

NG2 Glia Generate New Oligodendrocytes But Few Astrocytes in a Murine Experimental Autoimmune Encephalomyelitis Model of Demyelinating Disease

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The adult mammalian brain and spinal cord contain glial precursors that express platelet-derived growth factor receptor α subunit (PDGFRA) and the NG2 proteoglycan. These “NG2 cells” descend from oligodendrocyte precursors in the perinatal CNS and continue to generate myelinating oligodendrocytes in the gray and white matter of the postnatal brain. It has been proposed that NG2 cells can also generate reactive astrocytes at sites of CNS injury or demyelination. To test this we examined the fates of PDGFRA/NG2 cells in the mouse spinal cord during experimental autoimmune encephalomyelitis (EAE)—a demyelinating condition that models some aspects of multiple sclerosis in humans. We administered tamoxifen to *Pdgfra-CreER^{T2}:Rosa26R-YFP* mice to induce yellow fluorescent protein (YFP) expression in PDGFRA/NG2 cells and their differentiated progeny. We subsequently induced EAE and observed a large (>4-fold) increase in the local density of YFP⁺ cells, >90% of which were oligodendrocyte lineage cells. Many of these became CC1-positive, NG2-negative differentiated oligodendrocytes that expressed myelin markers CNP and Tmem10/Opalin. PDGFRA/NG2 cells generated very few GFAP⁺-reactive astrocytes (1–2% of all YFP⁺ cells) or NeuN⁺ neurons (<0.02%). Thus, PDGFRA/NG2 cells act predominantly as a reservoir of new oligodendrocytes in the demyelinated spinal cord.

Introduction

Oligodendrocytes (OLs) synthesize CNS myelin, which is required for fast saltatory transmission of action potentials. In multiple sclerosis (MS), OLs die, possibly through autoimmune attack, and demyelination results. During this and other demyelinating diseases there is spontaneous regeneration of lost OLs and myelin. The replacement OLs are believed to originate from adult oligodendrocyte precursors (OLPs), which are widespread and abundant in the adult CNS (French-Constant and Raff, 1986; Wolswijk and Noble, 1989; Pringle et al., 1992; Butt et al., 2005; Wilson et al., 2006). Adult OLPs, also known as “NG2 cells,” coexpress platelet-derived growth factor receptor α subunit (PDGFRA) and the NG2 proteoglycan (Nishiyama et al., 2009; Wilson et al., 2006) and are antigenically similar to OLPs in the perinatal CNS. Perinatal OLPs generate OLs *in vitro* and in the

early postnatal period *in vivo* (Raff et al., 1983; Hall et al., 1996; Zhu et al., 2008; Guo et al., 2009). They also generate a subset of protoplasmic astrocytes during perinatal development (Zhu et al., 2008; Guo et al., 2009). Adult OLPs continue to divide and generate new myelinating OLs in the healthy adult mouse CNS (Horner et al., 2000; Dawson et al., 2003; Aguirre and Gallo, 2004; Dimou et al., 2008; Rivers et al., 2008), though at a steadily decreasing rate with age (Lasiene et al., 2009; Psachoulia et al., 2009). They do not appear to generate astrocytes during normal adulthood (Dimou et al., 2008; Rivers et al., 2008). There is evidence that NG2 cells can generate small numbers of neurons during adulthood, although this remains controversial (Aguirre and Gallo, 2004; Dayer et al., 2005; Tamura et al., 2007; Rivers et al., 2008; Guo et al., 2010).

Several studies have indicated that adult NG2 cells can generate remyelinating OLs following experimental demyelination in rodents (Gensert and Goldman, 1997; Keirstead et al., 1998; Redwine and Armstrong, 1998; Watanabe et al., 2002; Polito and Reynolds, 2005; Zawadzka et al., 2010). There is also circumstantial evidence that NG2 cells generate remyelinating OLs during MS in humans (Nishiyama et al., 2009; Wilson et al., 2006). However, neither in rodents nor in humans has it been demonstrated unequivocally that NG2 cells can regenerate OLs in conditions of chronic demyelination. Moreover, it is not known whether the reported multilineage differentiation potential of NG2 cells is realized—or augmented—in the abnormal environment of the injured CNS. To establish the differentiation fates of PDGFRA/NG2 cells during MS-like pathology we administered tamoxifen (Tam) to adult *Pdgfra-CreER^{T2}:Rosa26R-YFP* double-transgenic mice to induce YFP labeling of PDGFRA-expressing cells, then in-

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duced experimental autoimmune encephalomyelitis (EAE) by immunizing with emulsified myelin oligodendrocyte glycoprotein (MOG) peptide. This caused widespread demyelination along the neuraxis. We subsequently identified YFP-labeled PDGFRA/NG2 cells and their differentiated progeny by immunohistochemistry. Our lineage tracing study provides direct evidence that PDGFRA/NG2 cells generate new OLs in the demyelinated spinal cord. By comparison, PDGFRA/NG2 cells produced very few astrocytes and practically no neurons. A significant fraction (2–10%) of YFP-labeled cells could not be identified with a battery of antibodies against neurons, glia, neural stem/progenitor cells, or vascular or immune system cells.

We also report that Tam pretreatment resulted in significantly reduced locomotor disability in female but not male mice with EAE.

Materials and Methods

Induction of EAE. All animal work conformed to local ethical committee guidelines and the Animals (Scientific Procedures) Act 1986 and was specifically approved by the United Kingdom Government Home Office. *Pdgfra-CreERT²* BAC transgenic mice have been described (Rivers et al., 2008). They were made by pronuclear injection of C57BL/6-CBA F1 hybrids and maintained on the *Rosa26R-YFP* (*R26R-YFP*) reporter background (Srinivas et al., 2001). Cre recombination was induced by administering tamoxifen (Tam) (Sigma, 40 mg/ml), dissolved in corn oil (Sigma, C8267), by sonication for 45 min at 30°C. Adult mice were given 300 mg/kg body weight by oral gavage on 4 consecutive days starting 14 d before EAE induction (Fig. 1). Our *Pdgfra-CreERT²* line expresses Cre exclusively in PDGFRA-immunoreactive precursors (Rivers et al., 2008) but not in differentiated OLs, which do not express PDGFRA (Hall et al., 1996; Butt et al., 1997). Cre-mediated recombination is completely absent in the absence of Tam and continues in the spinal cord for at most 10 d following Tam induction (Psachoulia et al., 2009). The efficiency of Cre recombination (proportion of PDGFRA⁺ cells that became YFP⁺) in the adult spinal cord was ~30%, and this fraction remained stable between 14 and 42 d post-Tam. This was slightly lower than we found previously in the adult forebrain (~45–50%) (Rivers et al., 2008).

EAE was induced in 14- to 18-week-old [postnatal day ~110 (~P110)] male and virgin female *Pdgfra-CreERT²:R26R-YFP* mice by immunizing with emulsified MOG peptide (amino acids 35–55) together with Freund's adjuvant [1 mg/ml MOG peptide, 2.5 mg/ml *Mycobacterium tuberculosis* in 50% (v/v) incomplete Freund's adjuvant, 50% (v/v) PBS], injected subcutaneously on days 0 and 7 (i.e., 14 and 21 d post-Tam). In addition, 0.1 ml of *Pertussis* toxin (300 ng/ml) was injected intraperitoneally on days 0 and 2. Mock-immunized animals received the same inoculum without MOG peptide. The time line of the experiments is illustrated in Figure 1A. We analyzed three groups of mice that had received (1) Tam in corn oil followed by mock-EAE immunization ("Tam-only"), (2) corn oil followed by EAE inoculum ("EAE-only"), and (3) Tam followed by EAE inoculum ("Tam+EAE").

Locomotor testing. All mice were evaluated daily for signs of locomotor disability on a 7 point scale (supplemental Table S1, available at www.jneurosci.org as supplemental material). Mice showing severe spasticity or a score >5 were killed immediately by a humane method.

Tissue processing. Mice were perfused intracardially with 4% (w/v) paraformaldehyde (PFA) in PBS at room temperature (~20°C). Spinal cords were dissected and postfixed in 4% PFA overnight at 4°C. Tissue was cryoprotected in 20% (w/v) sucrose at 4°C overnight, embedded in OCT compound, frozen, and stored at -80°C until sectioning. Spinal

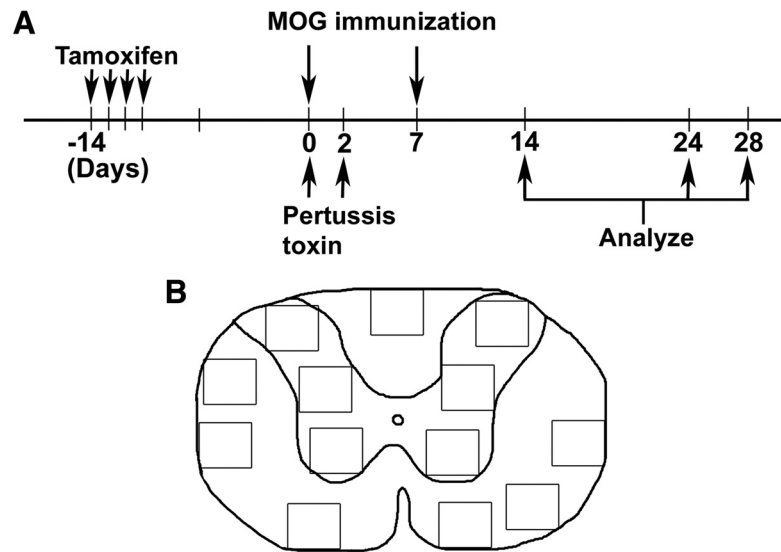


Figure 1. *A*, Timeline of the experiments. Mice were given tamoxifen (300 mg/kg) in corn oil by oral gavage on 4 consecutive days, starting 14 d before EAE induction. MOG peptide (amino acids 35–55) together with Freund's adjuvant was injected subcutaneously on days 0 and 7. *Pertussis* toxin (300 ng/ml) was injected intraperitoneally on days 0 and 2. Animals were analyzed on days 14 and 24 (males) or 28 (females). *B*, Diagram of the spinal cord indicating locations of sample boxes used for cell counts.

cords at the cervical, thoracic, and lumbar levels were sectioned at 30 μ m nominal thickness and collected by floating on the surface of PBS.

Immunocytochemistry and microscopy. Floating spinal cord sections were pretreated with blocking solution [10% (v/v) sheep serum, 0.1% (v/v) Triton X-100 in PBS], incubated with primary antibodies overnight at 4°C, then secondary antibodies for 1 h at 20–25°C. Details of primary antibodies are given in supplemental Table S2 (available at www.jneurosci.org as supplemental material). Solochrome cyanine dye was used to visualize myelin. The following secondary antibodies were used in blocking solution: Alexa Fluor 488 goat anti-rat IgG (Invitrogen, 1:500), Alexa Fluor 567 goat anti-mouse IgG1, Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen, 1:1000), and Cy3-conjugated donkey anti-guinea pig IgG (Millipore Bioscience Research Reagents, 1:500). Fluorescein-conjugated isolectin B4 from *Bandeiraea (Griffonia) simplicifolia* (ILB4, Vector Labs, 1:100) was used to label microglia and endothelial cells. Cell nuclei were visualized by poststaining with Hoechst 33258 (Sigma, 1:1000). Sections were transferred to Superfrost Plus slides and dried in air, mounted in DAKO mounting medium under coverslips, and examined in a PerkinElmer Ultraview confocal microscope.

Two sections from each level (cervical, thoracic, and lumbosacral) of the spinal cord were analyzed for every animal. Seven arbitrary nonoverlapping sample fields were counted in the white matter and six fields in the gray matter of every section (Fig. 1B) and data were pooled per section, then per animal. Data from white matter (WM) and gray matter (GM) were kept separate. WM and GM boxes were distinct and nonoverlapping with an area of 0.11 mm² (Fig. 1B). However, if a WM box straddled WM and GM—which sometimes happened in lumbosacral spinal cord—the cells in GM were easily excluded since GM and WM could be visually distinguished. The general strategy was to place at least one WM box in each of the dorsal, dorsolateral, lateral, and ventral funiculi and to place two GM boxes in each of the dorsal horn, ventral horn, and intermediate GM areas. Since EAE results in multiple diffuse areas of demyelination instead of necessarily sharp delineated lesions, we decided to take this nonselective approach to field selection. Data were plotted and statistical analyses performed using GraphPad Prism 5.0 software. All data are plotted as mean \pm SEM.

Results

Locomotor analysis of *Pdgfra-CreERT²:R26R-YFP* mice following EAE induction

Our EAE paradigm (Fig. 1) produced locomotor disability beginning in the second week after initial immunization. Our locomo-

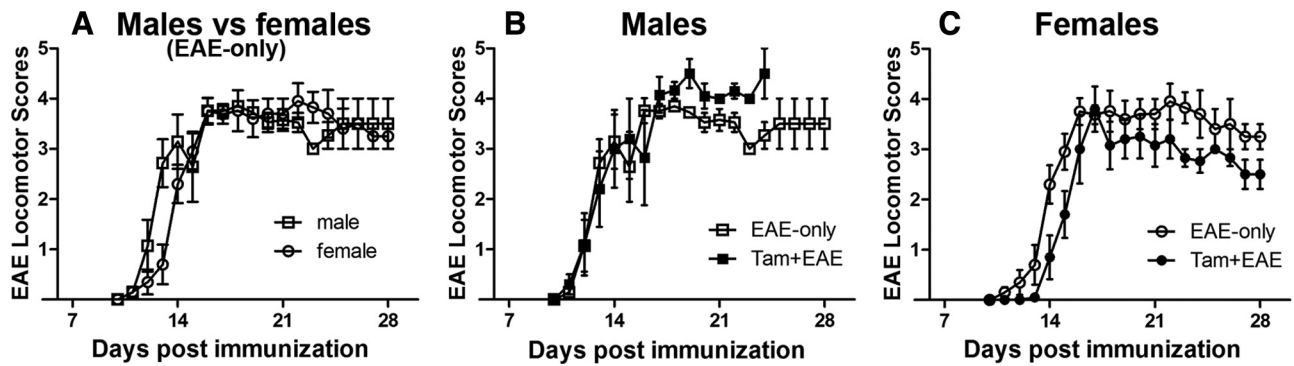


Figure 2. Time course of EAE symptoms in male and female mice. **A**, Locomotor function of all mice deteriorated over the course of the study, with EAE-only control males ($n = 7$) responding on a similar course as control females ($n = 10$). **B**, A trend toward greater disability was apparent in Tam + EAE ($n = 5$) compared with EAE-only ($n = 7$) males, although the difference did not reach statistical significance. However, Tam + EAE males reached greater disability faster than EAE-only males and were humanely killed 24 d after EAE induction (24 dpi). **C**, In contrast, Tam + EAE females ($n = 10$) performed significantly better than EAE-only females ($n = 10$) ($p < 0.0001$). Also, the time of onset of symptoms was significantly delayed, by around a day, in Tam + EAE females. All data are represented as mean \pm SEM. Two-way ANOVA was used to analyze data.

tor scoring criteria are shown in supplemental Table S1 (available at www.jneurosci.org as supplemental material). A progressive worsening of locomotion was observed following the onset of symptoms (Fig. 2A), with a few (9 of 32) mice showing a relapsing–remitting phenotype (not evident, because the data are pooled; Fig. 2A). Male and female mice (EAE-only) showed similar progression curves (Fig. 2A). There was a tendency for males to show earlier onset of symptoms, but this did not reach statistical significance.

Since it was necessary to administer Tam to our mice to fate-map PDGFRA/NG2 cells during EAE, we asked whether Tam itself might have an effect on disease progression (Tam+EAE mice). Several Tam+EAE males were very severely affected (5 on the locomotor scale), which was never seen in the control group that received corn oil without Tam (EAE-only males) (Fig. 2B). Moreover, all of the Tam+EAE males had to be humanely killed before 28 d post-EAE immunization because of the severity of their symptoms; this curtailed the experiment before a statistically significant difference between Tam+EAE and EAE-only males was established. Nevertheless, there was a trend toward more severe outcome in Tam+EAE male mice (Fig. 2B). In contrast, Tam treatment decreased the severity of EAE symptoms in Tam+EAE females (Fig. 2C). Neither male nor female Tam-only mice displayed any locomotor disability.

Fates of PDGFRA cells during EAE

Different EAE induction protocols result in different disease courses and this can be mouse strain specific. We therefore checked whether immunization with MOG peptide caused demyelination in our *Pdgfra-CreER^{T2}:R26R-YFP* mice. Myelin histochemistry (with solochrome cyanine) and myelin basic protein (MBP) immunohistochemistry revealed demyelinated plaques/lesions all along the rostral–caudal extent of the spinal cord (Fig. 3A,B), accompanied by a robust inflammatory response (Fig. 3C,D).

To determine the fates of PDGFRA cells following EAE immunization, we examined spinal cord tissue on 14 d postimmunization (14 dpi), when most animals had begun to show locomotor symptoms, and also on 28 dpi (females) or 24 dpi (males). No significant differences were noted among these time points (nor between males and females) by any of our immunolabeling criteria, so data for all time points and both sexes were subsequently pooled.

In Tam+EAE mice there was an ~ 4.5 -fold increase in YFP⁺ cells in the white matter and an ~ 2 -fold increase in the gray

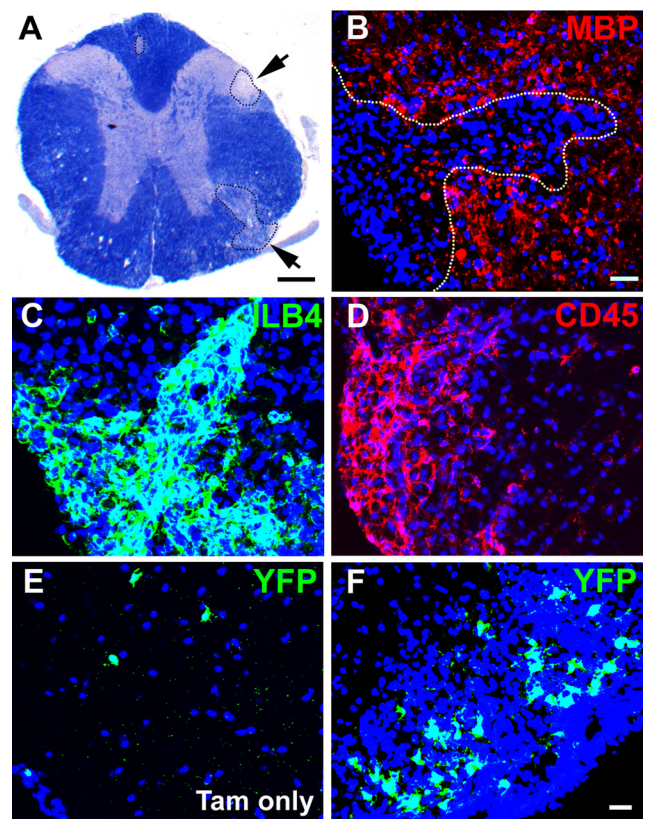


Figure 3. MOG immunization causes inflammation and demyelination. **A–F**, Following MOG immunization, demyelinated plaques, visible by local reductions of solochrome staining intensity (**A**, arrows) or loss of MBP immunoreactivity (dotted line in **B**) were seen at all spinal cord levels. Also, massive inflammation marked by CD45 (**C**) and ILB4 (**D**) labeling indicated activation of microglia and macrophages and infiltration by blood-borne immune cells. Following EAE induction there was an increase in YFP⁺ (*Pdgfra*-derived) cells (**F**) in Tam+EAE compared with Tam-only spinal cords (**E**). Images were taken from thoracic–lumbar spinal cord at 24 dpi in Tam+EAE animals unless indicated otherwise. Sections **B–F** are counterstained with Hoechst dye (blue). Scale bars: 200 μ m (**A**), 20 μ m (**B, F**).

matter of the spinal cord (Figs. 3E,F, 4I). These are average figures; there was variation among individual mice—from 2- to 7-fold in white matter, for example. Since there were more YFP⁺ cells in gray matter to begin with, this resulted ultimately in a roughly even distribution of YFP⁺ cells in the gray and white matter of Tam+EAE animals (Fig. 4I). Most YFP⁺ cells in

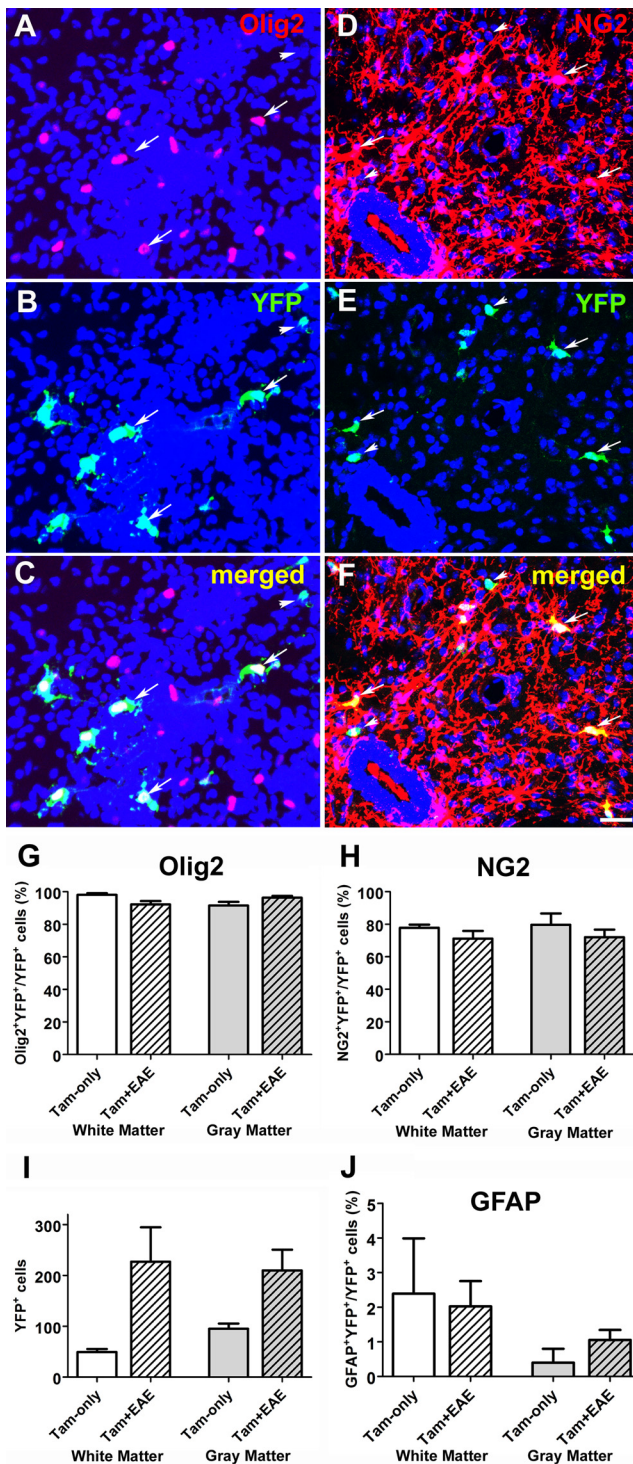


Figure 4. A large proportion of YFP⁺ cells belong to the OL lineage. Immunolabeling for OLIG2 (**A**) and YFP (**B**) revealed many colabeled cell (**C**), indicating they belonged to the OL lineage. This image was taken near a white matter lesion at 14 d dpi. Also, many YFP⁺ cells (**E**) coexpressed NG2 (**D, F**). The image was taken in the gray matter at 28 dpi. Arrows indicate double-labeled cells and arrowheads indicate single-labeled cells. **G**, Quantification showed that 92–98% of all YFP⁺ cells were OLIG2⁺. There were no detectable differences between white or gray matter or between Tam + EAE and Tam-only groups ($n = 5$ /group). **H**, NG2 cells comprised ~78% of all YFP⁺ Tam-only cells and ~72% in Tam + EAE white and gray matter ($n = 3$ /group). **I**, As expected, there was a large increase in YFP⁺ cells in the white and gray matter following EAE induction. Data in **I** are shown as average numbers of YFP⁺ cells counted/animal. **J**, Approximately 2% and 1% of all YFP⁺ cells in the white and gray matter, respectively, were colabeled with GFAP ($n = 5$ /group). All data are pooled from 14 d and 24 d/28 d post-EAE immunization (28 d and 38 d/42 d following tamoxifen administration) and represented as mean \pm SEM. One-way ANOVA was used to compare data across groups. Scale bar, 20 μ m.

Tam+EAE spinal cords were OLIG2⁺, identifying them as OL lineage cells ($92 \pm 2\%$ in white matter, $96 \pm 2\%$ in gray) (Fig. 4A–C,G). In Tam-only animals $98 \pm 1\%$ of YFP⁺ cells were also OLIG2⁺ in white matter, compared with $92 \pm 1\%$ in gray (Fig. 4G). A large fraction of YFP⁺ cells was also NG2⁺, both in Tam+EAE mice ($71 \pm 5\%$ in white matter, $72 \pm 5\%$ in gray) and Tam-only mice ($78 \pm 2\%$ in white, $80 \pm 6\%$ in gray) (Fig. 4D–F,H).

Approximately 2% of all YFP⁺ cells in the white matter of Tam-only tissue (5/229 cells in 30 sections) and Tam+EAE tissue (18/970) were GFAP⁺ (Fig. 4J). In gray matter, ~1% (9/898) of YFP⁺ cells were GFAP⁺ in Tam+EAE mice, compared with ~0.4% (2/466) in Tam-only animals. Because GFAP is expressed at a low level or not at all in protoplasmic astrocytes in gray matter, we also coimmunolabeled for YFP and S100 β , which is expressed by astrocytes and a subset of oligodendrocyte lineage cells. We did not detect YFP⁺, S100 β ⁺ double-labeled cells in TAM+EAE mice, even in or around lesions. It was originally reported that the *Rosa* promoter has undetectable activity in mature astrocytes (Malatesta et al., 2003) but we have found that both fibrous and protoplasmic astrocytes are strongly labeled in P50 *Fgfr3-CreER^{T2}:Rosa26-YFP* animals (Young et al., 2010). Hence the use of the *Rosa26-YFP* reporter does not preclude our ability to detect astrocytes. Approximately 1.4% (10/716) of YFP⁺ cells in Tam-only spinal cords were NeuN⁺ and ~0.2% (4/2147) in Tam+EAE cords (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). A small number of new neurons generated from the SVZ have been previously reported in a subpopulation of multiple sclerosis lesions (Chang et al., 2008). However it is unclear whether these were generated from NG2 cells. Together, the evidence indicates that PDGFRA/NG2 glia generate very few, if any, astrocytes or neurons in the EAE spinal cord.

Production of OLs from PDGFRA cells during EAE

In agreement with previous data (Ligon et al., 2006), we did not detect any NG2⁺ cells that were not also OLIG2⁺ in Tam+EAE or Tam-only spinal cords (data not shown). Since more YFP⁺ cells were OLIG2⁺ than NG2⁺ (e.g., 92% versus 71%, respectively, in Tam+EAE white matter; Fig. 4G,H), it follows that a subset of OLIG2⁺ cells (~21% in this example) were NG2-negative, differentiated OLs. The proportion was similar in Tam-only white matter (~20%). Since there were ~4.5 times more YFP⁺ cells on average in Tam+EAE white matter compared with Tam-only white matter, it is clear that many new differentiated OLs are generated in response to EAE induction.

To look for direct evidence of remyelinating OL production, we double-immunolabeled Tam+EAE spinal cords for YFP and one of the following markers of differentiated/myelinating oligodendrocytes: adenomatous polyposis coli (APC, recognized by monoclonal CC1), 2',3'-cyclic nucleotide phosphodiesterase (CNP), MBP, Tmem10/Opalin or Ermin/Juxtannodin. APC is found in oligodendrocyte cell bodies and proximal processes, CNP and MBP in cell bodies, processes and myelin sheaths. Tmem10/Opalin is expressed at the onset of myelination in the OL cell body, processes and at the internodes (Golan et al., 2008). Ermin/Juxtannodin is an oligodendrocyte cytoskeletal-related protein that is found in the cytoplasmic tongue processes and terminal loops of compact myelin (Zhang et al., 2005; Brockschneider et al., 2006). Its temporal expression profile closely follows that of MBP. YFP is a large cytoplasmic protein that is physically excluded from compact myelin, so it is difficult to correlate YFP expression with myelin wraps. Nevertheless, we

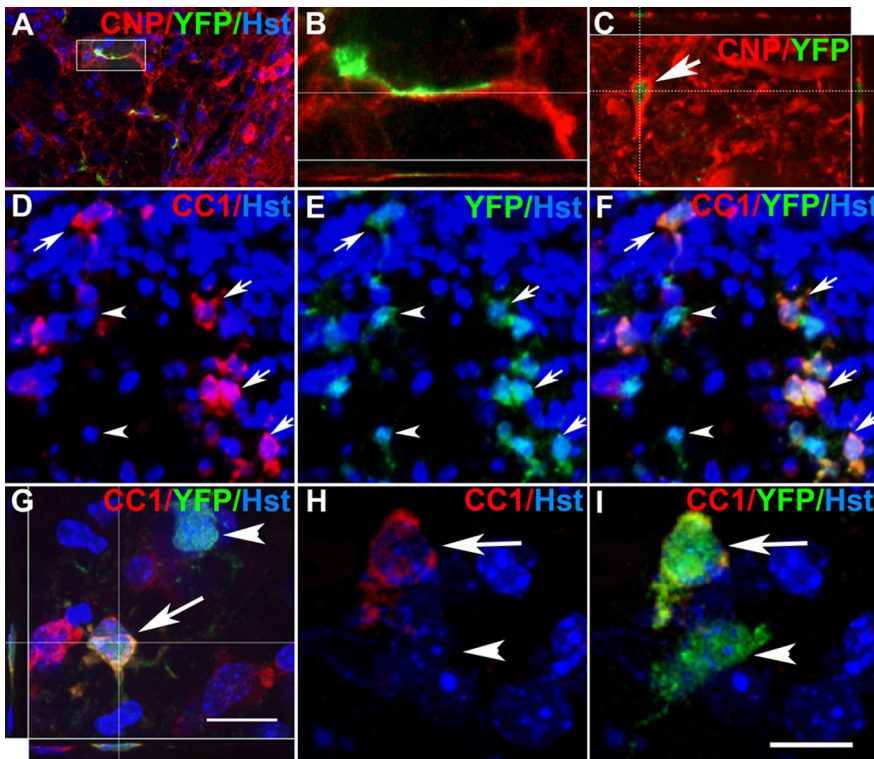


Figure 5. PDGFRA/NG2 cells give rise to mature OLs. **A–C**, A proportion of YFP⁺ cells colabels for CNP. The box in **A** is shown at higher magnification (single scan) in **B** along with an XZ plane through the presumptive myelin sheath. Another CNP⁺, YFP⁺ cell in the YZ and XZ planes is shown in **C**. Multiple CC1⁺, YFP⁺ oligodendrocytes are observed following EAE induction (**D–F**). These are shown at higher magnification in **G–I**. Orthogonal views are shown in **G**. Arrows indicate double-labeled cells and arrowheads single-labeled YFP⁺ cells. The CC1 images were taken from 24 dpi tissue and the CNP images from 28 dpi tissue. Sections are counterstained with Hoechst dye (Hst, blue). Scale bar, 20 μ m.

found many YFP⁺ cell bodies and processes that also colabeled for CNP (Fig. 5A–C), APC (Fig. 5D–I) or Opalin (Fig. 6C–E). We estimated that in Tam+EAE animals 35 \pm 12% of 404 YFP⁺ cells, and in Tam-only animals, 21 \pm 7% of 183 YFP⁺ cells were also CC1⁺ (mean \pm SEM, 30 sections from three mice). Both YFP⁺/Opalin⁺ and YFP⁺/Ermin⁺ cells could be found in close apposition to Neurofilament-positive profiles (Fig. 6A,B,F–H) but these were not numerous, presumably because YFP is excluded from the myelin sheaths. Consequently, we cannot be certain that the new OLs all form myelin; it is possible that some or many of them are differentiated but not myelin-forming either because of the lack of suitable axons or because the pathological conditions of EAE inhibit myelination. To address this issue we would need a reporter transgene that specifically labels myelin sheaths but such a reporter is not yet available.

Unidentified YFP⁺ cells

In both Tam-only and Tam+EAE spinal cords there was a subset of YFP⁺ cells that was not accounted for by immunolabeling for NG2, OLIG2, GFAP or NeuN. These unidentified cells were rare in Tam-only white matter (\sim 2% of YFP⁺ cells) but became more numerous during EAE (\sim 13% of YFP⁺ cells) (Fig. 4G). In gray matter, \sim 13% of YFP⁺ cells in Tam-only mice and \sim 9% in Tam+EAE mice were unaccounted for (Fig. 4G). This contrasts with our previous study, in which we could colabel essentially all YFP⁺ cells in the gray and white matter of the normal adult forebrain with antibodies against OLIG2, NG2, or NeuN (Rivers et al., 2008). We considered the possibility that our present study might have failed to detect 100% of OL lineage cells with our

OLIG2 antibody for technical reasons. However, we found that the YFP⁺, OLIG2[−] cells also failed to label for SOX10 in triple-label experiments (see supplemental Fig. S2, available at www.jneurosci.org as supplemental material), arguing against a purely technical problem and suggesting that they might be non-OL lineage cells.

We tried, unsuccessfully, to identify these OLIG2-negative cells with a battery of antibodies against blood-borne cells (T-cells, B-cells, monocytes, macrophages, neutrophils and granulocytes), vascular cells (endothelial cells and pericytes), CNS-resident microglia, neural stem cells, neural progenitor cells, or immature neurons (supplemental Table S2, supplemental Fig. S3, available at www.jneurosci.org as supplemental material). A few YFP⁺ cells could be immunolabeled for the Schwann cell myelin marker Protein zero (P₀) but these were rare relative to the OLIG2[−] YFP⁺ cells (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). Moreover, they were almost always found close to the pial surface whereas most of the OLIG2[−], YFP⁺ cells were in the interior of the cord. The YFP⁺, OLIG2[−] cells therefore remain unidentified.

Discussion

The main purpose of our study was to investigate the fates of NG2 cells in a demyelinating/remyelinating disease model, EAE. Using Cre-lox methodology in transgenic mice, we found that there was a significant increase ($>$ 4-fold) in the overall number of OL lineage cells (YFP⁺, OLIG2⁺) in the demyelinating cord and a parallel \sim 4-fold increase in the number of differentiated OLs (YFP⁺, OLIG2⁺, NG2[−]; Fig. 4G,H). This is an underestimate of the increase that occurs within lesion areas, since our counting method did not discriminate between lesions and adjacent normal-appearing white matter, thus “diluting” the increase within lesions. New YFP⁺ OLs could be coimmunolabeled with antibody CC1, an accepted OL differentiation marker. The number of YFP⁺, CC1⁺ OLs (\sim 35% of YFP⁺ cells) in Tam+EAE mice (40 d post-Tam) exceeded the number of YFP⁺, OLIG2⁺, NG2[−] cells (\sim 20% of all YFP⁺ cells). This discrepancy might be down to the fact that there is some overlap between NG2 and CC1 expression in newly differentiating OLs. Nevertheless, there clearly was a lot of OL differentiation from PDGFRA/NG2 cells in Tam+EAE mice. We presume that the newly formed OLs are engaged in remyelinating axons that had become demyelinated during the course of EAE. A generally accepted hallmark of remyelinating OLs is that they generate thinner myelin sheaths (less wraps) than developmentally generated OLs. To detect myelin directly would require electron microscopy (EM), but we have been unable to identify YFP-labeled myelin sheaths unambiguously by EM immunohistochemistry, because YFP is physically excluded from compact myelin (Rivers et al., 2008).

Even in Tam-only spinal cord \sim 20% of YFP⁺, OLIG2⁺ cells in white matter were NG2-negative at 40 d post-Tam and a sim-

ilar fraction in gray matter, so new OLs are generated in the healthy adult spinal cord. This was confirmed by the fact that ~21% of YFP⁺ cells coimmunolabeled with antibody CC1 in Tam-only spinal cord. We and others have previously shown that new myelinating OLs continue to be formed for an extended period after birth (at least 8 months) in the forebrain gray and white matter (Dimou et al., 2008; Rivers et al., 2008; Lasienne et al., 2009; Psachoulia et al., 2009).

Apart from remyelinating OLs, many hypertrophic “reactive” astrocytes appear in the demyelinating/remyelinating spinal cord (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). These GFAP⁺ astrocytes tend to accumulate at the periphery of demyelinated lesions where they form a dense glial “scar.” This could be a barrier to inward migration of NG2 cells and thus might inhibit repair, especially after multiple episodes of demyelination at the same locus such as occurs in relapsing-remitting MS. The origin of reactive astrocytes has been controversial, some reports suggesting that they are generated from NG2 cells (Alonso, 2005; Tatsumi et al., 2005; Horvath et al., 2006; Leoni et al., 2009). However, the data presented here do not support that view, for we found that only small numbers of YFP⁺, GFAP⁺ astrocytes were generated from *Pdgfra-CreER^{T2}*-expressing cells, either in the normal healthy spinal cord or after EAE induction. This accords with another recent study of cell generation in a mouse model of acute focal demyelination (stereotaxic injection of ethidium bromide or lyssolecithin into spinal cords of *Pdgfra-CreER^{T2}:R26R-YFP* mice) (Zawadzka et al., 2010). The latter study also demonstrated extensive oligodendrocyte regeneration from PDGFRA/NG2 cells but little reactive astrocyte production. However, Zawadzka et al. (2010) found that reactive astrocytes surrounding gliotoxin-induced lesions were YFP-labeled in *Fgfr3-CreER^{T2}:R26R-YFP* mice, in which parenchymal astrocytes and ependymal zone (EZ) cells but not PDGFRA/NG2 cells are labeled (Young et al., 2010). Therefore it is likely that most reactive astrocytes in and around areas of demyelination are formed by activation and migration of EZ stem cells and/or by multiplication of preexisting parenchymal astrocytes. Further fate mapping experiments with astrocyte- and EZ-specific Cre lines would be required to distinguish these possibilities.

The lack of significant astrocyte production from PDGFRA/NG2 glia in our current study and that of Zawadzka et al. (2010) seems at odds with previous reports (Alonso, 2005; Magnus et al., 2007; Sellers et al., 2009; Zhao et al., 2009). However, all of the latter studies involved stab or cut injury models, not demyelination. The fates of stem/precursor cells will be influenced by the

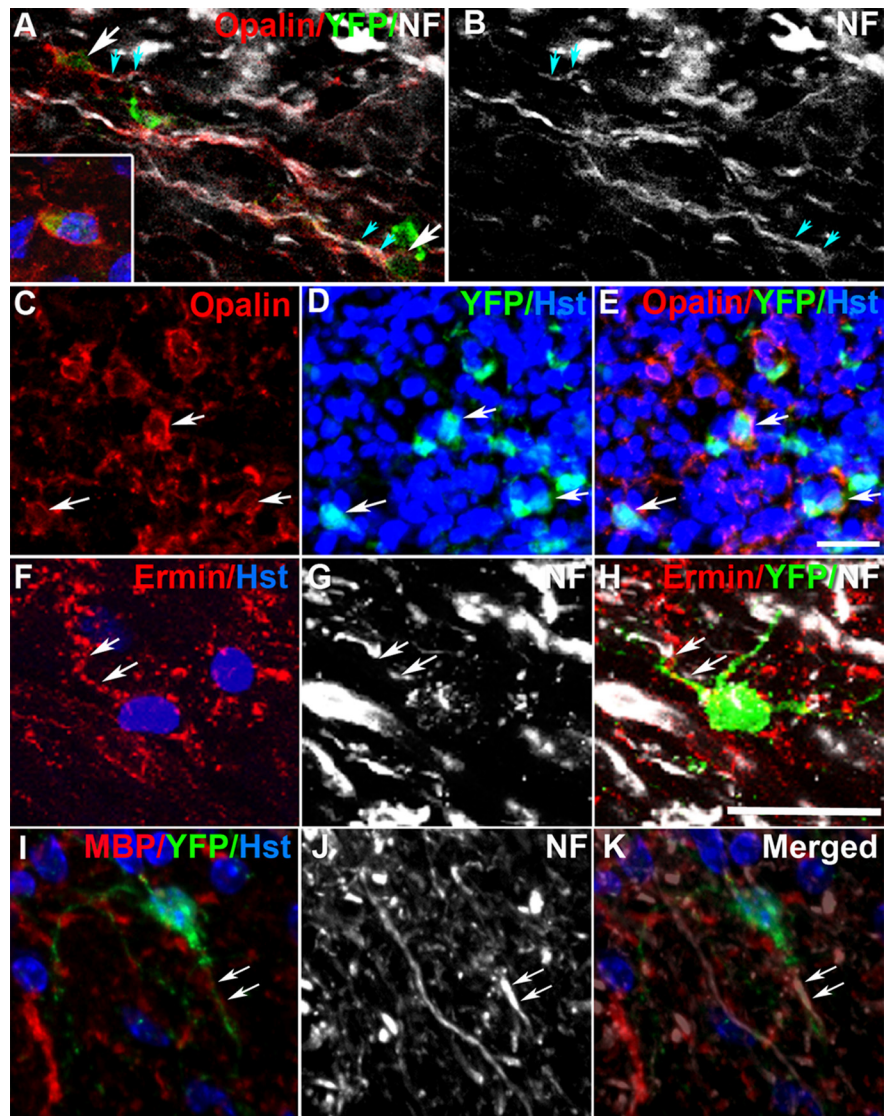


Figure 6. PDGFRA/NG2 glia give rise to myelinating cells. Processes from Opalin⁺, YFP⁺ cells in close apposition to Neurofilament (NF) profiles (**A, B**, arrows). Many Opalin⁺, YFP⁺ double-labeled cells were observed following EAE induction (**C–E**), some of which were found closely apposed to Neurofilament (NF) immunolabeling (**A, B**). Since Opalin is expressed in late-stage, myelinating OLs, this suggests that PDGFRA/NG2 cells give rise to remyelinating OLs in EAE. YFP⁺ cells that were colabeled for Ermin/Juxtandoin were also found in close association with NF⁺ profiles (**F–H**). MBP⁺ processes of YFP⁺ cells were sometimes found entwined with NF⁺ processes (**I–K**). Arrows indicate double-labeled cells. Images **A, B**, and **F–K** are from longitudinal sections through 28 dpi spinal cords, other images are of transverse sections. Sections are counterstained with Hoechst dye (Hst, blue). Scale bar, 20 μ m.

local environment near the injury site and this is likely to depend on the nature of the insult. It is possible that we might have underestimated astrocyte production specifically within lesions, for the same reason that we underestimated OL production (the dilution effect of our counting method, described above). However, note that the study of Zawadzka et al. (2010) did not suffer from this dilution effect because they counted cells specifically within focal lesions. Another potential source of disparity between our study and others who reported astrocyte production from NG2 cells is in the experimental approach or its interpretation. Some previous studies have relied on coexpression of antigens to infer lineage relationships and have concluded, on the basis of coexpression of NG2 and GFAP, that NG2 glia start to express GFAP on their way to generating astrocytes. However, it also seems possible that GFAP⁺ astrocytes start to express NG2

and/or OLIG2, perhaps as part of a dedifferentiation program, before going on to generate more astrocytes.

In our own study, only ~30% of PDGFRA⁺ cells in the spinal cords of *Pdgfra-CreER^{T2}:R26R-YFP* mice recombined and expressed YFP. It is therefore conceivable that the recombined cells are not representative of the population as a whole and that the low level of astrocyte production is an artifact of this selectivity. We have tried to discover whether Cre recombination marks a particular subgroup of OLPs. For example, in the forebrain there are distinct subsets of adult OLPs that are (1) mitotically active or quiescent and (2) developmentally derived from ventral or dorsal neuroepithelium. We have shown that the OLPs that undergo Cre recombination are equally likely to fall into any of these subgroups (Rivers et al., 2008; Psachoulia et al., 2009). We therefore think it likely that the lack of complete recombination in our experiments is simply a function of the relatively low activity of CreER^{T2} (compared with constitutive Cre), the narrow window of opportunity for recombination following Tamoxifen treatment and possibly a low level of *Cre* transcription from the *Pdgfra* promoter (discussed by Young et al., 2010). Inefficient cell labeling is not unusual with CreER lines (Dimou et al., 2008; Guo et al., 2009).

Unexpectedly, we found a significant minority (2–10%) of YFP⁺ cells in the spinal cords of healthy as well as EAE mice that did not colabel with antibodies against OLIG2, SOX10 or NG2. These cells therefore seem not to belong to the OL lineage. They also did not label with any of a battery of reagents selected to identify resident or infiltrating immune system cells (microglia, macrophages, neutrophils, T- or B-lymphocytes), vascular mural cells (endothelial cells or pericytes), astrocytes or neurons. Each of these cell types could be detected in the EAE spinal cord but none was YFP⁺. We also failed to label any YFP⁺ cells for markers of immature neural precursor/stem cells. Rare YFP⁺ Schwann cells were detected but these could not account for the majority of “mystery” cells. Therefore, the YFP⁺ non-OL lineage cells remain unidentified. Given the presence of some of these mystery cells in healthy mice that received Tam but no EAE inoculum, we presume that they are a normal cell type of the mouse spinal cord. We did not previously detect any such cells in the adult mouse forebrain so they seem to be CNS region-specific.

An unexpected finding was that Tam administration reduced locomotor deficits in female mice with EAE, even though the first EAE inoculum was administered 10 d after the final dose of Tam. Perhaps Tam produces a long-lasting effect on the immune system that moderates subsequent autoimmune attack. Alternatively, a low level of Tam might still have been present in our mice at the time of EAE induction and during the course of the disease. Acute Tam treatment is known to be protective during experimental ischemia or stroke (Kimmelberg et al., 2000, 2003; Zhang et al., 2007). This protection is due to an anti-oxidant effect of Tam treatment, not binding of Tam to estrogen receptors, and is evident at a very low dose of Tam compared with that used in our current study (a single dose of 5 mg/kg body weight, versus four doses of 300 mg/kg) (Zhang et al., 2007). Low dose Tam (5 mg/kg) has recently been reported to be beneficial following a spinal cord contusion injury in male rats (Tian et al., 2009). The beneficial effect noted in our EAE study was restricted to female mice; in males Tam appeared to have a damaging effect, advancing the onset of locomotor deficits and accelerating subsequent deterioration. Therefore, the action of Tam is likely to be complex, including both short and long term effects and mediated via estrogen receptors as well as other routes.

In conclusion, we have shown that in a MOG-induced EAE model of demyelinating disease, PDGFRA/NG2 cells in the adult mouse spinal cord generate elevated numbers of OL lineage cells, including differentiated—presumably remyelinating—OLs, but very few astrocytes or neurons.

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