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

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O (OLP 'NG2 ') , . B B U , T$_C$ NG2 ( ) ~ 6 (P6), P60, P240 P540. I , T$_C$ <2 P6 ~9 P60 ~70 P240 P540. I , T$_C$ ~2 >150 . T ~50% , NG2 - . O NG2 . T , . W NG2 . W NG2 P240 (8).

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;

Recently, several groups have followed the fates of dividing NG2 cells by ‘Cre-lox’ technology in adult transgenic mice.

double heterozygous offspring for analysis. Genotyping was by PCR as previously described (Rivers ., 2008).

Heterozygous *G 2- C* and *E 1- C* BAC transgenic mice (Kessaris ., 2006) were separately crossed with homozygous *R 26R-YFP* or *R 26R-GFP* (Mao ., 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 *R* 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 1100 bp product for *R 26R-YFP* or *R 26R-GFP*, respectively.

Tamoxifen administration

Tamoxifen (Sigma) was dissolved at 40 mg/ml in corn oil by sonicating at 30°C for 1 h. It was administered to *P -C* ER^{T2}*:R 26R-YFP* double heterozygous mice by oral gavage on 4 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 h.

Tissue preparation and immunolabelling

Fig. 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 Materials and 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 BrdUþ, PDGFRAþ cells was counted in the corpus callosum and cerebral cortex and expressed as a percentage of the total number of PDGFRAþ cells (a,e). BrdUþ (green), PDGFRAþ (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 BrdUþ, cycling NG2 cells and white arrowheads indicate non-cycling cells. CC, corpus callosum; Ctx, cortex; Ra, PDGFRA. Scale bars: b–d and F–H, 20 μm.

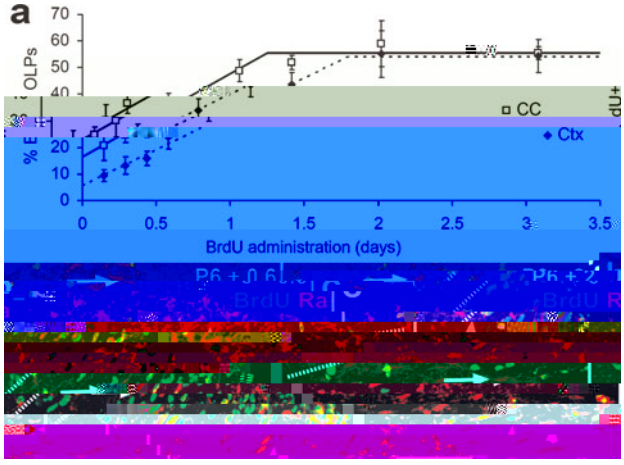

Fig. 2. Quiescent NG2 cells appear before the end of the first postnatal week. BrdU was administered for ~3 days starting on P6 by repeated subcutaneous injections. (These data are the same as those shown in red in Fig. 1a,e.) At various times after the start of BrdU administration, BrdUþ, PDGFRAþ cells were counted in the corpus callosum and cortex and expressed as a percentage of the total number of PDGFRAþ cells (a). BrdUþ (green), PDGFRAþ (red) NG2 cells could be detected readily in the grey and white matter at all BrdU labelling periods. Grey arrowheads indicate cycling, BrdUþ 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; Ra, PDGFRA. Scale bar: b,c, 60 μm.

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, 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 ., 2008). In that study we treated *P -C ER^{T2}:R 26R-YFP* mice with tamoxifen at P45 and followed the subsequent differentiation of labelled NG2 cells (YFPþ, PDGFRAþ) into oligodendrocytes (YFPþ, SOX10þ, 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 ., 2008). To ask whether oligodendrocyte production continues after P240 we have now administered tamoxifen to P240 *P -C ER^{T2}:R 26R-YFP* mice and have followed the subsequent appearance of differentiated YFPþ progeny for up to 100 days post-tamoxifen (P240 þ 100). We first determined that ~48% of all PDGFRAþ cells became YFP-labelled following tamoxifen administration (Fig. 4a–c) – very similar to the fraction that became labelled after tamoxifen administration at P45 (Rivers ., 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 ., 2008), which might indicate that tamoxifen is

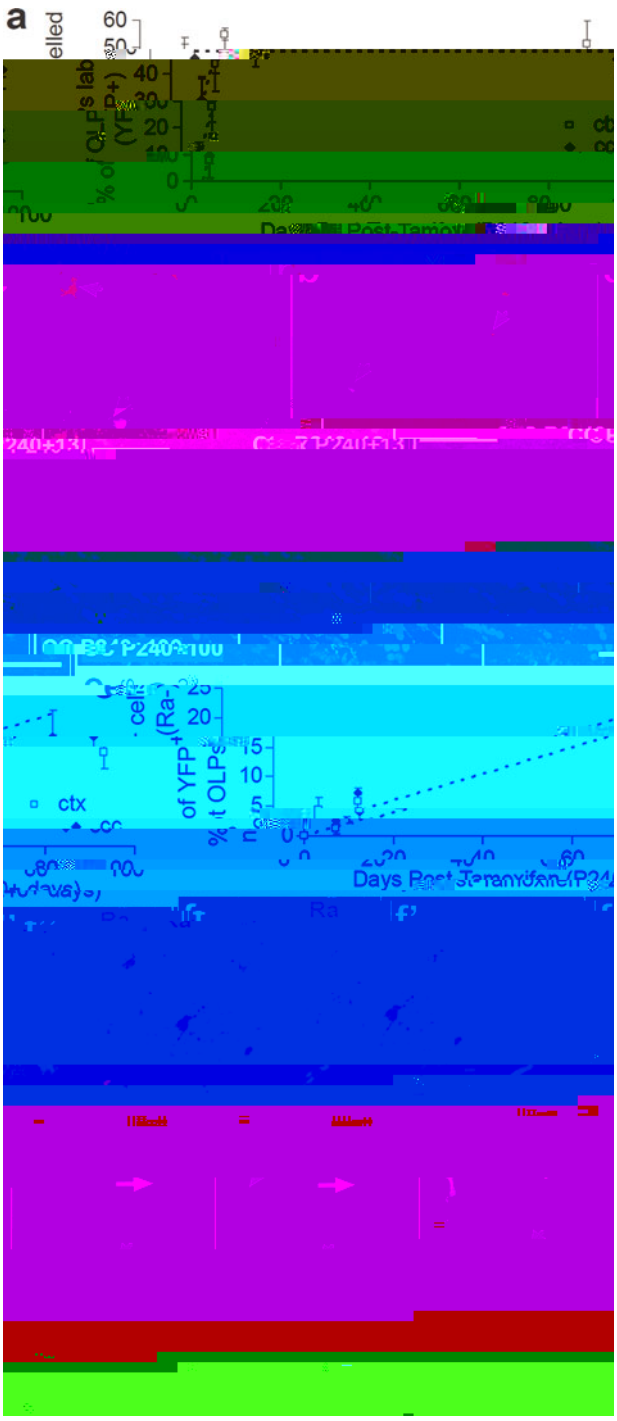

Fig. 4. NG2 cells continue to produce oligodendrocytes after 8 months of age. To trace the fate of NG2 cells in the mature brain, tamoxifen was administered to *P -C ER^{T2}:R 26R-YFP* mice starting on P240. (a) The proportion of PDGFRAþ cells that became YFP-labelled is plotted against time post-tamoxifen. Within ~10 days post-tamoxifen ~45% of PDGFRAþ (red) cells in the corpus callosum (b) and cortex (c) become stably labelled with YFP (green). Tracing the fate of YFPþ cells revealed that the great majority of YFPþ cells remained undifferentiated (PDGFRAþ), even 100 days post-tamoxifen (P240 þ 100) (d). The proportion of YFPþ cells that were differentiated (PDGFRA-negative) increased slowly with time (e). YFPþ, PDGFRA-negative cells were generated in both the corpus callosum (f) and cortex (g). (h) YFPþ

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 PDGFRAþ, as predicted (Fig. 4b,c).

The great majority of YFPþ 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 YFPþ, PDGFRA-negative cells in the grey and

white matter so that by P240 þ 100 approximately 18% of YFP þ cells in both corpus callosum and cortex were PDGFRA-negative, differentiated cells (Fig. 4e–g). The YFP þ, 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 8 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 þ) in the forebrain by Cre- fate mapping and found that they have multiple developmental origins in the VZ of the ventral and dorsal telencephalon (Kessaris ., 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 ., 2006). The MGE- and LGE-derived OLPs migrated widely throughout the developn,n623.46ine-349.2ncludopnthel74430.1(cort),)]TJT*whe62allsarose3-217.9-der(ted)-294.4(from)the cortical VZ3-215.rem46i(ted)

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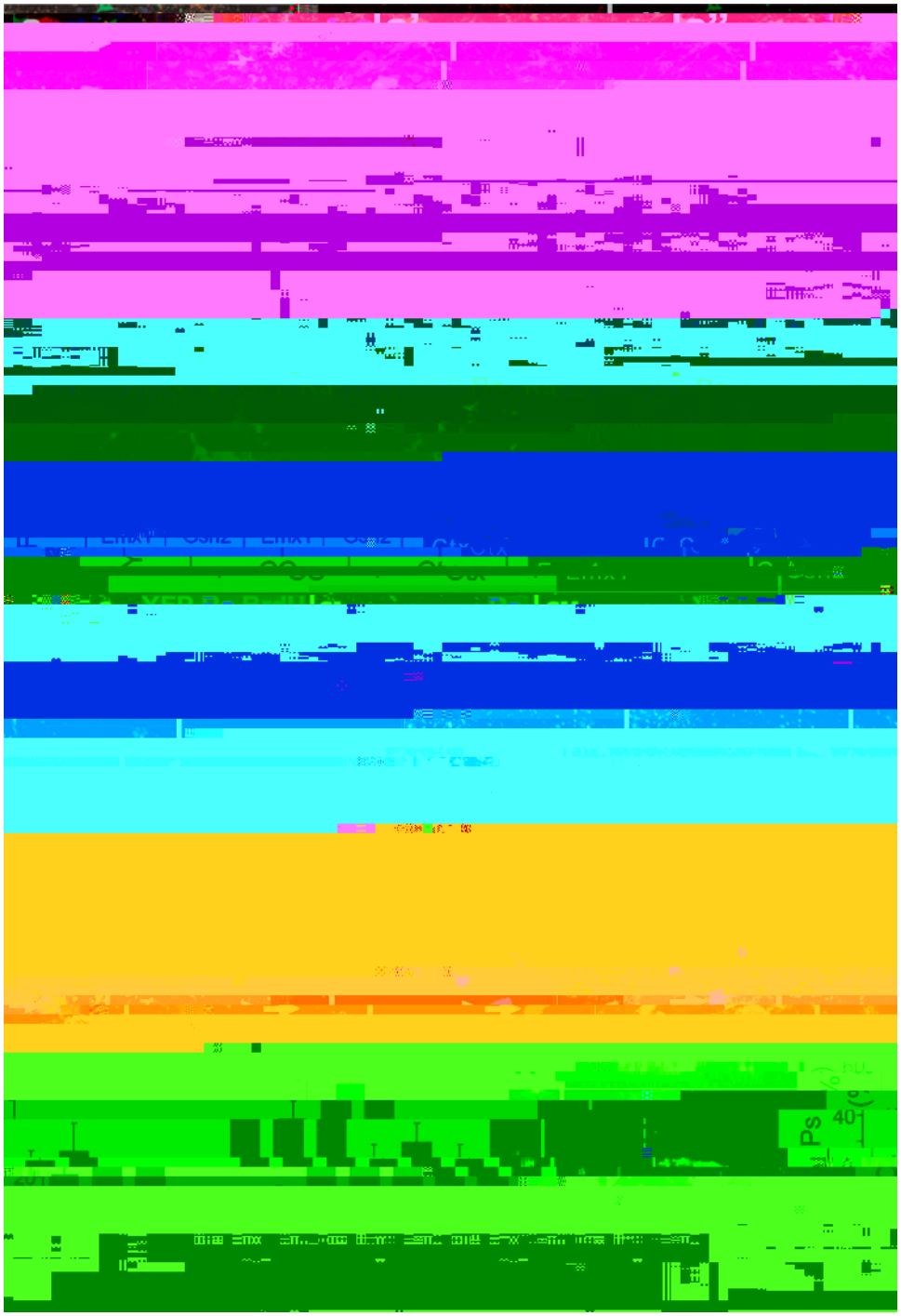

this, Kukley . (2008) found that some NG2 cells had synapses and sodium channels, whether or not they expressed PCNA, implying that both cycling and non-cycling cells are electrically active. Also, not all workers agree that NG2 cells can fire action potentials (Lin and Bergles, 2002), so these ideas need further scrutiny.

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 of 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

($T_C = 73 \pm 12$ days) predicts that at 100 days post-tamoxifen $41 \pm 4\%$ of YFP þ 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 þ, 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 3 months (Levison ., 1999). An age-related increase in the cell cycle of OLPs in the mouse spinal cord was also noted by Lasiene . (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 (Keirstead ., 1998; Redwine and Armstrong, 1998; Levine and Reynolds, 1999; Watanabe ., 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 and 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 .

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