Early Postnatal Proteolipid Promoter-Expressing Progenitors Produce Multilineage Cells *In Vivo*

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Proteolipid promoter (plp promoter) activity in the newborn mouse CNS is restricted to NG2-expressing oligodendroglial progenitor cells and oligodendrocytes. There are two populations of NG2 progenitors based on their plp promoter expression. Whereas the general population of NG2 progenitors has been shown to be multipotent *in vitro* and after transplantation, it is not known whether the subpopulation of plp promoter-expressing NG2 progenitors [i.e., plp promoter-expressing NG2 progenitors (PPEPs)] has the potential to generate multilineage cells during normal development *in vivo*. We addressed this issue by fate mapping Plp-Cre-ER^{T2}/Rosa26-EYFP (PCE/R) double-transgenic mice, which carried an inducible Cre gene under the control of the plp promoter. Expression of the enhanced yellow fluorescent protein (EYFP) reporter gene in PPEPs was elicited by administering tamoxifen to postnatal day 7 PCE/R mice. We have demonstrated that early postnatal PPEPs, which had been thought to be restricted to the oligodendroglial lineage, also unexpectedly gave rise to a subset of immature, postmitotic, protoplasmic astrocytes in the gray matter of the spinal cord and ventral forebrain, but not in white matter. Furthermore, these PPEPs also gave rise to small numbers of immature, DCX (doublecortin)-negative neurons in the ventral forebrain, dorsal cerebral cortex, and hippocampus. EYFP-labeled representatives of each of these lineages survived to adulthood. These findings indicate that there are regional differences in the fates of neonatal PPEPs, which are multipotent *in vivo*, giving rise to oligodendrocytes, astrocytes, and neurons.

Introduction

The proteolipid (plp) gene encodes myelin proteolipid protein in myelinating oligodendrocytes and a smaller isoform, DM-20, which, in the mouse, is expressed in embryonic neuroepithelial cells [embryonic day 9.5, (E9.5)], radial glial cells (E13.5), and progenitors expressing the proteoglycan NG2 (E14.5) (Ikenaka et al., 1992; Timsit et al., 1992, 1995; Spassky et al., 1998; Belachew et al., 2001; Delaunay et al., 2008; Tuason et al., 2008). These plp promoter-expressing embryonic progenitors are multipotent both *in vivo* and *in vitro*, giving rise to oligodendrocytes, astrocytes, and neurons (Le Bras et al., 2005; Delaunay et al., 2008). After birth, plp promoter activity has been reported to be maintained only in NG2+ progenitors and in the oligodendroglial lineage cells derived from them (Mallon et al., 2002; Leone et al., 2003; Pasquini et al., 2003; Hirrlinger et al., 2005; Le Bras et al., 2005; Baracskey et al., 2007). Whether these postnatal plp promoter-expressing progenitors (PPEPs) are multipotent has not been established.

There are two apparently distinct populations of actively proliferative NG2+ progenitor cells: those that express platelet-derived growth factor α receptors (PDGFR α +), but not the plp

promoter; and those in which the plp promoter is active (Mallon et al., 2002; Dawson et al., 2003; Ivanova et al., 2003). Additionally, substantial numbers of NG2+/PDGFR α + cells are present in postnatal gray matter, which undergo symmetric division while maintaining glutamatergic and GABAergic axonal inputs (Lin and Bergles, 2004; Kukley et al., 2008; Mangin et al., 2008). *In vivo* fate mapping using a constitutive NG2-Cre transgene demonstrated that NG2+ cells are precursors for both oligodendroglia and gray matter protoplasmic astrocytes, but not for neurons (Zhu et al., 2008a). Similar results were obtained by fate mapping adult tamoxifen-inducible Olig2-CreER^{T2} mice (Dimou et al., 2008). In contrast, *in vivo* fate mapping PDGFR α + cells in day 45 or 180 postnatal mice carrying a tamoxifen-inducible PDGFR α -CreER^{T2} transgene showed that these cells are precursors for myelinating oligodendroglia and small numbers of ventral forebrain especially piriform cortex projection neurons, but not for astroglia (Rivers et al., 2008). Thus, although previous studies have suggested that postnatal NG2+ oligodendroglial progenitor cells (OPCs) can give rise to astroglia and neurons (Raff et al., 1983; Rao et al., 1998; Dayer et al., 2005; Tamura et al., 2007), the contributions of the subpopulation of NG2+ cells that are PPEPs to postnatal astroglialogenesis and neuronogenesis *in vivo* remain unclear.

Two fate-mapping studies with Plp-CreER^{T2} mice were published in 2003 and demonstrated efficient oligodendroglial lineage labeling, but did not address whether other lineages arise from postnatal PPEPs (Doerflinger et al., 2003; Leone et al., 2003). To determine whether early postnatal PPEPs do give rise to astroglia or neurons, we administered tamoxifen to postnatal day 7 (P7) PCE/R double-transgenic mice to permanently label

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PPEPs and their progeny. Our results demonstrated that a subset of astrocytes and neurons, in addition to oligodendroglia, are generated from these early postnatal PPEPs.

Materials and Methods

Mice. The Plp-Cre-ER^{T2} mice (Doerflinger et al., 2003) and Rosa26-EYFP reporter line (Srinivas et al., 2001) were purchased from The Jackson Laboratory and maintained in C57BL/6 background. These two lines were crossed to obtain Plp-Cre-ER^{T2}/Rosa26-EYFP (PCE/R) double-transgenic mice. In PCE/R mice, Cre-ER^{T2} fusion protein is expressed in the cytosol under the control of plp promoter. After binding with tamoxifen, Cre recombinase translocates into the nucleus to mediate Cre-loxP recombination, thus eliciting permanent expression of the enhanced yellow fluorescent protein (EYFP) reporter gene in PPEPs and their progenies. All animal procedures were performed according to the Institutional Animal Care and Use Committee, University of California, Davis, and National Institutes of Health guidelines.

Tamoxifen and 5-bromo-2'-deoxyuridine or 5-ethynyl-2'-deoxyuridine treatments. Tamoxifen (TM) (T5648; Sigma-Aldrich) was dissolved in an ethanol/sunflower seed oil (1:19) mixture at a concentration of 10 mg/ml. At P7, PCE/R pups were injected twice, 6–8 h apart intraperitoneally with tamoxifen (75 μ g/g body weight). 5-Bromo-2'-deoxyuridine (BrdU) (B5002; Sigma-Aldrich) or 5-ethynyl-2'-deoxyuridine (EdU) (A10044; Invitrogen) (Buck et al., 2008) was prepared in sterile 1× PBS, pH 7.4, at a concentration of 10 mg/ml. Single or multiple intraperitoneal injections of EdU or BrdU (100 μ g/g body weight) were given at the times indicated in the figures or figure legends.

Tissue preparation. PCE/R mice were killed at 1 d ($n = 7$), 3 d ($n = 6$), 8 d ($n = 5$), 27 d ($n = 3$), or 53 d ($n = 3$) after TM injection [i.e. at P8, P10, P15, P30, and adult (P60)]. After anesthesia with ketamine (150 mg/kg body weight)/xylazine (16 mg/kg body weight), mice were perfused transcardially with 1× PBS and then with 4% paraformaldehyde (PFA) in PBS. Brain and spinal cord were collected, postfixed in 4% PFA in PBS for 1 h at room temperature (RT), then cryoprotected in 30% sucrose (v/v) prepared in 1× PBS at 4°C overnight, and then transferred to OCT compound for embedding. Anterior forebrain with lateral ventricle from bregma 0 mm to bregma 1 mm, posterior forebrain with hippocampus from bregma −0.9 mm to bregma −1.8 mm (Paxinos and Franklin, 2001), and the lumbar segment were cut transversely on a Leica cryostat (model CM3050 S) to prepare 12- to 14- μ m-thick sections, which were stored at −80°C until use. For the purpose of stereological counting, 40- μ m-thick cryosections were cut on a Leica cryostat (model CM3050 S), air-dried for 4 h at 37°C or RT overnight, and stored at −80°C until use.

Immunohistochemistry. Sections were air-dried for 30 min at RT, followed by incubation in normal serum blocking solution (dependent on the secondary antibodies used) at RT for at least 30 min (10% normal serum plus 0.1% Triton X-100 in 1× PBS). When a streptavidin (SA)–biotin detection system was used, the section was treated with a streptavidin–biotin blocking kit (SP-2002; Vector Laboratories) before normal serum blocking. Primary antibodies (supplemental Table 1, available at www.jneurosci.org as supplemental material) diluted in 5% normal serum plus 0.1% Triton X-100 in 1× PBS were incubated at 4°C overnight (12–20 h) or 37°C for 3 h, followed by three 20 min washes in PBS plus 0.1% Triton X-100. After incubation with secondary antibodies (for details, see supplemental Table 1, available at www.jneurosci.org as supplemental material) for 2 h at RT, the sections were washed three times (20 min each) in PBS plus 0.1% Triton X-100 at RT. For streptavidin–biotin detection system, FITC-, rhodamine X-, or Pacific blue-SA (supplemental Table 1, available at www.jneurosci.org as supplemental material), diluted in PBS, were incubated for 15 min at RT, followed by three 10 min washes in PBS plus 0.1% Triton X-100 at RT. Finally, Hoechst 33258 was used to label nuclei, and the sections were mounted with Vectashield mounting medium for fluorescence (Vector Laboratories; H-1000). Because intrinsic fluorescence of the EYFP reporter protein was weak, an antibody against EYFP was used to amplify the EYFP signal.

For BrdU immunostaining, after all the immunostaining steps except Hoechst staining were completed, the sections were postfixed with 2%

PFA in 1× PBS at RT for 15 min, and then denatured in 2N HCl at 37°C for 45 min. After three 5 min washes in PBS, the sections were incubated with BrdU antibody (supplemental Table 1, available at www.jneurosci.org as supplemental material) diluted in 5% normal serum plus 0.1% Triton X-100 at 4°C overnight or 37°C for 3 h. For detection of EdU, an EdU imaging kit (C10084; Invitrogen) was used per the manufacturer's instructions.

For immunostaining for stereological counting, a modified protocol was used. Briefly, the 40- μ m-thick cryosections were incubated with blocking solution (10% normal serum plus 0.5% Triton X-100 in 1× PBS) for 4–5 h at RT. Primary antibodies were diluted in 10% normal serum plus 0.1% Triton X-100 and incubated at 4°C for 48 h. Secondary antibody incubations were at RT for 4 h. Hoechst 33258 was used to label nuclei, and the sections were mounted with Vectashield mounting medium (Vector Laboratories; H-1000). We ascertained that, after tissue processing and staining procedure, the 40- μ m-thick sections typically underwent 20% shrinkage, to reach an approximate final thickness of 32 μ m, still sufficient for the stereological counting.

vGLUT1 mRNA in situ hybridization. A 30-mer oligonucleotide probe 5'-GCACGGGAACAAGGGAGGAGCTTGCATCTT-3' targeted mouse vesicular glutamate transporter 1 mRNA was synthesized and 5' labeled with digoxigenin by Integrated DNA Technologies. The 12- μ m-thick sections were air dried at RT for 30 min. Sections were incubated in 0.3 Triton X-100/DEPC–PBS for 5 min, followed by two 5 min washes in DEPC–PBS. Sections were then digested in 1 μ g/mg proteinase K for 10 min at RT, followed by two 5 min washes in DEPC–PBS, and incubated for 10 min in acetylating solution (2.33 ml of triethanolamine from Sigma-Aldrich plus 500 μ l of acetic anhydride from Sigma-Aldrich plus 1 ml of HCl, volume up to 200 ml in water), and then washed two times (5 min each) with DEPC–PBS. Probe was added in hybridization buffer (50% formamide plus 0.3 M NaCl plus 20 mM Tris-HCl, pH 8.0, plus 5 mM EDTA plus 10 mM NaPO₄, pH 8.0, plus 10% dextran sulfate plus 1× Denhardt's plus 0.5 mg/ml yeast RNA) to a final concentration of 1 μ g/ml. The probe was then denatured at 65°C for 5 min and immediately cooled on ice. Sections were incubated at 37°C overnight, and then washed in 2× SSC at 55°C for 15 min, 1× SSC, four times for 15 min at 55°C, and 1× SSC at RT for 15 min, followed by the immunohistochemistry protocol. We used goat anti-digoxigenin antibody and a rhodamine-conjugated-donkey anti-goat secondary antibody to visualize the *in situ* signal.

Microscopy and stereological quantification. A Nikon Eclipse C1 confocal laser-scanning microscope was used to image FITC (488 nm laser line excitation), rhodamine X (561 nm laser line excitation), and Hoechst 33258 or Pacific blue (404 nm laser line excitation). Optical sections ($z = 0.45$) were acquired using 20× [numerical aperture (NA), 0.75], 40× (NA, 1.30), or 60× (NA, 1.40) oil objective lens with Nikon EZ-C1 software, version 3.40. The Nikon EZ-C1 3.20 FreeViewer was used to create single-channel views, merged views, and orthogonal views of the images, and Photoshop CS3 was used to combine the images, which were exported from EZ-C1 3.20 FreeViewer without any manipulation of contrast. We considered two antigens as colocalized only if colocalization extended from the top to bottom of the z -plane images.

For cell counting, three to four sections from each brain or spinal cord (three to five animals for each time point) were examined. The dorsal cortex area (D-ctx) (depicted in Fig. 1B) refers to the cerebral cortex above the corpus callosum that extends from the midline laterally to the tip of hippocampus CA3, including retrosplenial cortex, parietal association cortex, trunk region, and part of barrel field of primary somatosensory cortex. The ventral cortex area (V-ctx) (depicted in Fig. 1B) refers to cerebral cortex located ventrally down from the rhinal fissure, including amygdala and piriform cortex. Fimbria (Fi) and hippocampus (Hip) counting areas were the whole Fi and Hip area, respectively.

Stereological unbiased estimates of volumes and cell numbers were obtained by using a computer interfaced with an Olympus BX61-DSU microscope equipped with a motorized stage, running StereoInvestigator software, version 8.24 (MicroBrightField). The regions of interest were traced at low magnification (4× with NA of 0.16 for brain; 10×, NA of 0.4 for spinal cord), and the counting was conducted at 60× oil (NA, 1.42). Every 12th section was selected for counting. Counting grids randomly placed by the software were applied onto D-ctx (300 × 300; x and

y size; in micrometers), V-ctx (300 × 300), fimbria (80 × 80), hippocampus (200 × 200), and spinal cord gray matter (200 × 200). The counting frames were set at 100 × 100 (x and y size; in micrometers) in D-ctx, V-ctx, hippocampus, and spinal cord gray matter, and at 40 × 40 in fimbria. In most instances, 30–40 counting sites were evaluated in one section for the region of interest. The optical dissector and guard zone were set at 15 and 5 μ m, respectively. Cells immunoreactive for a specific marker were counted only if they overlapped with the Hoechst 33258 nuclear staining. Cell densities were calculated by dividing the total cell number in question by the total volume (in cubic millimeters) counted. All counting data were expressed as mean $\pm$ SD.

Results

Early postnatal OPCs are predominantly undifferentiated and are selectively targeted by Cre-mediated recombination

By immunostaining oligodendroglial progenitors and immature/mature oligodendrocytes with antibodies against NG2 and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), respectively, we determined that almost all (~96%) oligodendroglial lineage cells in P8 cerebral cortex were undifferentiated NG2+ progenitors (supplemental Fig. 3A, arrowheads and inset, available at www.jneurosci.org as supplemental material). Microvessel endothelial cells also expressed NG2 but were easily distinguished from parenchymal NG2+ progenitors by their morphology (supplemental Fig. 3A, arrows, available at www.jneurosci.org as supplemental material). A few CNPase+ immature/mature oligodendrocytes were present in close proximity to the corpus callosum (cc) (supplemental Fig. 3B, inset, arrowhead, available at www.jneurosci.org as supplemental material). Even in the fimbria, in which myelination starts at around P5, a large proportion of oligodendroglial lineage cells (~87%) continued to be NG2+ at P8 (supplemental Fig. 3C, available at www.jneurosci.org as supplemental material). The basic helix-loop-helix (bHLH) transcription factor Olig2 is required for NG2 specification (Ligon et al., 2006). Our data showed that almost all NG2 cells expressed Olig2 throughout the P8 CNS, including dorsal cortex, ventral cortex, and hippocampus, with 99.3% (± 0.7 ; $n = 1089$), 99.6% (± 0.4 ; $n = 972$), and 98.6% (± 1.2 ; $n = 560$) of NG2 cells being Olig2-positive, respectively (supplemental Fig. 3D,E, available at www.jneurosci.org as supplemental material).

Previous studies have shown that tamoxifen-induced Cre translocation into the nucleus and reporter gene induction occur within 12–24 h (Danielian et al., 1998; Hayashi and McMahon, 2002; Leone et al., 2003; Zervas et al., 2004; Ganat et al., 2006). Additional Cre-mediated recombination is unlikely to occur beyond this period, but the recombination reporter gene (in our study, EYFP) then remains constitutively expressed in the cells targeted by the Cre transgene and their progeny. To determine

Figure 1. A subpopulation of early postnatal NG2+ oligodendrocyte progenitor cells is targeted by Cre-mediated recombination. **A**, Experimental design: two tamoxifen intraperitoneal injections at P7 and analysis at P8. **B**, Diagram showing anatomical structures of mouse forebrain examined in our study in partial coronal section. **C**, Percentage of EYFP+ cells that expressed NG2 in different areas. Error bars indicate SD. **D–F**, Double immunohistochemical staining shows that a subset of NG2+ (red) progenitors expressed the reporter gene, EYFP (green), in dorsal cortex (**D**), hippocampus (**E**), and fimbria (**F**). The boxed areas in **D–F** are shown at higher magnification. The arrowheads point to EYFP+/NG2+ cells. The blue channel in the images shows Hoechst 33258+ nuclei. S-GM, Spinal cord gray matter. Scale bars: 50 μ m (applied to all).

the cell population targeted by tamoxifen-inducible Cre-mediated recombination, tamoxifen was administered twice, 6–8 h apart, to PCE/R double-transgenic mice at P7. One day later, at P8, in agreement with the Cre recombinase expression (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), plp promoter-driven, Cre-mediated reporter gene EYFP expression was restricted to oligodendroglial lineage cells, and almost all of this EYFP expression was in NG2 progenitors. In the dorsal and ventral cerebral cortex (see Materials and Methods), 92 and 94% of EYFP-tagged cells were NG2 cells, respectively (Fig. 1C,D; supplemental Fig. 3G, available at www.jneurosci.org as supplemental material); pairs of EYFP+/NG2+ cells that presumably had recently undergone mitosis were frequent (Fig. 1D, inset). In the fimbria, 90.5% of EYFP+ cells were NG2+ and spindle-shaped (Fig. 1F, inset, arrowheads). Most strikingly, all of the EYFP expression in hippocampus was restricted to NG2 cells (Fig. 1E). In spinal cord, in which oligodendroglial maturation begins earlier than in forebrain, 27% of EYFP+ cells expressed NG2 in the spinal gray matter (Fig. 1C;

Table 1. Cre-mediated recombination rate in NG2 cell in different regions

Region	Percentage of NG2 cells that are EYFP+ [% (%)]
D-ctx	8.6 (2.5) ^a
V-ctx	14.7 (3.1)
Hip	17.3 (4.0)
Fi	14.3 (3.2)
S-GM ^b	11.3 (3.5)

^aMean (±SD).^bS-GM, Gray matter of spinal cord.**Table 2. Recombination rates in the forebrain dorsal cortex at different ages (three to five PCE/R mice at each time point)**

Age	NG2 density	Recombination rate [% (%)]
P8 ^a	23,750 (1707) ^b	8.6 (2.5)
P15 ^a	16,181 (1578)	2.6 (0.3)
P45 ^c	8186 (1130)	0.34 (1.1)
P70 ^c	4436 (283)	0

^aTwo intraperitoneal injections of tamoxifen (75 μg/g body weight) 6–8 h apart; analysis at 1 d after first injection.^bMean (±SD).^cFive consecutive days of tamoxifen intraperitoneal injections (75 μg/g body weight), twice a day (10 total injections); analysis 1 d after last injection.

supplemental Fig. 3F, available at www.jneurosci.org as supplemental material), the remaining cells being CNPase+ oligodendrocytes (supplemental Fig. 3H, arrowheads, available at www.jneurosci.org as supplemental material), whereas only ~5% of EYFP+ cells were NG2 cells in spinal cord white matter.

We also noticed that a few NeuN+ mature neurons in the caudate and putamen between the external and internal capsule of the posterior forebrain (from bregma –1 mm backward) ectopically expressed plp promoter (supplemental Fig. 1J, available at www.jneurosci.org as supplemental material), assessed by Cre recombinase immunostaining, and thus became EYFP-labeled 1 d after tamoxifen administration (supplemental Fig. 1K1–K3, available at www.jneurosci.org as supplemental material) in PCE/R mice, but these neurons were nonproliferative based on BrdU incorporation (data not shown).

Twenty-four hours after tamoxifen, the recombination rates among the total NG2+ cells varied in different CNS anatomical structures (Table 1). For example, in the spinal cord gray matter and ventral cortex, the recombination efficiencies were 11.4 and 14.7%, respectively. It has been shown that there are two populations of NG2+ cells based on their plp promoter activity: PPEPs and plp promoter-nonexpressing progenitors (Mallon et al., 2002). Given this scenario, the tamoxifen-induced recombination is likely to have been considerably higher among PPEPs than that among the general NG2 population.

We also explored the recombination rate at different ages from early postnatal to adult, focusing on the dorsal cortex. One day after tamoxifen treatment, the recombination rate decreased from 8.6% at P8 to 2.6% at P15 (Table 2). Even using a more intensive tamoxifen paradigm, the recombination rate in P70 adult mice was 0% (Table 2). Thus, our data showed the number of PPEPs decreased over time. Since PPEPs are defined by their expression of the PLP promoter, virtually all of the abundant NG2+ cells in the adult dorsal cortex were non-PPEPs (Table 2). Additional studies will be required to determine whether PPEPs represent a discrete stage in development of NG2+ cells or, instead, are a distinct cell population with fates different from those of non-PPEP NG2+ cells.

Phenotypic features and proliferative potential of plp promoter-expressing progenitors

Because the transcription factor Mash1 specifies cells that can give rise to both neurons and oligodendroglial lineage cells in embryonic (Parras et al., 2007) and postnatal brain (Parras et al., 2004), we asked whether these EYFP+ progenitors expressed Mash1. At P8, 1 d after tamoxifen administration, 75 ± 9 and 87 ± 6% of EYFP+ progenitors expressed Mash1 in the dorsal cortex (Fig. 2A) and ventral cortex (not depicted). We also found that a minority (~15% in hippocampus, 12.5% in fimbria, 14.3% in dorsal cortex, and 35% in ventral cortex) of EYFP+ cells expressed nestin, for example, in hippocampus (Fig. 2B) and dorsal and ventral cortex (picture not shown). Most interestingly, 100% of the EYFP+ cells were labeled with an antibody against the bHLH transcription factor Olig2 in all areas we assessed, including dorsal cerebral cortex (Fig. 2C). Almost all of the EYFP+ PPEPs expressed epidermal growth factor receptor (EGFR) (Fig. 2D) and PDGFRα (Fig. 2E). However, none of the PPEPs expressed doublecortin (DCX) and polysialylated isoforms of the neural cell adhesion molecule (PSA-NCAM) (data not shown), both of which are neuronal progenitor markers.

Next, we asked whether PPEPs have a proliferative potential similar to that of the general NG2 population. We used BrdU and Ki67 immunostaining to determine the proliferative potential of PPEPs and of the general population of NG2+ cells in postnatal P8 mice. Two hours after BrdU administration, BrdU+ PPEPs (EYFP+/NG2+/BrdU+ cells) were easily seen throughout all the areas we assessed; 21.5 and 25% of PPEPs were BrdU positive (Fig. 3A, C) in the dorsal and ventral cortex, respectively. In the fimbria (Fig. 3B) and hippocampus (not including the dentate gyrus), 28.5 and 10.1% of EYFP+/NG2+ PPEPs incorporated BrdU (Fig. 3C). These data, compared with that obtained for the general NG2+ population (supplemental Fig. 3I, available at www.jneurosci.org as supplemental material), indicated that the proliferative potential of PPEPs is similar to, although a little less than, that of overall NG2+ cells. Consistent with BrdU incorporation, similar results were also obtained by immunostaining with an antibody against Ki67 (Fig. 3C–E), a cell cycle-associated protein expressed from G₁ through the end of M phase (Gerdes et al., 1983). Together, these data indicate that early postnatal EYFP+ PPEPs have some features of type-C transit-amplifying cells, including antigenic expression and proliferative potential (Aguirre et al., 2004; Menn et al., 2006).

EYFP-tagged PPEPs generate mature oligodendrocytes

Previous studies using oligodendroglial stage-specific antibodies and BrdU labeling have suggested that oligodendrocytes are derived from NG2 progenitors (Polito and Reynolds, 2005). Here, we present direct *in vivo* fate-mapping evidence that postnatal NG2+/Olig2+ are, indeed, precursors for mature oligodendrocytes throughout the CNS. We illustrate this issue by focusing on the hippocampus and fimbria. At P15, 8 d after tamoxifen injection, EYFP+ cells with typical morphology of oligodendrocytes were present in hippocampus and fimbria. In hippocampus, these cells had larger, more rounded perikarya and fewer radial branched processes than NG2+ PPEPs (Fig. 4A1–A3, arrows), whereas in the white matter of the fimbria, they had round- to oval-shaped somas with many parallel fine myelinating processes (Fig. 4C, arrows and boxed area). These cells were perfectly colabeled with adenomatous polyposis coli (APC) gene product, a marker for mature oligodendrocytes (Bhat et al., 1996) (Fig. 4A1–A3, C), and were not stained with the astrocyte markers, brain lipid binding protein (BLBP) and glial fibrillary acidic pro-

tein (GFAP) (data not shown). Some NG2+ PPEPs that stained weakly for APC were also observed (Fig. 4A1–A3, wavy arrows) and were likely in transition from NG2+ progenitors to mature oligodendrocytes. We further identified these mature oligodendrocytes in the hippocampus with additional markers for mature oligodendrocytes, glutathione-S-transferase Pi isoform (GST-Pi) (Fig. 4B) and aspartoacylase (ASPA) (data not shown) (Tansey and Cammer, 1991; Madhavarao et al., 2004).

The extent to which PPEPs gave rise to mature oligodendrocytes varied in different areas we assessed at P15. In white matter, for example, in the fimbria, almost all of the EYFP+ PPEPs generated APC+ mature oligodendrocytes, whereas in the dorsal and ventral cerebral cortex, only 44.3 and 25.9% of EYFP+ PPEPs produced oligodendrocytes (Table 3). Both EYFP+/NG2+ PPEPs and EYFP+/APC+ mature oligodendrocytes in all areas continued to express Olig2 at P15. Together, these observations directly demonstrated that PPEPs serve as precursors of mature oligodendrocytes.

Some astrocytes are generated from EYFP-tagged PPEPs

It is well documented that OPCs can generate both oligodendrocytes and type 2 astrocytes under appropriate conditions *in vitro* (Raff et al., 1983). We investigated whether EYFP-tagged NG2+/Olig2+/PDGFR α + PPEPs give rise to astrocytes developmentally *in vivo* in the PCE/R double-transgenic mice. PCE/R mice were given tamoxifen at P7, and CNS tissues were analyzed 8 d later, at P15, to provide sufficient time for EYFP-tagged NG2+/Olig2+ progenitors to transit to astroglial lineage cells.

A large number of cells with dense EYFP expression in small, round cell bodies were observed in the spinal gray matter and ventral forebrain, for instance, piriform cortex, amygdala, and hypothalamus. These EYFP+ cells had complex bushy distal processes (Fig. 5), a morphology reminiscent of that of gray matter protoplasmic astrocytes (Bushong et al., 2004). Confirming this impression, immunostaining of coronal sections from forebrain and transverse section from spinal cord showed that these EYFP+ cells expressed S100- β (Fig. 5C; supplemental Fig. 4C, available at www.jneurosci.org as supplemental material) (Raponi et al., 2007), BLBP, and GFAP (Fig. 5A, B; supplemental Fig. 4A, B, available at www.jneurosci.org as supplemental material). These cells were negative or only faintly stained with an antibody against NG2 in their distal processes (supplemental Fig. 4D, arrowhead, available at www.jneurosci.org as supplemental mate-

Figure 2. Phenotypic features of PPEPs. Plp promoter-expressing progenitors expressed Mash1 (**A**), Nestin (**B**), Olig2 (**C**), EGFR (**D**), and PDGFR α (**E**). The diagram at the top left indicates the locations where the images in **A–E** were obtained. **B**, **D**, and **E** show orthogonal view, whereas **A** and **C** show projection view. Cells in the boxed area in **A** and **C**, and at crosshair in **B**, are shown at higher magnification. The arrowheads point to double-positive cells. Scale bars: **A–C**, 50 μ m; **D**, **E**, 10 μ m.

rial) and did not express CNPase or the neuronal marker, NeuN (data not shown). We thus concluded that these bushy process-bearing EYFP+ cells were astroglia that had been derived from plp promoter-expressing progenitors.

Some of the EYFP+ astrocytes were in close doublets (Fig. 5A, boxed area a1), and most of them were distributed in clusters (Fig. 5A, arrowheads; supplemental Fig. 4A, boxed area a1, available at www.jneurosci.org as supplemental material), observa-

Figure 3. Proliferative potential of EYFP+ PPEPs. Two hours before being killed at P8, mice were injected with BrdU. Many BrdU+ (blue) PPEPs (i.e., BrdU+/EYFP+/NG2+ cells) were present in the gray matter, dorsal cortex (**A**), and white matter, fimbria (**B**). Consistent with the BrdU results, Ki67+ (red)/EYFP+ PPEPs were distributed in dorsal cortex (**D**) and fimbria (**E**). Cells a1, a2 in **A**; d1, d2, d3 in **D**; and boxed areas in **B** and **E** were shown at higher magnification accordingly. The arrows point to proliferative PPEPs in all images. **C**, Percentages of proliferating EYFP+ PPEPs in different areas evaluated by BrdU and Ki67, respectively. Error bars indicate SD. S-GM, Spinal cord gray matter. Scale bars: 50 μ m (applied to all).

tions that support the hypothesis that these EYFP+ astrocytes were generated *in situ* from EYFP+ PPEPs. To test this hypothesis, we exploited EdU pulse labeling. When a single dose of EdU was given to P7 PCE/R mice at the same time as the first tamoxifen injection and the mice were analyzed at P15, occasional EYFP+/BLBP+ astrocytes that were EdU+ and close to each other (Fig. 5D, arrowhead and higher magnification) were seen, arguing that EdU+/EYFP+/PPEPs had bequeathed EdU to EYFP+/BLBP+ astroglia derived from them. Together, these observations support the postnatal derivation of astroglia from local PPEPs subpopulation.

Since Olig2 was strongly expressed in EYFP+ PPEPs (Fig. 2C), we asked whether Olig2 persisted in the astrocytes after they had

been generated from PPEPs. Immunostaining showed that Olig2 expression in these EYFP+ astroglia was much fainter (supplemental Fig. 4G, arrowheads and higher magnification, available at www.jneurosci.org as supplemental material) than that in oligodendroglial lineage cells (Fig. 2C; supplemental Fig. 4G, arrows, available at www.jneurosci.org as supplemental material) or had become undetectable.

To exclude the possibility that the formation of astroglia from PPEPs was a transient event induced by tamoxifen, we used the same tamoxifen paradigm as for the P15 group but killed the mice at P60. In these adult mice, EGFP+/GFAP+ cells were present in ventral forebrain (supplemental Fig. 4H, available at www.jneurosci.org as supplemental material) and spinal gray matter

Figure 4. EYFP-tagged PPEPs generate mature oligodendrocytes. **A1–A3**, Triple-positive EYFP (green), NG2 (blue), and APC (red) cells in hippocampus CA3 region. The arrowheads (for example, cell a1) in **A1–A3** point to examples of EYFP+/NG2+/APC− progenitors; the arrows (cell a2), EYFP+/NG2−/APC+ mature oligodendrocytes; whereas wavy arrows (cell a3) point to EYFP+/NG2+/APC+ transitional stage. Cells marked with a1, a2, and a3 are shown at higher magnification below **A1–A3**. Mature oligodendrocytes were also identified by another mature oligodendrocyte marker, GST-Pi, in hippocampus (**B**). **C**, EYFP/APC double staining in fimbria showed virtually all of the EYFP+ cells were APC+ mature oligodendrocytes, and the arrows point to examples of mature oligodendrocytes with parallel myelinating processes. Blue in **B** and **C** are Hoechst 33258+ nuclei. All images obtained from P15 mice. Scale bars: 50 μ m (applied to all).

(data not shown), and had the typical morphology of mature protoplasmic astrocytes, with small round somas and complex bushy processes (supplemental Fig. 4H, H1, arrowheads and boxed area, available at www.jneurosci.org as supplemental material). Some of these EYFP+ astrocytes extended an endfoot to a blood vessel (supplemental Fig. 4H1, arrow, available at www.jneurosci.org as supplemental material), suggesting a normal physiological role. These data indicated that the generation of astroglia from PPEPs was a developmental process, rather than a transient event.

We used stereology to estimate the prevalence of EYFP+ astrocytes generated from EYFP-tagged PPEPs in the ventral cortex of forebrain (V-ctx) (indicated in Fig. 1B) and gray matter of the spinal cord. To quantify EYFP+ astrocytes at P15, we used BLBP as an astrocytic marker to count astrocytes, since S100- β was also expressed in a large proportion of oligodendroglial cells especially in mature oligodendrocytes (Hachem et al., 2005; our unpublished data) and GFAP was expressed in only a subpopulation of the gray matter astrocytes (Bushong et al., 2002). The preva-

Table 3. Percentage of EYFP+ cells that express indicated markers in different regions at P15

Region	NG2 [% (%)]	APC [% (%)]	BLBP [% (%)]	HuC/D [% (%)]
D-ctx	42.9 (10.7) ^a	44.3 (16)	0	11.9 (3.6)
V-ctx	40.1 (1.8)	25.9 (6.8)	23.2 (7.1)	9.0 (2.6)
Hip	55.1 (10.5)	41.2 (11.2)	0	4.1 (1.7)
Fi	5.6 (1.1)	92.4 (6.1)	0	0
S-GM ^b	7.7 (1.2)	71.3 (2.0)	20.5 (3.7)	0

^aMean ($\pm$ SD).

^bS-GM, Gray matter of spinal cord.

lence of PPEPs-derived, EYFP+/BLBP+ astrocytes was similar in ventral cortex of forebrain and gray matter of spinal cord; 23.2 and 20.5% of EYFP+ cells were of the astroglial lineage (i.e., EYFP+/BLBP+ in the ventral cortex and spinal gray matter, respectively) (Table 3). However, 15.9% ($\pm$ 3.5%, SD) and 12.4% ($\pm$ 2.5%) of total BLBP+ astrocytes expressed EYFP in the ventral cortex and spinal cord gray matter, respectively. Considering that we used inducible Cre mice to fate map PPEPs postnatally (tamoxifen at P7), and the likelihood of incomplete recombination of the reporter gene in the PPEPs, our data are consistent with a previous study by Zhu et al. (2008a). The distribution pattern of EYFP+ astrocytes in adult (P60) transgenic PCE/RE mice was similar to that in P15 mice, which also indicates that the

Figure 6. EYFP-tagged PPEPs produce immature astrocytes. **A** and **B** show triple immunostaining for EYFP (green), vimentin (Vim) (red), and GFAP (blue) of P10 and P15 spinal cord gray matter (S-GM), respectively. Cells labeled with a1–b2 are shown at higher magnification and display an astroglial maturation progression evaluated by vimentin and GFAP staining, with a1 cell being most immature and b2 cell being most mature. The asterisk in **B** shows central canal. Scale bars: 50 μ m (applied to all).

formation of astrocytes from EYFP-tagged plp promoter-expressing progenitors was not a transient event, but a normal development phenomenon, and that a substantial proportion of all gray matter astroglia were derived from NG2+ PPEPs.

The data presented here are the first to definitively demonstrate that NG2+ PPEPs give rise to a large number of astrocytes postnatally. This generation of astrocytes from PPEPs was an unexpected finding, since NG2+/PDGFR α + PPEPs have been reported to be oligodendrocyte restricted (Mallon et al., 2002), and astrogenesis from NG2+ cells has been reported to occur only in the late embryonic stage (Zhu et al., 2008a).

NG2+ PPEPs give rise to immature, postmitotic astrocytes

To determine whether astrocytes generated from EYFP-tagged PPEPs were still mitotically active, we analyzed coronal sections from P10, P15, and P60 mice, 3, 8, and 53 d after tamoxifen injection, respectively; 2 h before being killed, mice were given a single injection of EdU. Triple immunostaining of P10 and P15 spinal cord showed that there were virtually no EYFP+/BLBP+ cells labeled with EdU (supplemental Fig. 4*I*, cell c1, available at www.jneurosci.org as supplemental material) (3 of 286 from three mice at P10; 2 of 198 from five mice at P15), although there were many EYFP+/BrdU+ PPEPs (supplemental Fig. 4*I*, cell c2, available at www.jneurosci.org as supplemental material), indicating that EYFP+ astrocytes generated from NG2 cells were postmitotic.

Some newly generated, immature EYFP+ astrocytes had few or no processes as expected for immature astrocytes (Fig. 5*A*, boxed area a1). Others had more but nonbushy processes (supplemental Fig. 4*E*, cell e1, available at www.jneurosci.org as supplemental material), whereas in more advanced cells, the processes were more complex (supplemental Fig. 4*E*, cell e2, available at www.jneurosci.org as supplemental material). EYFP+ cells typical of mature protoplasmic astrocytes were also present at P15, both in forebrain (supplemental Fig. 4*F*, cell,

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Figure 5. Early postnatal PPEPs give rise to gray matter protoplasmic astrocytes. At P15, 8 d after tamoxifen treatment, a subpopulation of EYFP+ cells (green) were labeled with the astroglial markers BLBP (**A**), GFAP (**B**), and S100- β (**C**). The higher magnification in boxed area a1 in **A** shows an example of EYFP+/BLBP+ astrocytes with few processes that were newly generated from EYFP+ PPEPs. EYFP+/BLBP+ astrocytes are usually distributed in clusters (**A**, arrowheads) or in doublets (**D**, arrowhead). EdU incorporation experiment showed that newly formed EYFP+/BLBP+ astrocytes were EdU immunoreactive with a single injection at P7 (arrowhead and higher magnification image in **D**). **B** is orthogonal view showing *x*–*z* and *y*–*z* axes, and the others are projection view. All pictures were from ventral cortex of forebrain. Scale bars: **A**, **D**, 50 μ m; **B**, **C**, 20 μ m.

Figure 7. A subpopulation of neurons are produced from EYFP-tagged PPEPs. EYFP+ (green) neurons in the P15 ventral cortex are stained with neuronal marker NeuN (**A**, red) and HuC/D (**B**, red). **A**, Orthogonal views of neuron at the crosshair, showing colocalization of EYFP with NeuN. The boxed area in **B** shows clustered EYFP+ neurons. **C**, EYFP+/NeuN+ neurons from P60 adult had the appearance of pyramidal neurons, and the cell marked with arrowhead is shown at higher magnification in **C1** (EYFP, green; NeuN, red). The arrowheads and arrows in **C1** point to apical (marked with asterisk) and basal dendrites, respectively, whereas the wavy arrows point to axon extending to subcortical white matter. **D**, PPEP-derived neurons (EYFP+/HuC/D+) are EdU (red) positive in the P15 ventral cortex. **D1** depicts five consecutive 1 μ m confocal images in the z-dimension of the neuron indicated by arrowhead in **E**, showing colocalization of EYFP, HuC/D, and EdU. Scale bars: **A**, **D**, 30 μ m; **B**, **C**, 50 μ m.

available at www.jneurosci.org as supplemental material) (Fig. 5B,C) and in spinal cord (Fig. 6B, cell b2); these had the bushy or densely ramified processes characteristic of spongiform mature protoplasmic astroglia (Bushong et al., 2004). In adult P60 mice, virtually all of the EYFP+ astrocytes had this bushy morphology (supplemental Fig. 4H,H1, available at www.jneurosci.org as supplemental material). Since vimentin expression in rodent CNS development precedes that of GFAP (Dahl et al., 1981; Schnitzer et al., 1981), we evaluated vimentin as a marker for labeling immature astrocytes. In the gray matter of spinal cord at P10, most of the EYFP+ astrocytes expressed high levels of vimentin, with no or at most weak GFAP expression (Fig. 6A). By P15, most EYFP+ astrocytes were strongly labeled with GFAP, whereas vimentin immunoreactivity was faint (Fig. 6B, cell b1) or undetectable (Fig. 6B, cell b2). At this time point, however,

ependymal cells around the central canal were strongly vimentin+ (Fig. 6B, asterisk). Cells marked with a1, a2, a3 (Fig. 6A), and b1, b2 (Fig. 6B) illustrate this astroglial maturation progress, with a1 being immature and b2, mature astrocytes. Together, these results demonstrate that EYFP-tagged PPEPs in the neonatal CNS generate immature, but mostly postmitotic, astrocytes.

EYFP-tagged PPEPs generate a subset of neurons

It is proposed that NG2+ progenitors can give rise to neurons when maintained in an appropriate culture medium (Kondo and Raff 2000; Belachew et al., 2003; Nunes et al., 2003) or after transplantation (Aguirre et al., 2004; Liu et al., 2007). Does this lineage relationship also take place during normal *in vivo* development, and do PPEPs contribute to this neurogenesis? When PCE/R double-transgenic mice were given two injections of tamoxifen at P7 and killed 8 d later (P15), we found occasional EYFP+ cells with relative large cell bodies ($11.6 \pm 1.2 \mu\text{m}$; $n = 50$) and zero to three visible processes scattered in the dorsal and ventral (including amygdala and piriform cortex) forebrain cerebral cortex and hypothalamus. Some of these cells were stained with an immature neuronal marker, β -III tubulin (also Tuj1) (supplemental Fig. 5A, available at www.jneurosci.org as supplemental material). Almost all of them were also labeled with an antibody against another immature neuronal marker, RNA-binding protein HuC/D (Fig. 7B; supplemental Fig. 5C, available at www.jneurosci.org as supplemental material), which is initially expressed when neuroblasts exit the mitotic cycle and persists in adult neurons (Okano and Darnell, 1997). EYFP+/HuC/D+ cells often appeared in clusters $<25 \mu\text{m}$ apart (Fig. 7B, boxed area; supplemental Fig. 5C, boxed area b, available at www.jneurosci.org as supplemental material) or intermingled

with EYFP+ NG2 cells (Fig. 7B, arrow). Occasionally, two EYFP+ cells that coexpressed HuC/D antigen and were adjacent to each other (supplemental Fig. 5C, boxed area a, available at www.jneurosci.org as supplemental material) were observed, suggesting that they had been newly generated *in situ* from their ancestor EYFP+ PPEPs. Providing additional support to this suggestion, we sometimes observed EYFP+ cells close to each other, which displayed a transitional morphology from NG2 cells to neurons and were weakly stained with HuC/D (supplemental Fig. 5E1–E3, available at www.jneurosci.org as supplemental material). These large EYFP+ cells also colabeled with the pan-neuronal marker NeuN (Fig. 7A). Some also expressed microtubule-associated protein 2a (Map2a) (supplemental Fig. 5B–B2, available at www.jneurosci.org as supplemental material), a marker for mature neurons, but most EYFP+/HuC/D+ cells were negative for MAP2a at P15, suggesting

that they were immature. Most of the EYFP+/HuC/D+ neurons in the dorsal and ventral forebrain cortex had the appearance of pyramidal neurons, especially in adult P60 mice. In the dorsal cortex, they had triangular shaped cell bodies with typical pyramidal neuron morphology: (1) apex (Fig. 7C1, asterisk) pointing to pial surface; (2) a single thick Map2a+ apical dendrite (Fig. 7C1, arrowheads) extending toward the pial surface, with many laterally branched distal dendrites; (3) the base giving rise to horizontally oriented dendrites (Fig. 7C1, arrows); (4) a single axon pointing to subcortical white matter (Fig. 7C1, wavy arrow). Because of the synaptic distribution of vesicular glutamate transporter 1 (vGLUT1), it was difficult to colocalize vGLUT1 protein with specific EYFP+ pyramidal neurons (data not shown). Instead, we used vGLUT1 mRNA *in situ* hybridization to identify glutamatergic neurons. Our data showed that the neurons generated from PPEPs in the dorsal and ventral cortex were glutamatergic pyramidal neurons (supplemental Fig. 5F–H, available at www.jneurosci.org as supplemental material). We quantified the percentage of EYFP+ neurons among all the neurons using stereological counting. Our data showed that 0.88 and 0.65% of total neurons were EYFP+ in the dorsal and ventral cortex. In contrast to the high expression of Olig2 in PPEPs, the EYFP+ neurons lost Olig2 expression after generation from PPEPs, assessed by EYFP, HuC/D, and Olig2 triple immunostaining (supplemental Fig. 5D, arrowhead, available at www.jneurosci.org as supplemental material).

If neurons are arising postnatally from mitogenic PPEPs, then we would expect to detect EdU-labeled neurons after neonatal administration of EdU. Considering that the number of NG2+ progenitors reaches a peak in the first postnatal week (Nishiyama et al., 1996), we injected EdU once daily at P1, P4, and P5 and administered tamoxifen at P7. We detected the newly generated EdU+/HuC/D+ neurons in the subgranular zone (SGZ) of hippocampus (supplemental Fig. 6B, arrowhead, available at www.jneurosci.org as supplemental material), which served as an internal positive control of postnatal neurogenesis and also validated the efficacy of EdU labeling. As expected, we also observed EYFP+/HuC/D+ neurons that were EdU immunoreactive in P15 forebrain, for example, in the amygdala (Fig. 7D, arrowhead and higher magnification), piriform cortex (supplemental Fig. 6D, arrowhead, available at www.jneurosci.org as supplemental material), and hypothalamus (supplemental Fig. 6C, arrowhead, available at www.jneurosci.org as supplemental material). This result indicates that EYFP+/HuC/D+/EdU+ neurons were formed from EYFP+ PPEPs that became labeled with EdU between P1 and P5.

At P8 in the hippocampus, all of EYFP-tagged cells were NG2+ (Fig. 1C,E); 8 d after tamoxifen treatment, 41.2% of EYFP+ cells had matured into APC+ oligodendrocytes (Table 3), whereas 55.1% of EYFP+ cells retained their undifferentiated NG2 status (Fig. 4A1–A3, Table 3). Interestingly, we observed that 4.1% of EYFP+ cells did not express the oligodendroglial marker, APC, nor were they NG2+, and they were also negative for astrocytic markers BLBP and GFAP. Immunohistochemistry revealed that all of these EYFP+ cells were double-labeled with antibodies against the neuronal markers: HuC/D (Fig. 8A; supplemental Fig. 7B, available at www.jneurosci.org as supplemental material) and NeuN (Fig. 8C), and some of them were positive for Tuj1 (supplemental Fig. 7A1–A3, arrowhead, available at www.jneurosci.org as supplemental material) and MAP2a (supplemental Fig. 7C1–C3, available at www.jneurosci.org as supplemental material). The nuclei of these EYFP+/HuC/D+ neurons were smaller (7.9 ± 1.4 ; $n = 102$) than those in cerebral cortex and resembled those of granule cells in the dentate gyrus and

olfactory bulb, two widely accepted adult neurogenic regions. This small nuclear size suggested that the EYFP+ neurons generated from EYFP-tagged PPEPs in hippocampus were interneurons. To evaluate this possibility, we immunostained P15 tissues using an antibody against the GABA-synthesizing enzyme, glutamic acid decarboxylase 67 (GAD67). Results showed that these EYFP+ neurons were GAD67 positive (Fig. 8D; supplemental Fig. 7D, available at www.jneurosci.org as supplemental material), supporting their GABAergic interneuronal identity. A total of 1.12% of GAD67+ GABAergic interneurons in the hippocampus were EYFP+. These interneurons were also immunoreactive for somatostatin (Fig. 8B), but not parvalbumin (supplemental Fig. 7F, available at www.jneurosci.org as supplemental material) or calretinin (supplemental Fig. 7E, available at www.jneurosci.org as supplemental material). Together, our data showed that EYFP-tagged PPEPs generate GABAergic interneurons in the hippocampus *in vivo*.

It is possible that the EYFP+ neurons were generated locally from NG2+/Olig2+/PDGFRa+ PPEPs. Alternatively, they might have been derived from migrating neuroblasts that arose in the subventricular zone (SVZ) of anterior forebrain. The latter seems unlikely, as there were no GFAP+ type-B cells or BLBP+ radial glial cells in the SVZ labeled with EYFP along the axis of anterior forebrain (bregma 0 mm to bregma 1 mm) at P8 (supplemental Fig. 2A1–A3, arrowheads, available at www.jneurosci.org as supplemental material) through P15, although there were EYFP+/NG2+ cells in the ventral part of SVZ (supplemental Fig. 2F2, boxed area, available at www.jneurosci.org as supplemental material). Furthermore, no EYFP+/NeuN+ neurons were found in coronal sections of P60 olfactory bulb (supplemental Fig. 2C1–C4, arrowheads, available at www.jneurosci.org as supplemental material). However, there was an accumulation of EYFP+ neurons in dorsal cortex from P10 through P60 with the same tamoxifen paradigm as P8 (supplemental Fig. 2B, available at www.jneurosci.org as supplemental material), whereas virtually no NeuN+ neurons labeled with EYFP were present in the dorsal cortex (supplemental Fig. 2B1, arrowheads, available at www.jneurosci.org as supplemental material) at P8. Although there were DCX+ neuroblasts in the dorsal–lateral SVZ (supplemental Fig. 2D1–D3, available at www.jneurosci.org as supplemental material) and DCX+ neurons in cerebral cortex (supplemental Fig. 2E1,E2, arrowheads, available at www.jneurosci.org as supplemental material), no EYFP+ neurons were DCX-positive (supplemental Fig. 2D3,E1–E4, arrows, available at www.jneurosci.org as supplemental material). Since DCX is required for neuroblast migration (Francis et al., 1999; Nacher et al., 2001; Bai et al., 2003), this result suggests that EYFP+ neurons were generated from NG2+ PPEPs *in situ* (Dayer et al. 2005), rather than from EYFP+ neuroblasts migrating from SVZ. These data demonstrate for the first time that endogenous postnatal PPEPs generate neurons in specific areas of forebrain.

Discussion

Identity and characterization of early postnatal plp promoter-expressing progenitors

Since no EYFP+ cells were seen in PCE/R mice with sunflower oil treatment (supplemental Fig. 1A1–B2, available at www.jneurosci.org as supplemental material), nor with tamoxifen treatment of Rosa26-EYFP mice lacking Cre transgene, we concluded that expression of EYFP in PCE/R mice was controlled by the plp promoter and tamoxifen, with no “leaky expression” of the reporter gene. One day after tamoxifen treatment, expres-

Figure 8. EYFP-tagged PPEPs generate hippocampal GABAergic interneurons. PPEP-derived neurons in the P15 hippocampus express reporter gene EYFP (green) and are labeled with neuronal marker, HuC/D (**A–A3**, red) and NeuN (**D–D2**, red). The boxed areas in **A** are depicted at higher magnification in **A1–A3**. The EYFP+ neurons expressed the GABAergic interneuron marker, GAD67 (**D**, crosshair, and higher magnification, red). The arrows in **D** point to examples of GAD67+/EYFP– neurons. **B–B3** show the neurons generated from EYFP-tagged PPEPs (EYFP, green; HuC/D, blue) in the hippocampus were somatostatin (SOM) (red)-expressing interneurons. **B** and **D** are orthogonal views with *x–z* and *y–z* axes. Blue in **A**, **C**, and **D** are Hoechst+ nuclei. The location of each picture is indicated in the schematic drawing of the hippocampus. Scale bars: **A**, **D**, 50 μ m; **B**, **C**, 10 μ m.

sions of nuclear Cre recombinase and EYFP were restricted to NG2+/Olig2+ progenitors and APC+ mature oligodendrocytes (supplemental Fig. 1C,D, available at www.jneurosci.org as supplemental material). Although plp promoter activity has been reported in radial glia at E13.5 (Delaunay et al., 2008), there was no detectable Cre recombinase or reporter gene expression in BLBP+/GFAP+ astroglia and in BLBP+, 3CB2+ radial glia (supplemental Fig. 1E–I, available at www.jneurosci.org as supplemental material) in neonatal PCE/R mice. This result, which is consistent with the study by Doerflinger et al. (2003), indicates that postnatal astrocytes and radial glia do not express plp promoter activity. However, we observed ectopic expression of plp promoter in some mature neurons in the caudate–putamen between external and internal capsule (supplemental Fig. 1J–K3, available at www.jneurosci.org as supplemental material).

Our study demonstrated that early postnatal PPEPs became EYFP-labeled 24 h after tamoxifen. The majority of these PPEPs also expressed Mash1, a marker for postnatal bipotential oligodendrocyte/neuron progenitors (Parras et al., 2004, 2007; Kim et al., 2007), Olig2, EGFR, PDGFR α , and to a much lesser extent, nestin. Their proliferative potential (Fig. 3) suggested that the PPEPs in the neonatal mice were type C-like transit-amplifying progenitors (Aguirre et al., 2004; Menn et al., 2006).

The PPEP subpopulation and the general NG2+ population

NG2+ cells are evenly distributed in the CNS of neonatal and adult rodents. NG2+ cells are highly heterogeneous in their origins (Bouslama-Oueghlani et al., 2005), morphology and electrophysiological properties (Chittajallu et al., 2004; Káradóttir et al., 2008), antigen expression (Karram et al., 2008), cell fates (Rivers et al., 2008; Zhu et al., 2008a), and plp promoter activity (Mallon et al., 2002). In our study, we focused on the fate of plp promoter-expressing NG2+ progenitors (PPEPs). Our data argue that PPEPs are an intrinsically unique subset of NG2+ cells: (1) Although the general NG2+ cell population is abundant in adult CNS (Dawson et al., 2003) (Table 2; supplemental Table 2, available at www.jneurosci.org as supplemental material), PPEPs decreased virtually to zero by adulthood in forebrain dorsal cortex (Table 2). (2) Using NG2-CreER^{T2} mice, and obtaining a 5% recombination rate after tamoxifen, Nishiyama's group showed that postnatal NG2+ cells generated only oligodendrocytes (Zhu et al., 2008b). However, we unexpectedly found that the PPEP subset of NG2+ cells also generated astrocytes postnatally. Thus, using a PCE/R transgene, we fate mapped a different subset of NG2+ cells than the 5% of NG2+ cells that underwent recombination using the NG2 promoter in the Nishiyama lab (Tables 2, 3). (3) There were still a large number of PPEPs in ventral cortex

of adult forebrain (~9% of recombination rate in NG2+ cells), and we also found, by fate mapping adult PCE/R mice, that continued neurogenesis from PPEPs persisted in ventral adult forebrain, whereas no EYFP+ neurons were generated in the adult dorsal forebrain (our unpublished data). This is consistent with a recent study showing that NG2+/PDGFR α + progenitors generate neurons only in the adult ventral forebrain (Rivers et al., 2008), although there is also a 50% recombination efficiency in NG2+/PDGFR α + progenitors in dorsal forebrain. Our data, coupled with that of other groups, argue that PPEPs are a unique subpopulation within the general population of NG2 cells and that *plp* promoter expression in NG2 cells correlates with the capability for astrogenesis, neurogenesis, in addition to traditional oligodendrogenesis.

Early postnatal PPEPs generate protoplasmic astroglia in CNS gray matter

The lack of GFAP+/NG2+ cells *in vivo* (Skoff, 1990; Fulton et al., 1992) led many investigators to conclude that the observation by Raff et al. (1983) of astrogenesis from OPCs was a culture artifact. More recent, but still inconclusive, support for the results of Raff et al. came from transplantation studies, in which oligodendrocyte progenitors (presumably NG2+/PDGFR α +) generated astrocytes in their new host (Liu et al., 2007), and by the detection of GFAP+/NG2+ or GFAP+/Olig2+ cells in CNS injury models (Alonso 2005; Tatsumi et al., 2008) (our unpublished data). Here, we present more direct *in vivo* evidence that NG2+ PPEPs give rise to a subset of astrocytes during normal postnatal development. Our data are consistent with recent fate-mapping results obtained using NG2-Cre mice (Zhu et al., 2008a). Importantly, since we used an inducible rather than constitutive Cre, we were able to unequivocally demonstrate postnatal astrogenesis from NG2+ PPEPs. Also, by using thymidine analogues to tag cells undergoing mitosis, we showed that the immature astroglia initially generated from these PPEPs were already postmitotic.

In our experiments, no EYFP+ astrocytes were detected in CNS white matter and dorsal forebrain, despite the presence of numerous, actively proliferating PPEPs in these locations. This regional difference may depend on the intrinsic properties of PPEPs, since NG2+ cells are heterogeneous in their physiological properties between gray matter and white matter (Chittajallu et al., 2004). Alternatively, environmental cues in different regions might dictate different fates of these PPEPs.

Whether astrogenesis from PPEPs persists into adulthood is still elusive. It has been reported that astrocytes are still derived from a dividing progenitor population (supposed to be NG2+ OPCs) in adult spinal cord (Horner et al., 2000). But according to recent studies, no astrocyte production from adult PDGFR α +/NG2+ glia or Olig2+ progenitors was detected in the adult forebrain (Dimou et al., 2008; Rivers et al., 2008). Whether there exist fate differences of PPEPs between adult spinal cord and forebrain or between different ages needs to be further investigated.

Neuronal fate of early postnatal *plp* promoter-expressing progenitors in the forebrain

Several previous studies have shown that neurons, including both projection neurons and interneurons, can be formed from OPCs, both *in vitro* (Kondo and Raff, 2000; Nunes et al., 2003) and *in vivo*, in regions including neocortex (Dayer et al., 2005; Tamura et al., 2007), ventral cerebral cortex (Rivers et al., 2008), and under transplantation condition in hippocampus (Aguirre et al., 2004). We now present the first *in vivo* fate-mapping evidence showing that early postnatal NG2+ PPEPs gave rise to pyramidal

neurons in cerebral cortex and interneurons in hippocampus. EYFP+/HuC/D+ neurons first appeared in very low number 2–3 d after tamoxifen injection, and their numbers increased approximately threefold over the following 3 weeks, and then at a much slower pace until adulthood (supplemental Fig. 2B, available at www.jneurosci.org as supplemental material), thus suggesting derivation of neurons from NG2+/EYFP+ PPEPs is most rapid in the neonatal period. The observation of EdU+/EYFP+/HuC/D+ neurons also supported the hypothesis that neurons are generated from NG2+/EYFP+/EdU+ PPEPs during early postnatal development. Our data indicate that the EYFP+ neurons derived from PPEPs do not express DCX, a protein that plays an important role in migration of cortical interneurons (Francis et al., 1999; Nacher et al., 2001; Bai et al., 2003; Friocourt et al., 2007). In contrast, previous fate mapping in neonatal GFAP-CreER^{T2} mice indicated that neurons that express DCX are generated postnatally from GFAP+ neural stem cells in SVZ (Ganat et al., 2006). The results of Ganat et al., in combination with our data, suggest that there are two disparate sources of progenitors for neurogenesis/astrogenesis in early postnatal forebrain. In postnatal forebrain gray matter, NG2+ OPCs receive glutamatergic and GABAergic synaptic inputs from local neurons (Gallo et al., 2008). These synaptic junctions are retained during mitosis and inherited by daughter cells (Kukley et al., 2008). The developmental roles of these synaptic inputs on NG2+ OPCs are still unclear. It is tempting to postulate that signaling via these synapses influences the fates of NG2+ OPC.

We consider it unlikely that the postnatal generation of EYFP+ neurons from PPEPs was an artifact attributable to fusion of EYFP+ glia with neurons. Although fusions between neurons and microglia have been reported (Ackman et al., 2006), we are unaware of evidences of fusions between neurons and oligodendroglial lineage cells or other macroglial progenitors, and observed no instances of EYFP+ microglia. We cannot exclude the possibility that the cerebral cortical EYFP+ neurons we observed, although DCX negative, were derived from NG2+ PPEPs in SVZ (supplemental Fig. 2F–F2, available at www.jneurosci.org as supplemental material) (Aguirre et al., 2004), rather than from *in situ* generation in cerebral cortex. However, our studies do provide the first unequivocal fate-mapping evidence to support neurogenesis from PPEPs in the neonatal cerebral cortex (Fagel et al., 2006; Ahmed et al., 2008).

In conclusion, our genetic fate-mapping findings demonstrate that CNS PPEPs remain multipotent at the end of the first postnatal week, generating oligodendrocytes, astrocytes, and neurons. Our results also reveal that PPEPs in different CNS regions and different developmental stage have different fates. Additional experiments will be needed to explore whether there exist certain specific local signals and/or environment cues that control these differing fates in different regions, and/or an intrinsic heterogeneity of these PPEPs.

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