	60	240	540
CC	GF	0.54 ± 0.02	0.47 ± 0.02	0.46 ± 0.06	0.45 ± 0.03
	m	0.31 ± 0.02	0.05 ± 0.01	0.006 ± 0.001	0.006 ± 0.001
	Tc	1.7 ± 0.2	9 ± 2	72 ± 21	75 ± 18
Ctx	GF	0.55 ± 0.03	0.38 ± 0.02	0.39 ± 0.03	-
	m	0.27 ± 0.01	0.021 ± 0.002	0.0051 ± 0.001	0.0023 ± 0.0005
	Tc	2 ± 0.2	18 ± 3	76 ± 28	170 ± 54

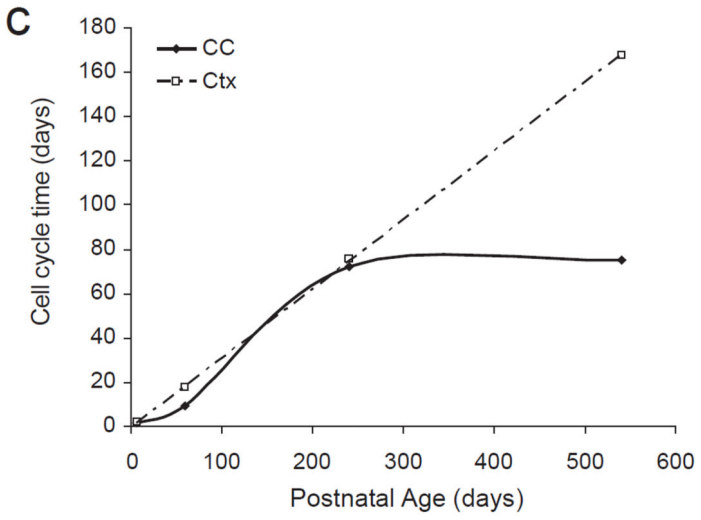


Figure 3.

The NG2 cell cycle slows down during postnatal life. BrdU was administered to mice via their drinking water starting on P6, P60, P240 or P540 and the fraction (percentage) of PDGFRA+ NG2 cells that was BrdU+ was plotted versus the BrdU labelling period. (**a**) Calculation of cell cycle time (T_C). The gradient (m) of the linear rising part of the graph was determined by the method of least squares (see Methods). The labelling index at plateau is the fraction of the population that is actively cycling (the growth fraction, GF). If the whole population were cycling GF would be 100%; in our experiments GF was closer to

50%. (**b**) Table of GF, m and T_C for NG2 cells in the corpus callosum and cortex at the ages examined. (**c**) In the cortex there was a linear relationship between age and T_C. In the corpus callosum T_C reached a plateau after ~P240. CC, corpus callosum; Ctx, cortex

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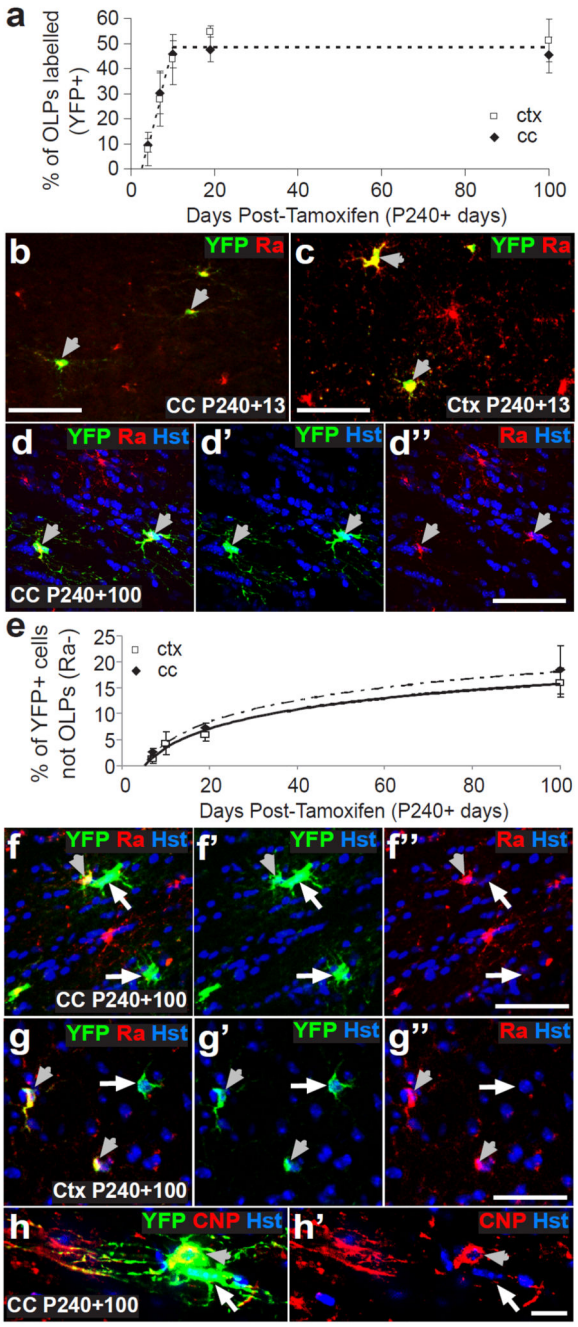

Figure 4.

NG2 cells continue to produce oligodendrocytes after 8 months of age. To trace the fate of NG2 cells in the mature brain, tamoxifen was administered to *Pdgfra-CreER*^{T2} : *Rosa26R-YFP* mice starting on P240. (**a**) The proportion of PDGFRA+ cells that became YFP-labelled is plotted against time post-tamoxifen. Within ~10 days post-tamoxifen ~45% of PDGFRA+ (red) cells in the corpus callosum (**b**) and cortex (**c**) become stably labelled with YFP (green). Tracing the fate of YFP+ cells revealed that the great majority of YFP+ cells remained undifferentiated (PDGFRA+), even 100 days post-tamoxifen (P240+100). The

proportion of YFP+ cells that were differentiated (PDGFRA-negative) increased slowly with time (**e**). YFP+, PDGFRA-negative cells with the morphology of differentiating oligodendrocytes were generated in both the corpus callosum (**f**) and cortex (**g**). (**h**) YFP+ cells (green) with the morphology of oligodendrocytes were found to co-stain for the differentiated oligodendrocyte marker CNPase (red). Grey arrowhead indicates a YFP+, PDGFRA+ NG2 cell, white arrow indicates a YFP+, PDGFRA-negative oligodendrocyte. CC, corpus callosum; Ctx, cortex. Scale bars: **b-d**, 35 μm; **f-g**, 30 μm.

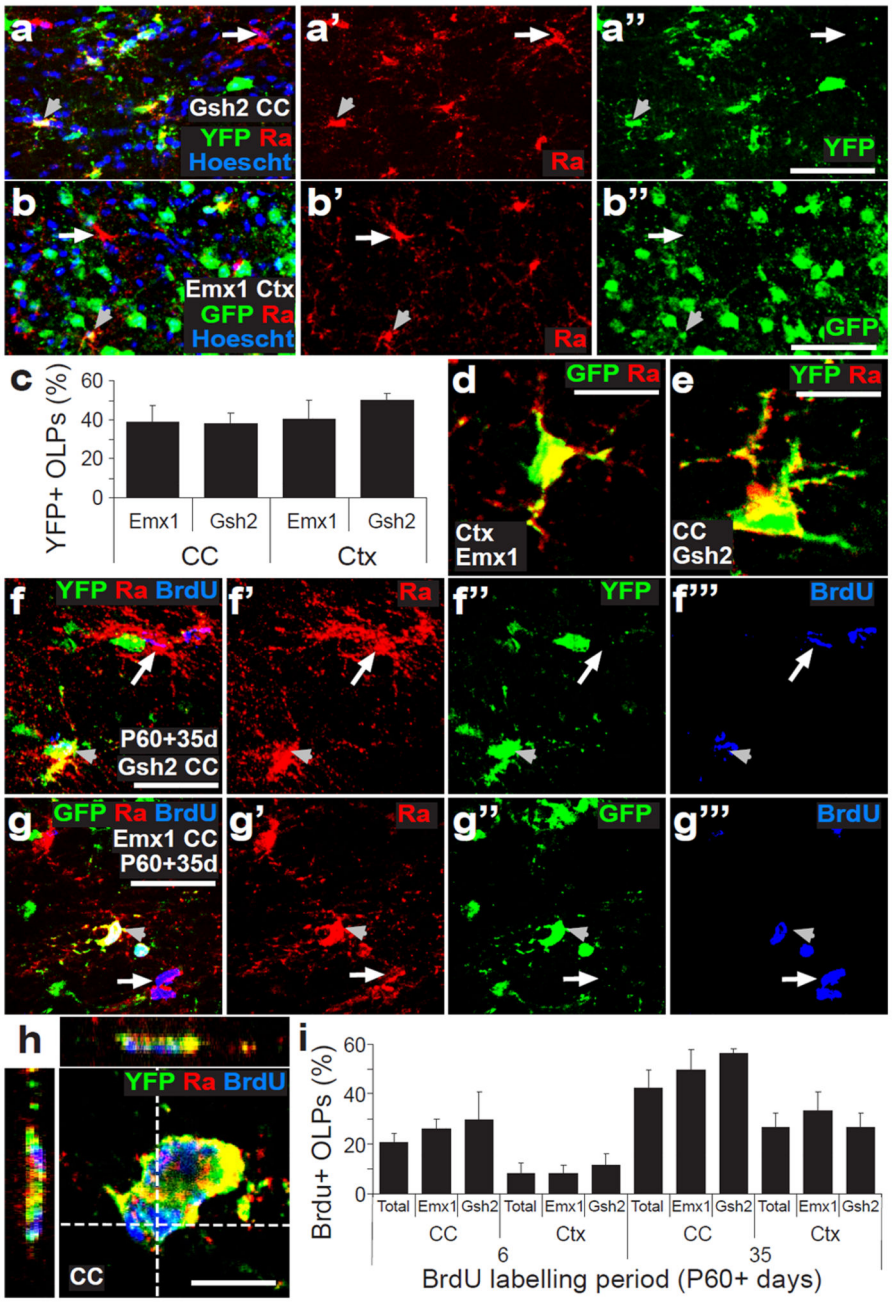

Figure 5.

NG2 cells derived from both the dorsal and ventral VZ contribute to both the cycling and non-cycling populations. By crossing *Gsh2-iCre* or *Emx1-iCre* transgenic mice with Cre-sensitive *Rosa26R- GFP* reporter mice we were able to trace GFP+ (green), PDGFRA+ (red) NG2 cells that originated from either the embryonic LGE/MGE or the cortical VZ, respectively (**a, b**). (**c**) Numbers of GFP+, PDGFRA+ cells in the corpus callosum or cortex (3 sections from each of 6 mice) were expressed as a percentage of all PDGFRA+ cells in the same region. *Gsh2*- and *Emx1*-derived NG2 cells were found in approximately equal

numbers in both corpus callosum and cortex (**d, e**). To determine whether *Gsh2*- and/or *Emx1*- derived NG2 cells were dividing, BrdU was administered via the drinking water for 6 or 35 days starting on P60 and brain sections were triple immunolabelled for BrdU (blue), PDGFRA (red) and GFP (green) (**f, g**). GFP+, PDGFRA+ cells were scored as BrdU-positive or –negative by examining confocal images with orthogonal projections (**h**). Numbers of BrdU+, GFP+, PDGFRA+ cells in corpus callosum and cortex were expressed as a percentage of all GFP+, PDGFRA+ cells in the same area. Both *Gsh2*- and *Emx1*-derived PDGFRA/NG2 cells contributed approximately equally to the cycling and non-cycling sub-populations. Grey arrowheads indicate (GFP+, PDGFRA+) double-positive (**a, b**) or (BrdU+, GFP+, PDGFRA+) triple-positive (**f, g**) cells. White arrows indicate a PDGFRA+ single-positive (**a, b**) or (GFP+, PDGFRA+) double-positive (**f, g**) cell. CC, corpus callosum; Ctx cortex. Scale bars: **a-b**, 40 μm; **d-e**, 10 μm; **f-g**, 17 μm; **h**, 6 μm.

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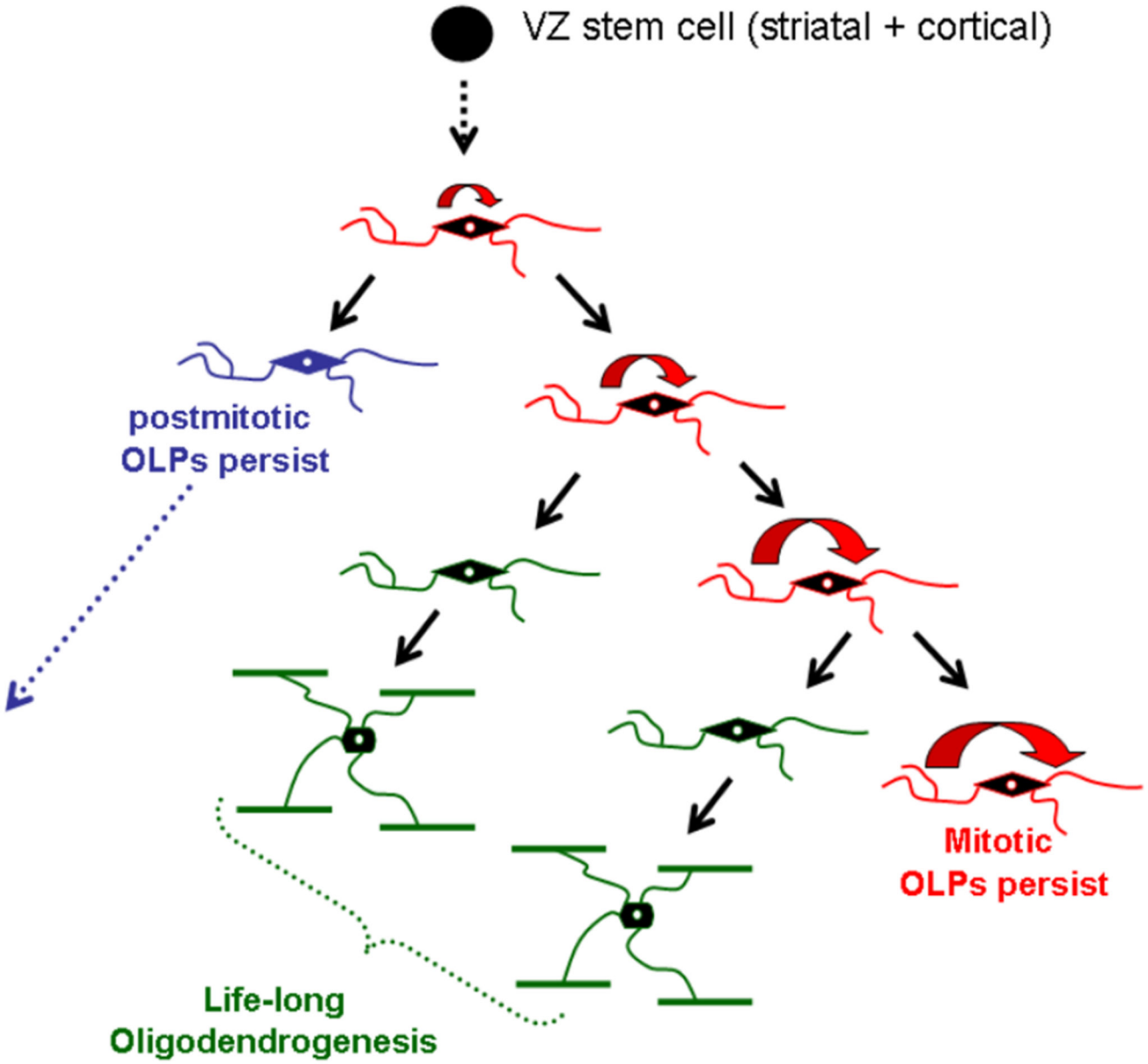

Figure 6.

The birth and behaviour of NG2 glia. From data presented in this paper and Kessaris *et al.,* (2006), we conclude that adult forebrain OLPs/ NG2 cells are initially derived from embryonic VZ stem cells located in both the dorsal (cortical) and ventral (LGE/MGE) telencephalon. In this paper we show that their embryonic origin does not have any bearing on their mitotic activity as adult NG2 cells. A population of quiescent NG2 cells, comprising approximately half of all NG2 cells, appears some time before the end of the first postnatal week and persists throughout life. The function of these cells is unknown. The other half of the NG2 cell population remains in cycle throughout life, although their cell cycle length increases with age (e.g. from ~2 days at P6, to ~70 days at 8 months and >150 days at 18 months in the cortex). One major function of the proliferating OLPs is to generate new

oligodendrocytes throughout life. However, the rate of oligodendrogenesis slows progressively with age, roughly in parallel with the decelerating cell cycle.

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