Supporting Information

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SI Materials and Methods

Isolation of Boundary Cap Cells. Cell preparations containing boundary cap cells (BC) were obtained as described previously (1). Briefly, meninges from embryonic day (E)12.5 *Krox20*$^{Cre/+}$, *R26R*$^{YFP/+}$ embryos were microdissected and dissociated in a mix of collagenase (Sigma-Aldrich) and trypsin (Invitrogen) at 37 °C for 10–20 min. Single-cell populations were obtained by mechanical trituration. The dissociation process was ended by adding 10% FBS (Gibco-BRL).

BC Culture. ***Amplification of cells dissociated from meninges.*** Cells obtained from dissociated meninges were seeded at semiclonal concentration (25,000 cells/mL) in a serum-free medium consisting of NeuroBasal supplemented with 2% B27 (Gibco), 1% Glutamax, heparin (8 μg/mL) (Sigma), and 1% penicillin-streptomycin in the presence of FGF2 (40 ng/mL) and EGF (20 ng/mL) (Sigma), referred to as "proliferation medium." Cells were grown as floating spheres at 37 °C/5% CO_2 and passaged every 10 d.

FACS sorting. At the second passage (P2), YFP$^+$ cells were sorted by FACS (Becton Dickinson) on the basis of YFP expression. Spheres were dissociated with ATV (0.05% trypsin, 0.1% glucose, and 0.5 nM EDTA) and DNase for 10 min at 37 °C. Dissociated cells were washed in DMEM containing 2% BSA (Sigma) and 0.5 mM EDTA, collected after FACS sorting in DMEM containing 4% BSA, and washed twice in DMEM prior to plating.

Amplification of FACS-sorted YFP$^+$ cells. FACS-sorted YFP$^+$ cells were seeded at semiclonal concentration (25,000 cells/mL) in proliferation medium and serially passaged up to P9.

Cell characterization. At each passage, cells from dissociated floating spheres were plated on polyornithine/laminin (Sigma) -coated coverslips (50,000 cells per well) in proliferation medium and fixed 24 h later with 2% paraformaldehyde (PFA) for immunocytochemistry.

Neural Precursor Cell Culture. Primary neural precursor cells (NPC) were obtained from E12.5 C57Bl6 embryos (Elevage Janvier). Forebrains were dissected free of meninges and enzymatically dissociated using ATV. Collected cells were resuspended in DMEM/F12 medium (1/1) supplemented with N2 supplements (1%), B27 (0.5%), insulin (25 g/mL), glucose (6 mg/mL), Hepes (5 mM), bFGF2 (20 ng/mL), and EGF (20 ng/mL).

Differentiation Protocols. Differentiation of amplified FACS-sorted YFP$^+$ cells was achieved by plating YFP$^+$ cells on polyornithine/laminin-coated coverslips (20,000 cells per well) under various conditions before fixation with 2% PFA. Experiments were performed in triplicate and repeated with two different isolates.

Neural crest differentiation medium. Neurobasal medium containing 5–10% FBS.

NPC differentiation medium. Neurobasal medium containing 2% FBS.

Noggin medium. DMEM/F12 (1:1; Invitrogen) supplemented with N2 (Sigma), heparin (2 μg/mL; Sigma), B27 (2%; Gibco), Noggin (50 ng/mL; R&D Systems), SB431542 (10 μM; Sigma), and FGF2 (10 ng/mL; Sigma).

Purmorphamine medium. DMEM/F12 (1:1) supplemented with N2 (Sigma), heparin (2 μg/mL; Sigma), B27 (2%; Gibco), Purmorphamine (5 mM; Calbiochem), and retinoic acid (100 nM; Sigma).

Glial differentiation medium. DMEM/F12 (1:1) supplemented with N1, B27, T3 (60 ng/mL; Sigma), cAMP (1 μM; Sigma), biotin (100 ng/mL; Sigma), PDGF (10 ng/mL; R&D Systems), NT3 (10 ng/mL; R&D Systems), and IGF1 (10 ng/mL; R&D Systems).

BC Transduction. BC (5×10^4) were transduced with HIV-CMV-GFP (12 ng of p24 antigen), which allowed strong and stable cytoplasmic GFP expression and unambiguous tracing after grafting (2). Expression of GFP was detected 48–72 h posttransduction.

Immunocytochemistry. Cells and tissues were rehydrated in PBS, 4% BSA for 10 min at room temperature (RT). For intracellular staining, cells were incubated with primary antibodies for 1 h at RT in the presence of 0.01% Triton X-100 for permeabilization. Cells were washed three times with PBS and then incubated with fluorescence-conjugated secondary antibodies for 45 min at RT. The following primary antibodies were used: anti-GFP (chick, 1/500, Aves Labs; rabbit, 1/2,000, Chemicon International; rat, 1/100, R&D Systems), anti-Sox9 (goat IgG, 1/1,000; R&D Systems), anti-Sox2 (mouse IgG, 1/1,000; R&D Systems), rabbit anti-S100 (1/100; Dako), anti-Sox10 (goat IgG, 1/1,000; R&D Systems), anti-Nestin (mouse IgG, 1/1,000; Chemicon International), anti-PSA-NCAM (mouse IgM, 1/1,000; AbCys), anti-Ki67 (mouse IgG1, 1/100; BD Biosciences), anti-GFAP (mouse IgM or rabbit, 1/1,000; AbCys), anti-p75 (rabbit, 1/100; Chemicon), anti-β3-tubulin (mouse IgG2b, 1/800; Sigma), anti-NeuN (mouse IgG, 1/100; Chemicon), anti-SMA (mouse IgG2a, 1/400; Sigma), anti-Nkx2.2 (mouse hybridoma IgG2b; Developmental Studies Hybridoma Bank), anti-Olig2 (rabbit, 1/500; Millipore), anti-O4 [mouse hybridoma (3)], anti-GalC [mouse IgG3 hybridoma (4)], and anti-CNPase (mouse IgG1, 1/100; Sigma). Species-specific secondary fluorescence-conjugated antibodies were applied for 1 h in addition to Hoechst 33342 (Sigma). Coverslips were mounted with Fluoromount and analyzed under a fluorescence DBM Leica microscope (Leica Microsystems) using Explora Nova image acquisition software. Cell counts were performed with ImageJ software 1.39v (National Institutes of Health). Statistical analysis was performed using the Student's t test with $P < 0.01$ for significance with SigmaStat software (SYSTAT, Erkrath, Germany).

RT-PCR Assay. Total RNAs were isolated with TRIzol reagent (Invitrogen) from embryonic neural tube and meninges, adult mouse spinal cord, and short- and long-term BC cultures, and used (1 μg per sample) to synthesize cDNA with random-hexamer primers. cDNA synthesis was performed at 50 °C for 50 min in a final volume of 20 μL, according to the manufacturer's instructions for ThermoScript (Invitrogen). PCR was performed using GoTaq Green Master Mix (Promega) according to the manufacturer's standard protocol in a final volume of 25 μL. Ubiquitously expressed β-actin mRNA was used as a reference. To exclude contaminating genomic DNA, reverse transcriptase-omitted samples amplified with β-actin primers were used as negative control. PCR products were resolved by electrophoresis on 2% agarose gels, and bands were visualized with ethidium bromide under UV light. At least three independent experiments were performed for every set of RT-PCR analyses. Primer details are listed in Table S2.

Transplantation. Newborn mice [postnatal day (PN)0–PN2] were cryoanesthetized and grafted with 10^5 cells from short-term amplified FACS-sorted YFP$^+$ cells in 1 μL DMEM. Glass pipettes were directed 1 mm lateral and 1 mm caudal to Bregma (detected through the newborn skin). Cells were injected in the subventricular zone (SVZ) at a depth of 1 mm and pups were returned to their mothers. Animals were euthanized by overanesthesia

(isoflurane followed by ketamine) 12 (n = 14), 28 (n = 30), or 42 (n = 20) d after engraftment.

Postmortem Analysis. For immunohistochemistry, animals were intracardially perfused with 2% PFA. Brains were postfixed for 1 h in the same fixative and cryoprotected overnight with 15% sucrose, embedded in PBS, 7% gelatin, and 15% sucrose, and frozen in isopentane cooled by dry ice. Immunohistochemistry was performed on 10-μm serial sagittal or coronal cryosections using the following primary antibodies: anti-GFP (rabbit, 1/250, Interchim; rat, 1/100, R&D Systems; chicken, 1/100, Aves Labs), anti-GFAP (mouse IgG or rabbit, 1/100; Dako), anti-PSA-NCAM (mouse IgG, 1/400; Abcys), anti-Ki67 (mouse IgG1, 1/100; BD Biosciences), anti-SMA (mouse IgG2a, 1/400; Sigma), anti-laminin (rabbit, 1/20; Chemicon), anti-PECAM1 (rat, 1/100; BD Biosciences), anti-MBP (myelin basic protein) (rat IgG or rabbit, 1/100; Millipore), anti-P0 [1/5, hybridoma (5)], anti-CC1 (mouse IgG, 1/100; Calbiochem), anti-Olig2 (rabbit, 1/500; Millipore), and anti-Caspr (rabbit, 1/800; generous gift from E. Peles, Weizmann Institute of Science, Rehovot, Israel).

For electron microscopy analysis, animals were perfused with 4% PFA and 2.5% glutaraldehyde (Electron Microscopy Sciences). Brains were removed and cut in 100-μm slices on a VT-1000S vibratome (Leica Microsystems). After localization of the engrafted GFP$^+$ cells under a fluorescence microscope, smaller slices containing the GFP$^+$ cells were postfixed with 2.5% glutaraldehyde for 2 h and 2% osmium tetroxide (Electron Microscopy Sciences) for 30 min. After dehydration, slices were embedded in epon. Ultrathin sections were viewed using a Philips CM 120-Bio-tween electron microscope.

1. Zujovic V, Thibaud J, Bachelin C, Vidal M, Coulpier F, Charnay P, Topilko P, Baron-Van Evercooren A (2010) Boundary cap cells are highly competitive for CNS remyelination: fast migration and efficient differentiation in PNS and CNS myelin-forming cells. *Stem Cells* 28:470–479.
2. Girard C, Bemelmans AP, Dufour N, Mallet J, Bachelin C, Nait-Oumesmar B, Baron-Van Evercooren A, Lachapelle F (2005) Grafts of brain-derived neurotrophic factor and neurotrophin 3-transduced primate Schwann cells lead to functional recovery of the demyelinated mouse spinal cord. *J Neurosci* 25:7924–7933.
3. Sommer I, Schachner M (1981) Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev Biol* 83:311–327.
4. Ranscht B, Clapshaw PA, Price J, Noble M, Seifert W (1982) Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proc Natl Acad Sci USA* 79:2709–2713.
5. Yoshimura K, Negishi T, Kaneko A, Sakamoto Y, Kitamura K, Hosokawa T, Hamaguchi K, Nomura M (1996) Monoclonal antibodies specific to the integral membrane protein P0 of bovine peripheral nerve myelin. *Neurosci Res* 25:41–49.

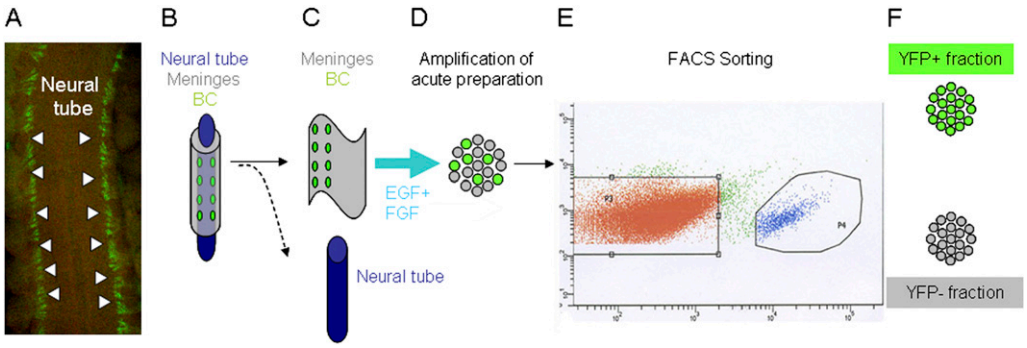

Fig. S1. Selection and amplification of YFP$^+$ cells. Schematic representation of the purification method. (*A*) Dorsal view of *Krox20*$^{Cre/+}$, *R26R*$^{YFP/+}$ embryonic neural tube illustrates BC-restricted location in situ (arrowheads). (*B* and *C*) Meninges containing YFP$^+$ cells were microdissected from E12.5 *Krox20*$^{Cre/+}$, *R26R*$^{YFP/+}$ mouse embryos and dissociated in enzyme mix. Age-matched neural tubes free of meninges were used as control. (*D*) Acute cell preparations were expanded in EGF/FGF as spheres for two passages before FACS. (*E*) Representative YFP-gated flow-cytometric analysis chart. (*F*) FACS-sorted YFP$^+$ and YFP$^-$ populations were expanded separately under semiclonal conditions.

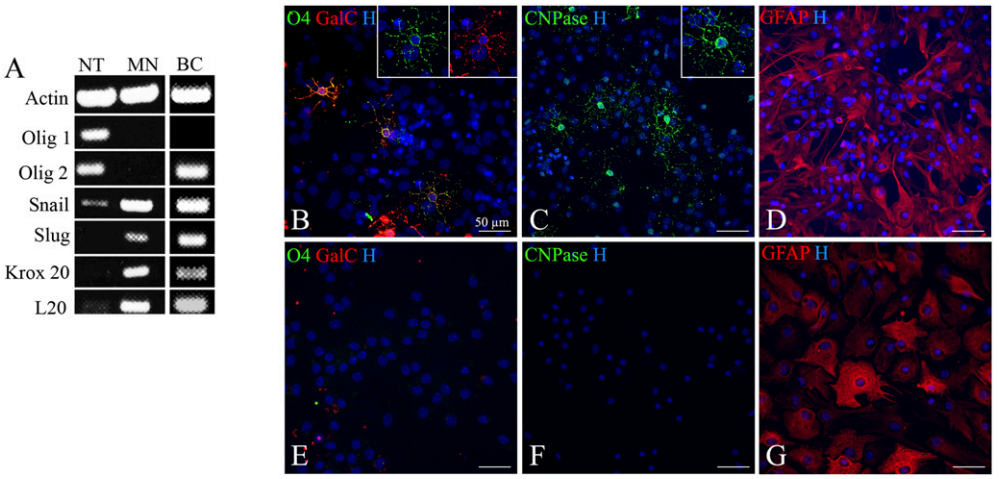

Fig. S2. Purity of BC preparations. (*A*) RT-PCR analysis of freshly isolated neural tube (NT) and meninges (MN) and BC progeny grown in proliferation medium for 8 d. Expression of Olig1 is restricted to NT, whereas that of Slug, Snail, Krox20, and L20 is restricted to the meninges and BC. (*B–G*) Differentiation potential of NPC (*B–D*) and BC (*E–G*) cultured in parallel in NPC differentiation medium. NPC gave rise to $O4^+/GalC^+$ (*B*) and $CNPase^+$ (*C*) oligodendrocytes and $GFAP^+$ cells (*D*). BC progeny were negative for O4 and GalC (*E*) or CNPase (*F*) but positive for GFAP (*G*). (Scale bars, 50 μm.)

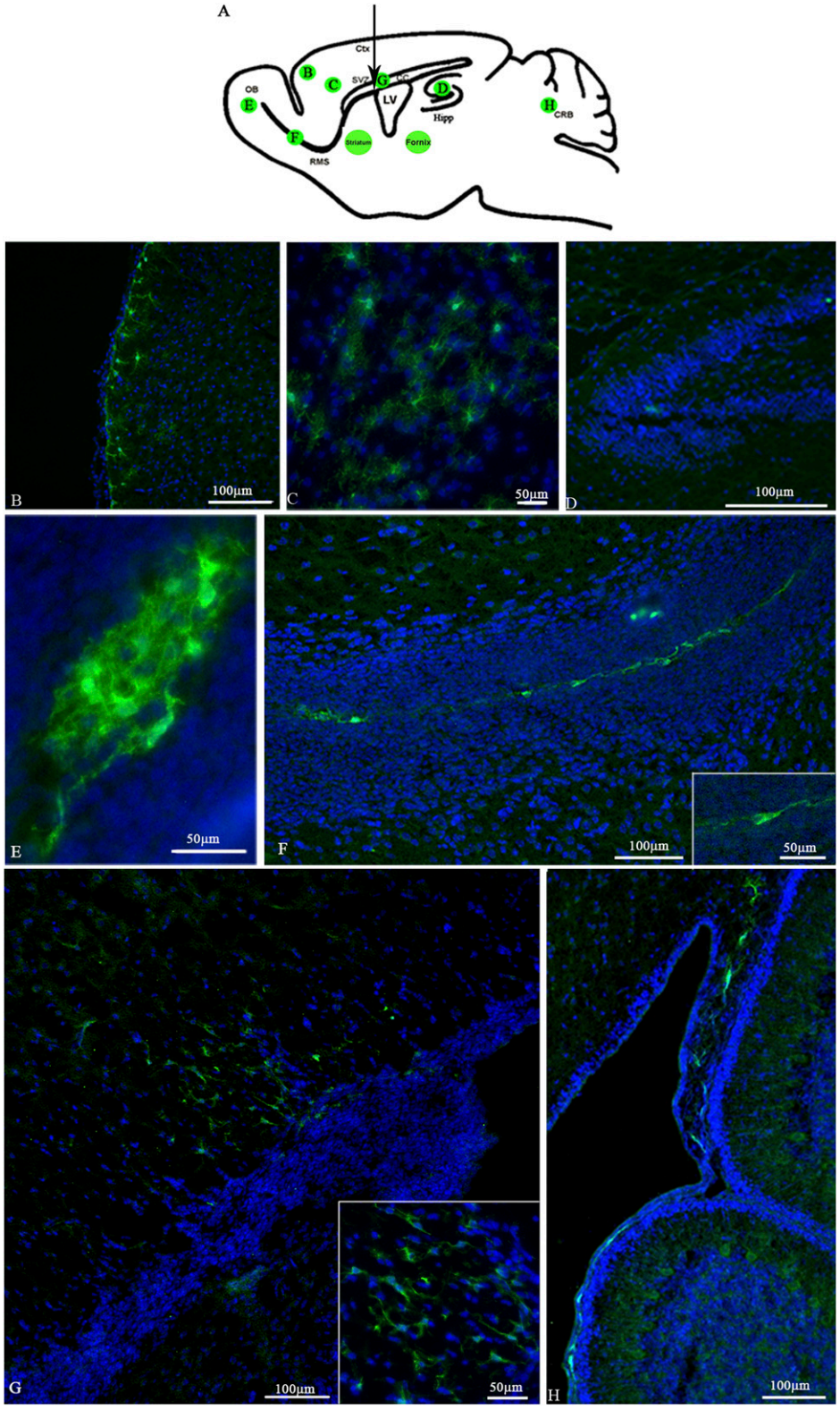

Fig. S3. Widespread distribution of FACS-purified YFP$^+$ cells after transplantation in the newborn *shiverer* SVZ. (*A*) Schematic representation of incorporation sites. The arrow indicates the injection site. Circles indicate the most frequent locations of donor cells, and letters correspond to the fields illustrated in *B*–*H*. (*B*–*H*) YFP$^+$ cells were found in meninges (*B*), cortex (*C*), dentate gyrus (*D*), olfactory bulb (*E*), rostral migratory stream (*F*), corpus callosum (*G*), and cerebellum (*H*). Insets in *F* and *G* illustrate the bipolar profile of migrating cells. Animals (n = 14) were transplanted at PN1–PN2 and analyzed at PN12. Sections were counterstained with Hoechst (blue).

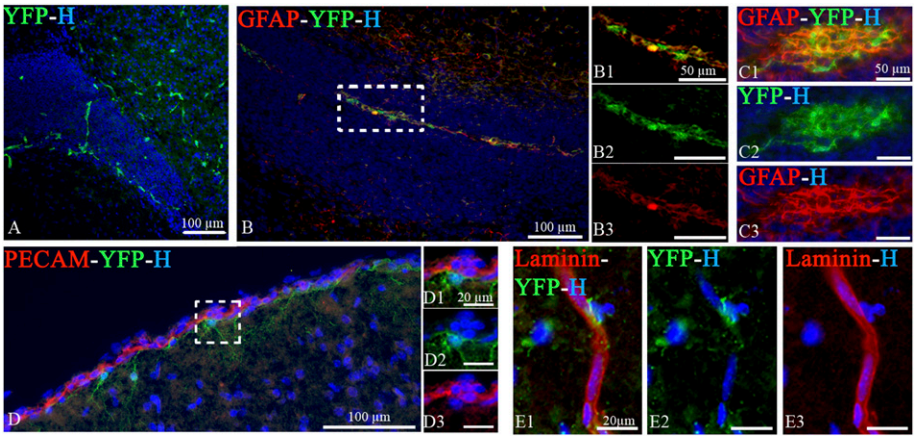

Fig. S4. Migration mode and vasculature interaction of grafted YFP$^+$ cells in the developing host brain. (*A*) YFP$^+$ cells migrate rostrally to the olfactory bulb but some migrate radially toward the subcortical white matter. (*B* and *C*) YFP$^+$ cells (green) are in close association with the GFAP$^+$ astrocyte network (red) en route toward (*B–B3*) or upon their arrival in (*C1–C3*) the olfactory bulb; *B1–B3* illustrate the enlarged dotted area in *B*. (*D–D3*) YFP$^+$ cells (green; *D–D2*, *E1*, and *E2*) are closely associated with blood vessels stained for PECAM1 (red; *D*, *D1*, and *D3*) in the meninges or for laminin (red; *E1* and *E3*) in the cortical parenchyma. H, Hoechst staining.

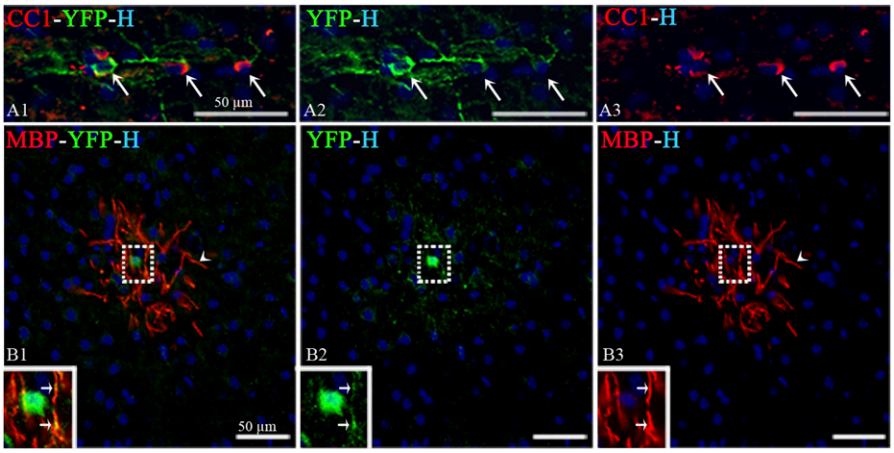

Fig. S5. BC differentiation into myelin-forming oligodendrocytes after long-term transplantation. (*A1–A3*) YFP$^+$ cells (green; *A* and *A2*) give rise to mature CC1$^+$ oligodendrocytes (red; *A1* and *A3*). (*B1–B3*) YFP$^+$ cells (green; *B1* and *B2*) expressing MBP (red; *B1* and *B3*) have features of myelin-forming oligodendrocytes. (*Insets*) Enlarged views of the dotted squares illustrating discrete coexpression of MBP and YFP in processes. The arrowhead points to the T-shaped myelin internode.

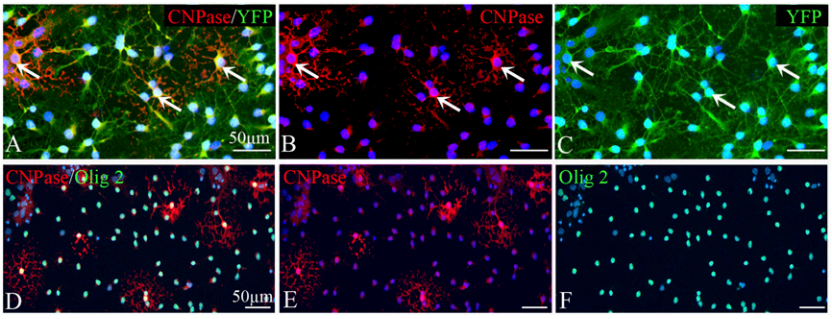

Fig. S6. Oligodendrocytes derived from the FACS-sorted positive fraction are all YFP$^+$. (*A–C*) BC progeny give rise to CNPase$^+$ oligodendrocytes (red; *A* and *B*) that are all YFP$^+$ (green; *A* and *C*). Arrows point to oligodendrocytes, and nuclei were Hoechst-labeled. (*D–F*) General view illustrating BC differentiation in CNPase$^+$(red, *D* and *E*)/Olig 2$^+$(green, *D* and *F*) oligodendrocytes in vitro.

Table S1. Summary of differentiation protocols and outcome. BC gave rise to different progeny depending on the differentiation protocol

Sequential treatment		Differentiation
Noggin 5 d	Glial differentiation 7 d	β3-tubulin$^+$ neurons
Purmorphamine 5 d	Glial differentiation 7 d	GFAP$^+$ astrocytes
Noggin + Purmorphamine 5 d	Glial differentiation 7 d	β3-tubulin$^+$ neurons GFAP$^+$ astrocytes
Noggin 3 d Purmorphamine 2 d	Glial differentiation 7 d	GFAP$^+$ astrocytes GalC$^+$ O4$^+$ CNPase$^+$ oligodendrocytes

Table S2. Primers

Gene	Sequence (5′-3′)
Snail sense	CCCACTCGGATGTGAAGAGATACC
Snail antisense	ATGTGTCCAGTAACCACCCTGCTG
Slug sense	GGCTGCTTCAAGGACACATTAGAAC
Slug antisense	GGTCTGCAGATGTGCCCTCA
Nestin sense	CAGCTGAGCCTATAGTTCAACGC
Nestin antisense	GAAACAAGATCTCAGCAGGCTGAG
Musashi sense	GGCTTCGTCACTTTCATGGACC
Musashi antisense	GGGAACTGGTAGGTGTAACCAG
Sox9 sense	CAAGTGTGTGTGCCGTGGATAG
Sox9 antisense	CCAGCCACAGCAGTGAGTAAGAA
Krox20 sense	CACCACTTCCACCTCCTCTC
Krox20 antisense	CTCACCGCCTCCACTTGCCC
L20 sense	TAGGGTCCGAGAGCTCCTGT
L20 antisense	CTCACACAACCGCTTTAGCC
gfap sense	GAGATCGCCACCTACAGGAA
gfap antisense	GCTCCTGCTTCGACTCCTTA
β3-tubulin sense	GGCCTCCTCTCACAAGTATGT
β3-tubulin antisense	CGCCCTCTGTATAGTGC
Olig2 sense	TAGTTTCGCGCCAGCAGCAG
Olig2 antisense	GGCGGTGGCTTCAAGTCATC
Olig1 sense	AAGGAGGACATTTCCAGACTT
Olig1 antisense	CTCTAAACAGGTGGGATTCA
PDGFRα sense	AGATAGCTTCATGAGCCGAC
PDGFRα antisense	GGAACAGGGTCAATGTCTGG
MBP sense	AAGCACACAGCAGACCCAAAGA
MBP antisense	AAGGATGCCGTGTCTCTGT
β-actin sense	TGACAGGATGCAGAAGGAGA
β-actin antisense	GCTGGAAGGTGGACAGTGAG