

Rat Optic Nerve Oligodendrocytes Develop in the Absence of Viable Retinal Ganglion Cell Axons

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Abstract. Retinal ganglion cell axons and axonal electrical activity have been considered essential for migration, proliferation, and survival of oligodendrocyte lineage cells in the optic nerve. To define axonal requirements during oligodendrogenesis, the developmental appearance of oligodendrocyte progenitors and oligodendrocytes were compared between normal and transected optic nerves. In the absence of viable axons, oligodendrocyte precursors migrated along the length of the nerve and subsequently multiplied and differentiated into myelin basic protein-positive oligodendrocytes at similar densities and with similar temporal and spatial patterns as in control nerves. Since transected optic nerves failed to grow radially, the number of oligodendrocyte lineage cells was reduced compared with control nerves. However, the mitotic indices of progeni-

tors and the percentage of oligodendrocytes undergoing programmed cell death were similar in control and transected optic nerves. Oligodendrocytes lacked their normal longitudinal orientation, developed fewer, shorter processes, and failed to form myelin in the transected nerves. These data indicate that normal densities of oligodendrocytes can develop in the absence of viable retinal ganglion axons, and support the possibility that axons assure their own myelination by regulating the number of myelin internodes formed by individual oligodendrocytes.

Key words: oligodendrocyte progenitor cells • axonal interactions • myelination • optic nerve • programmed cell death

MYELINATION of optic nerve axons requires migration of oligodendrocyte progenitor cells (OPCs)¹ into the optic nerve and proliferation and differentiation of OPCs into oligodendrocytes followed by axonal ensheathment and myelination. The appearance of OPCs, oligodendrocytes, and myelin in the optic nerve follows a reproducible temporal and spatial pattern. Precursor cells enter the rat optic nerve before birth and colonize the nerve in a chiasmal-to-retinal gradient during the first postnatal week (Small et al., 1987). Differentiated oligodendrocytes are first detected ~4 d after birth (P4) in the chiasmal end of the optic nerve, and populate the nerve in a chiasmal-to-retinal gradient between P4 and P7 (Colello et al., 1995). Myelin is first detected

around P6, and myelination progresses in a retinal-to-chiasmal gradient (Skoff et al., 1980; Colello et al., 1995). What regulates oligodendrocyte production and myelination in the optic nerve is unknown. It may be that myelination constitutively follows the colonization of the optic nerve by precursor cells. Alternatively, environmental factors, possibly derived from the retinal ganglion cell axons, may regulate oligodendrocyte production and myelination.

Since all appropriate axons are faithfully myelinated in the normal central nervous system, it seems likely that axons control some aspects of oligodendrocyte development or myelination. Axons could directly or indirectly control OPC migration or proliferation, oligodendrocyte differentiation or survival, or the number of myelin internodes formed by each oligodendrocyte. Viable axons have been proposed to play a direct and essential role in the generation and survival of newly formed oligodendrocytes in the developing rat optic nerve (Barres et al., 1992, 1993; Barres and Raff, 1993, 1994; Raff et al., 1993). In contrast, prolonged oligodendrocyte survival (up to 22 mo) has been reported following transection of P18 or older rat optic nerves (Kidd et al., 1990; Ludwin, 1990; McPhilemy

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; MBP, myelin basic protein; OPC, oligodendrocyte progenitor cell; PCD, programmed cell death.

et al., 1990), and oligodendrocytes can be generated in vitro from precursor cells in the absence of continuous axonal signals (Raff et al., 1983; Zeller et al., 1985; Dubois-Dalcq et al., 1986; Pfeiffer et al., 1993).

Three distinct stages of oligodendrocyte lineage cells have been characterized in the developing rodent brain (Trapp et al., 1997): OPCs that express the integral membrane chondroitin sulfate proteoglycan, NG2, and the α receptor for the PDGF (PDGF α R) (Levine and Nishiyama, 1996; Nishiyama et al., 1996a,b; Trapp et al., 1997); premyelinating oligodendrocytes that express myelin proteins and extend processes but do not ensheath axons (Warrington and Pfeiffer, 1992; Hardy and Friedrich, Jr., 1996; Trapp et al., 1997); and myelinating oligodendrocytes. The optic nerve offers several advantages for investigating oligodendrocyte development. The majority of precursor expansion and all of oligodendrocyte differentiation and myelination occur postnatally and can be easily assayed. Furthermore, the nerve is easily transected or enucleated without compromising the viability of the animal, and thus provides an ideal setting for investigating the role of viable axons in oligodendrocyte lineage development.

In this study, optic nerve transection and retina ablation paradigms were used to investigate the consequences of axonal loss on OPC migration and proliferation and on oligodendrocyte differentiation and survival. Immunocytochemical characterization and quantitative analysis of OPCs and oligodendrocytes in enucleated and transected optic nerves demonstrate that OPCs can colonize the optic nerve and produce oligodendrocytes in the absence of viable axons. Retinal ganglion cell axons appear essential for elaboration of the normal three-dimensional organization of oligodendrocytes and for myelination.

Materials and Methods

Optic Nerve Transection and Retinal Ablation

Newborn (P0) and 4-d-old (P4) Sprague Dawley rat pups were anesthetized with methoxyflurane, the left eye was gently raised with forceps, and the optic nerve was transected with microscissors between the retinal excavation and lamina cribrosa. Alternatively, the left cornea of P0 rats was cut, the vitreous body and the lens removed, and the retina was mechanically ablated. The right optic nerves served as nonoperated controls.

Tissue Preparations

Rat pups (P0–P7) were anesthetized as described above and perfused through the heart with 4% paraformaldehyde in 0.08 M Sorensen's phosphate buffer (pH 7.6). The optic nerves were removed and immersed in the fixative (3 d, 4°C). Longitudinal, free-floating sections (20- μ m thick) were cut on a freezing, sliding microtome. Nerves used for identification of oligodendrocytes were microwaved in citrate buffer (pH 6.0, three times for 3 min) before sectioning.

Immunoperoxidase Staining

Sections were placed in PBS containing 10% Triton X-100 and 1% H₂O₂ (30 min), and incubated in PBS containing 3% normal goat serum (30 min). Sections were exposed to primary antibodies at 4°C (NG2 antibodies overnight, myelin basic protein [MBP] or activated caspase-3 antibodies for 5 d). The sections were then placed in biotinylated goat anti-rabbit Ig or goat anti-mouse Ig (60 min), and peroxidase-conjugated avidin (60 min). Immunoperoxidase staining was developed by metal enhanced diaminobenzidine (Pierce). The sections were treated with 0.04% osmium

tetroxide (20 s), mounted in glycerol, and examined with a Zeiss Axiophot microscope.

Quantification of OPC Density

Progenitor cell density was quantified by counting NG2-labeled cell bodies in optic nerves from P0-transected and control optic nerves at P1, P2, P3, and P4 using a 40 \times objective lens, a rectile (10 \times 10 grid), and Zeiss Axiophot microscope. From four to five control and transected optic nerves at each age, two to three longitudinal sections of the entire optic nerve were examined from the chiasm to the lamina cribrosa. The number of OPCs per grid area was determined and the total area analyzed in each section was calculated. The optic nerve sections were divided into three equally sized segments (chiasm, mid, and retinal portions). For each portion, total areas and OPC numbers were calculated and expressed as OPCs/mm² tissue.

Explants of isolated retinal, chiasm, and intermediate regions of rat optic nerves were prepared from P0 and P4 rats as described previously (Ono et al., 1997). Explants were grown in collagen gels for at least 7 d and labeled with mAb O4 according to published protocols (Ono et al., 1997). The number of explants from each region of the nerve that contained oligodendrocytes at the end of the study was recorded, and the data from at least three separate experiments were pooled.

Measurement of OPC Length

To investigate the effect of nerve transection on progenitor cell process extension, the length of DAB-stained, bipolar NG2 cells was measured in P0-transected ($n = 26$ cells) and control ($n = 44$ cells) optic nerves at P1.

Bromodeoxyuridine Incorporation and Double-Labeling with NG2

Optic nerves were transected at P0 and the rat pups were killed at P2, 90 min after injection with bromodeoxyuridine (BrdU) (i.p.; 0.1 mg/g body weight) (Boehringer Mannheim). Sections were cut as described above and pretreated with 10% Triton X-100 in PBS (30 min), 2 N HCl (10 min), 4% sodium borate (10 min), and 3% goat serum in PBS (30 min). The sections were incubated with NG2 and BrdU antibodies overnight at 4°C, and were then exposed to biotinylated goat anti-rabbit Ig and Texas red-conjugated donkey anti-rat Ig (60 min), and FITC-conjugated avidin (60 min), and examined in a confocal microscope (Leica TCS-NT). To determine the effect of nerve transection on progenitor proliferation, the percentage of NG2-positive cells that were double-labeled by BrdU was compared in two to three sections from P0-transected ($n = 4$) and control ($n = 4$) optic nerves at P2.

Quantification of Oligodendrocyte Density

To investigate the effect of nerve transection on oligodendrocyte density, MBP-labeled cells were quantified in sections of P4-transected and control optic nerves at P5, P6, and P7, as well as in P0 retina-ablated and control optic nerves at P7. Two to three sections from four to five control and P4 retina-ablated optic nerves were analyzed at each age. Total areas of optic nerve sections analyzed were determined as described above, and oligodendrocyte density was expressed as cells/mm² of tissue. Retinal regions of P4, P5, and P6 nerves that contained <10 oligodendrocytes per 40 \times field were excluded from the analysis.

Measurement of the Radial Length of Premyelinating Oligodendrocytes

To examine the effect of axonal transection on oligodendrocyte process formation, the longest radial diameter of premyelinating oligodendrocyte cell bodies and associated processes was measured in sections from P4-transected ($n = 90$ cells) and control ($n = 52$ cells) optic nerves at P6. Sections were pretreated as described above for fluorescence immunostaining, incubated with proteolipid protein (5 d at 4°C), biotinylated goat anti-rat Ig (60 min), and FITC-conjugated avidin (60 min), and then examined in a confocal microscope. Analysis was restricted to cells that occupied clearly defined boundaries.

Double-Labeling with MBP and Activated Caspase-3 Antibodies

Sections were pretreated as described for immunoperoxidase staining above and incubated with MBP and activated caspase-3 antibodies (5 d at 4°C). The sections were then treated with biotinylated goat anti-mouse Ig and FITC-conjugated donkey anti-rabbit Ig (1 h), and then with Texas red-conjugated avidin (1 h) and the nucleic acid binding dye propidium iodide. The preparations were examined in a confocal microscope as described above.

Quantification of Dying Oligodendrocyte Density

To quantify the density of dying oligodendrocytes, the number of activated caspase-3-positive oligodendrocytes was counted in P4-transected and control optic nerves at P5, P6, and P7. Two to three sections from four to five control and P4-transected nerves were examined at each age. Only activated caspase-3-labeled cells with a clear oligodendrocyte-like shape or process morphology were counted. Optic nerve section area and dying oligodendrocyte density were determined as described above. Retinal areas of nerve sections not containing oligodendrocytes were excluded from the analysis.

Antibodies

Primary antibodies are well-characterized and were used at the following concentrations: rabbit polyclonal NG2 antibody, 1:15,000 (Trapp et al., 1997); mouse monoclonal MBP antibody, 1:8,000 (SMI-99; Sternberger Monoclonals, Inc.); rat monoclonal PLP antibody, 1:250 (Agmed, Inc.); rat monoclonal BrdU antibody, 1:5,000 (Harlan Bio); and rabbit polyclonal activated caspase-3 (CM1) antibody, 1:20,000 (Idun Pharmaceuticals, Inc.). Secondary antibodies were purchased from Vector Laboratories.

Statistical Analysis

Unpaired *t* test was used for statistical analysis of progenitor proliferation indices, progenitor cell length, radial diameter of premyelinating oligodendrocytes, and oligodendrocyte density in P0 retina-ablated nerves. Analysis of variance was used for statistical comparison of progenitor cell density, oligodendrocyte density, and dying oligodendrocyte density in controls and transected optic nerves. The data were expressed as means \pm SD.

Electron Microscopy

To verify that all axons degenerate after P0 retinal ablation and P4 transection, optic nerves were examined by electron microscopy. Retinal ablation and optic nerve transection were performed as described above, and the pups were anesthetized and perfused through the heart with 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.08 M Sorenson's buffer at 2, 14, 24, 48, and 96 h after treatment. Optic nerves were removed, immersed in the fixative (3 d at 4°C), postfixed with 1% osmium tetroxide (1 h), dehydrated, and embedded in Epon. Ultrathin sections were cut on an ultramicrotome (Ultracut E; Reichert), collected on Formvar-coated slot grids, counterstained with uranyl acetate and lead citrate, and examined in a Philips CM-100 electron microscope. Midportions of three optic nerves were examined at each time point.

Results

Oligodendrocyte Progenitors Appear in a Chiasmal-to-Retinal Sequence

Previous *in vitro* studies suggest that OPCs enter the chiasmal end of the rat optic nerve before birth, and populate the entire nerve by P9 (Small et al., 1987). However, direct demonstration of the distribution and morphology of OPCs in the intact and transected nerve has not been described. Therefore, sections of developing and transected optic nerve were labeled with antibodies specific for NG2, a sulfated proteoglycan that colocalizes with the PDGF re-

ceptor on OPCs *in vitro* and *in vivo* (Nishiyama et al., 1996a,b, 1997). Consistent with *in vitro* studies (Small et al., 1987), NG2 antibodies progressively stained more optic nerve cells in a chiasmal-to-retinal gradient between P0 and P4. Most NG2-positive cells were elongated (Fig. 1 A) or stellate-shaped (Fig. 1 B). Elongated cells were enriched at the migratory front of the NG2 cell population, always oriented parallel to developing axons, and their processes often terminated in growth cone-like extensions.

The densities of elongated and stellate-shaped NG2 cells were quantified in chiasmal, mid, and retinal thirds of P0-P4 optic nerves (Fig. 1 C). Collectively, there was a greater than sixfold increase in the density of NG2 cells between P0 and P4. In a chiasmal-to-retinal gradient along the developing optic nerve, elongated cells were detected before stellate cells, suggesting that the elongated cells were more immature and migratory. Elongated cells reached an optimal density (\sim 200-300 cells/mm²) in all three regions of the optic nerve at P3. Between P2 and P4, the density of stellate-shaped cells progressively increased at all levels of the nerve, suggesting a transition from elongated to stellate morphology during maturation.

To confirm that the distribution of NG2-positive cells accurately represented the location of oligodendrocyte precursors in the postnatal nerve, isolated explants of chiasmal, mid, and retinal thirds of P0 and P4 nerves were grown *in vitro* for 7 d and assayed for O4-positive cells. O4 recognizes sulphatide and other minor lipid components expressed by oligodendrocytes and some oligodendrocyte progenitors (Warrington and Pfeiffer, 1992). Greater than 96% (34/35) of chiasmal-derived explant contained O4-positive cells at both ages. In contrast, 44% (13/29) of mid and 25% of retinal (14/56) explants contained O4-positive cells in P0 explants, whereas \sim 80% of mid (10/12) and retinal (11/13) explants contained O4-positive cells in P4 explants. These data provide independent support for the migration of NG2-positive oligodendrocyte precursors along the postnatal optic nerve.

Similar Densities of OPCs Develop in Control and Transected Optic Nerves

To investigate whether the absence of axons influenced the developmental appearance, morphology, or density of OPCs, optic nerves were transected near the retina at P0 and analyzed at P1, P2, P3, and P4. The general distribution of NG2 cells was similar in sections from control and transected optic nerves. However, the morphology of NG2 cells was affected by nerve transection. Although NG2 cells could still be divided into two general morphologies, elongated (Fig. 1 D) and stellate (Fig. 1 E), the processes of OPCs were generally shorter than those in control nerve. For example, processes of elongated cells in transected nerves at P1 were significantly ($P < 0.0001$) shorter (66 ± 20 vs. 132 ± 52 μ m) and more tortuous than those in control nerves. Stellate cells in transected nerves extended fewer processes, and surface labeling of perikarya was more irregular (Fig. 1 E). These data suggest that the loss of viable axons and/or pathological changes that follow axonal transection alters the elaboration of normal OPC morphology. To investigate whether axons

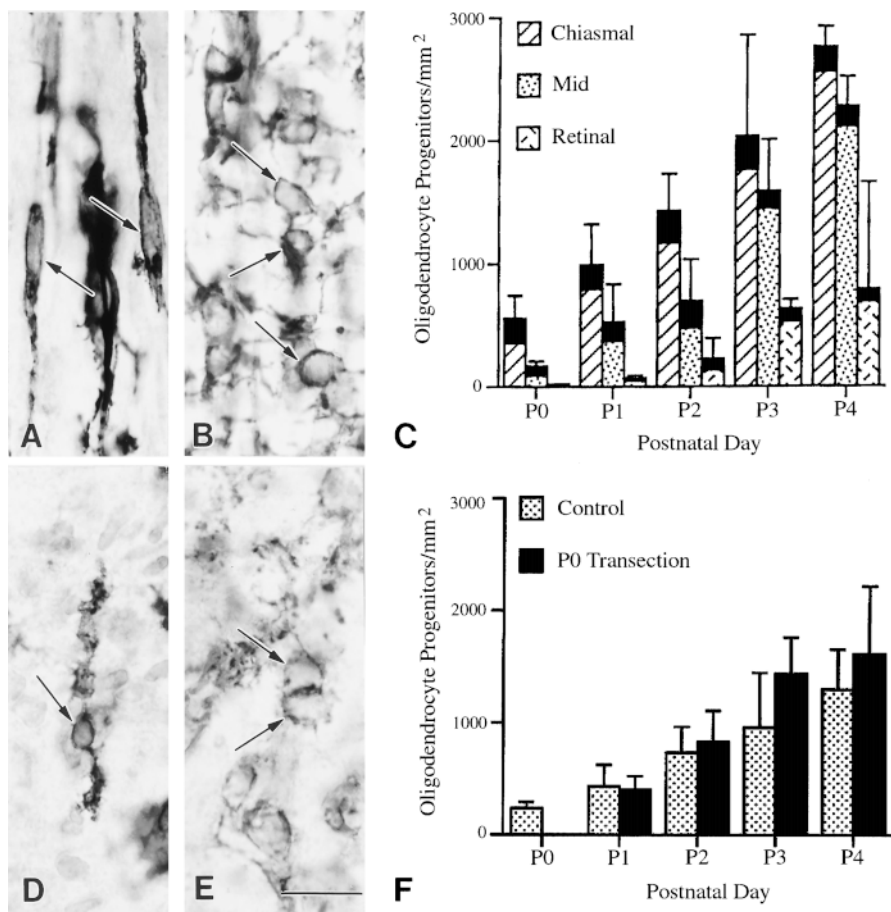


Figure 1. NG2 cells colonize the optic nerve in the absence of viable axons. In control nerves, NG2 cells displayed two morphologies: elongated (A) and stellate-shaped (B). Between P0 and P4, NG2 cells increased in a chiasmatal-to-retinal gradient. Elongated and stellate cells were quantified per mm² of tissue in the chiasmatal, mid, and retinal third of longitudinal sections from nerves at P0, P1, P2, P3, and P4 (C). Between P0 and P3, elongated cells (C, black boxes) were enriched at the leading edge of the migrating front, and their processes were always oriented parallel to developing axons (A). The density of NG2 cells increased sixfold between P0 and P4 (C), and stellate-shaped cells comprised 88% of the total NG2 cells at P4. The morphology and density of NG2 cells were determined following optic nerve transection at P0. Both elongated (D) and stellate-shaped (E) NG2 cells were detected in P1-, P2-, P3-, and P4-transected optic nerves, and NG2 cell distribution and density (F) were similar to those detected in P1-P4 control nerves. However, the processes extending from these NG2 cells were shorter and irregularly shaped. Bar, 20 μ m.

influence OPC density, the number of NG2-positive cells per unit area of tissue was compared in sections from P1, P2, P3, and P4 control and P0-transected optic nerves. At all ages, the density of NG2 cells/mm² of tissue was similar in control and transected optic nerve (Fig. 1 F).

The progressive increase in NG2-positive cells after P0 optic nerve transection suggested that viable axons were not required for OPC proliferation. A BrdU incorporation assay was used to compare levels of NG2 cell proliferation in transected and normal nerves. In P2 control (Fig. 2, A and B) and P2 optic nerves transected at P0 (Fig. 2 C), nuclei of both elongated and stellate NG2 were BrdU-positive after a 2-h pulse. Approximately 25% of all NG2-positive cells were BrdU-positive in both control and transected optic nerves (Fig. 2 D), indicating that viable axons and neuronal electrical activity are not essential for OPC proliferation *in vivo*.

Density and Timing of Oligodendrocyte Development Is Not Affected by Optic Nerve Transection

To investigate the influence of axons on the development of differentiated oligodendrocytes, the distribution and morphology of MBP-positive cells were compared at P5, P6, and P7 in normal and optic nerves transected at P4 (Fig. 3 A). In the normal optic nerve at the time of transection (P4), there was no detectable myelin, and MBP-positive oligodendrocytes were detected only in the chiasmatal

third of the nerve. These oligodendrocytes appeared in clusters of two to three cells, which extended multiple processes radially. 1 d later at P5, there was no detectable myelin, and MBP-positive oligodendrocytes were restricted to the chiasmatal half of control nerves (Fig. 3 A). These cells had a similar appearance to those at P4. In transected optic nerves at P5, there was no detectable myelin, and as in control nerves, the MBP-positive oligodendrocytes were restricted to the chiasmatal half of the nerve (Fig. 3 A). Compared with P5 control nerves, individual oligodendrocytes extended fewer and shorter processes (data not shown).

In control nerves at P6, MBP immunoreactive oligodendrocytes (Fig. 3 A) were present throughout the chiasmatal two thirds of the nerve. Although most MBP-positive cells had the appearance of premyelinating oligodendrocytes and occurred in clusters of two or three cells (Fig. 3 B), some extended processes that ran parallel to axons. In P4-transected nerves analyzed at P6, MBP-positive cells were also detected throughout the chiasmatal two thirds of the nerve (Fig. 3 A). The morphology of these MBP-positive cells differed from those in control nerves in that their processes were shorter and more asymmetrically distributed (Fig. 3 C).

In control nerves at P7, MBP-positive oligodendrocytes were detected along the entire length of the nerves. Myelin was detected along the entire optic nerve, with highest concentrations at the retinal end. The MBP-positive cell

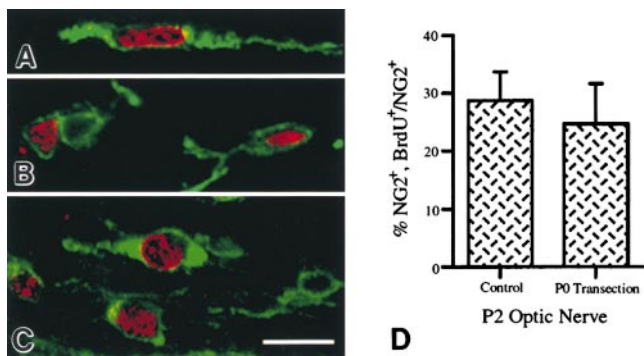


Figure 2. NG2 cell proliferation indices are similar in control and transected optic nerves. After a 90-min BrdU pulse at P2, the percentages of BrdU and NG2-positive cells were compared in control and P0-transected nerves. Sections were analyzed by confocal microscopy to assure precise identification of BrdU-labeled (A–C, red) NG2-positive cells (A–C, green). Both elongated and stellate-shaped NG2 cells incorporated BrdU in control (A and B) and transected (C) nerves. Quantification of NG2 and BrdU-positive cells as a percentage of total NG2 cells detected similar ($P = 0.31$) BrdU-labeling indices in control and transected nerves (D). Bar, 20 μm .

bodies were less clustered at P7 than the premyelinating oligodendrocytes at P6, and were generally located in longitudinally oriented chains parallel to the axons (Fig. 3 D). In P4 transected nerves analyzed at P7, myelin was not detected, although MBP-positive oligodendrocytes were distributed along the entire length of the optic nerve (Fig. 3 A). These oligodendrocytes were frequently clustered without any particular orientation (Fig. 3 E).

To investigate whether axons influence oligodendrocyte density in the optic nerve, the number of MBP-positive cells per unit area of tissue was compared in sections of P5, P6, and P7 control and P4-transected optic nerves (Fig. 3 F). In sections of P4 control nerves, MBP-positive cells were detected at a density of 380 cells/ mm^2 of tissue (Fig. 3 F). 1 d later at P5, the density increased to 700 cells/ mm^2 of tissue. At P5, the density of oligodendrocytes was significantly reduced in P4-transected nerves (361 cells/ mm^2 tissue). Although the overall distribution of oligodendrocytes increased in P6 control nerves, their density (622 cells/ mm^2 tissue) was similar to that observed at P5. At P6, the density of MBP-positive oligodendrocytes was similar in control (621 cells/ mm^2 tissue) and P4-transected (642 cells/ mm^2 tissue) nerves. Between P6 and P7, the density of oligodendrocytes increased and was similar in both control (1,157 cells/ mm^2 tissue) and transected (1,205 cells/ mm^2 tissue) optic nerves.

A consistent observation in transected optic nerves was an apparent reduction in the number and length of processes extending from premyelinating oligodendrocytes. Quantification of premyelinating oligodendrocyte process length in confocal microscopic images of control (Fig. 4 A) and P4-transected (Fig. 4 B) nerves at P6 detected a 50% reduction in the radial extensions of oligodendrocyte processes in the transected nerves (Fig. 4 C). The number of processes was clearly reduced in transected nerves, al-

though quantification was difficult due to the complexity of processes in normal nerves.

Optic Nerve Transection and Oligodendrocyte Cell Death

Cell death of premyelinating oligodendrocytes is a characteristic feature of oligodendrocyte lineage development (Barres et al., 1992; Raff et al., 1993; Trapp et al., 1997). Programmed cell death (PCD) requires the activation of a family of activator and effector cysteine protease enzymes referred to as caspases (Ellis et al., 1991; Alnemri et al., 1996; Thornberry and Lazebnik, 1998). Caspases are expressed as inactive proenzymes. Upon activation, caspases are cleaved into smaller catalytically active subunits. Activated caspase-3 is a downstream effector of PCD in a variety of cell types (Kuida et al., 1996; Cecconi et al., 1998; Namura et al., 1998; Srinivasan et al., 1998), including oligodendrocytes maintained in vitro (Gu et al., 1999). It is expressed as a 32-kD inactive proenzyme. The antibody used in this study selectively recognizes the enzymatically active p18 subunit of caspase-3 (Gu et al., 1999). To investigate whether P4 optic nerve transection influenced the extent of oligodendrocyte PCD, the number of dying oligodendrocytes was compared in sections from P5, P6, and P7 control and P4-transected nerves. Dying premyelinating oligodendrocytes were identified by fragmentation of myelin protein-positive processes, condensed nuclear chromatin, and the presence of activated caspase-3 (Fig. 5, A–C). Double-labeling immunocytochemistry colocalized activated caspase-3 and MBP in sections of control and transected nerves (Fig. 5, A and B). Double-labeled cells had the morphology of premyelinating oligodendrocytes. As described previously (Trapp et al., 1997), most (97%) had fragmented MBP-stained processes (Fig. 5) and condensed nuclear chromatin as detected by propidium iodide staining (data not shown). The remaining 3% of activated caspase-3-positive oligodendrocytes were probably in early stages of PCD before process fragmentation and nuclear chromatin condensation. Occasional caspase-3-positive myelinating oligodendrocytes were detected in control nerves. Activated caspase-3-positive NG2 cells were not detected in either control or transected nerves (data not shown). Activated caspase-3-positive, MBP-negative cells were also detected. These cells did not extend activated caspase-3-positive processes and they were often located next to vessels. At P5, there was a small but significant increase in the proportion of dying oligodendrocytes in transected optic nerves (Fig. 5 D). At P6 and P7, the percentage of activated caspase-3-positive oligodendrocytes was similar in control and transected optic nerves (Fig. 5 D), indicating that the absence of viable axons and axonal electrical activity did not significantly increase the extent of oligodendrocyte cell death, 2 and 3 d after P4 nerve transection.

Oligodendrocyte Lineage Develops throughout the Optic Nerve in the Absence of Axons

The studies described above independently investigated the role of axons in (a) OPC appearance following P0 optic nerve transection; and (b) oligodendrocyte appearance after P4 optic nerve transection. To investigate if OPCs

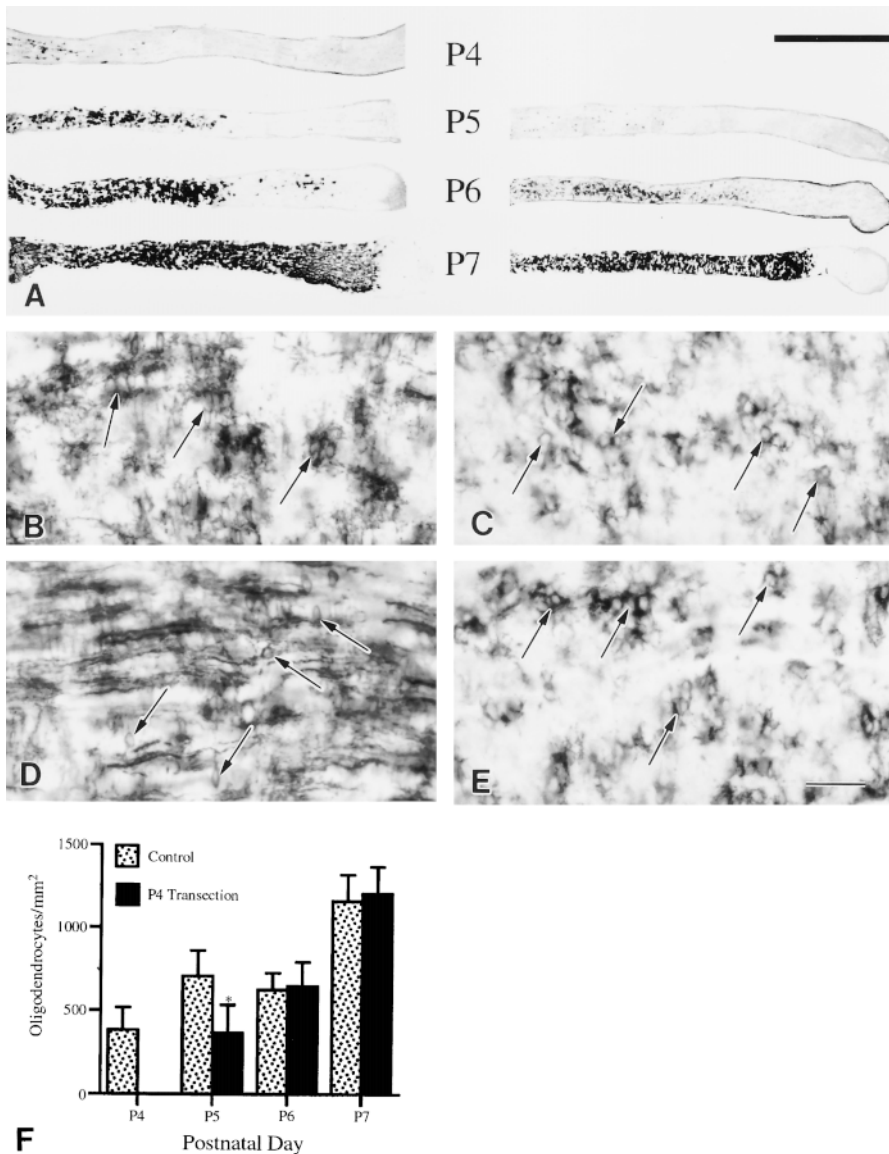


Figure 3. Oligodendrocytes populate the optic nerve in the absence of viable axons. Immunocytochemical detection of MBP-positive oligodendrocytes in sections from control (A, left column) and P4-transected (A, right column) optic nerves at P5, P6, and P7. At the time of transection (P4), oligodendrocytes were restricted to the chiasmal third of the nerve. In P5 control nerves, MBP-positive oligodendrocytes were detected within the chiasmal half of the nerve. P4 transection reduced the number but not the general distribution of MBP-positive cells in P5 nerves. At P6 and P7, the general distribution of MBP-positive oligodendrocytes was similar in control and P4-transected optic nerves. Oligodendrocytes in P6 control nerves (B) and transected nerves (C) occur in clusters of two to three cells. Processes extending from oligodendrocytes appear shorter and less complex in transected nerves at P6. At P7, myelin is abundant in control nerves (D) but not detected in P4-transected (E) nerves. Oligodendrocytes remain in clusters in P7 nerves transected at P4 (E). P4 nerve transection induced a reduction in the density of oligodendrocytes detected at P5, but not at P6 or P7 (F). Bars: 1 mm (A); 50 μ m (B–E). Arrows indicate oligodendrocyte cell bodies. Asterisk in F indicates $P < 0.0001$ based on Fisher's test.

can colonize the optic nerve and produce oligodendrocytes in the absence of viable axons, retinas of newborn (P0) rats were mechanically ablated and the optic nerves analyzed for oligodendrocytes using immunocytochemistry and MBP antibodies 1 wk later at P7. Compared with control nerves (Fig. 6 A), retina ablation at P0 drastically reduced the width of the optic nerve at P7 (Fig. 6 B), but these nerves retained their normal length, and thus provided a substantial substrate for OPC migration. In electron micrographs from midregions of nerves 14 h after P0 retinal ablation, all axons were in advanced stages of degeneration (data not shown). Axonal fragments were detected 24 h after ablation, were rare at 48 h, and undetected at 4 d. The marked decrease in optic nerve volume (Fig. 6 B) also supports total loss of axons in this experimental paradigm. In both control (Fig. 6 A) and P0 retinal-ablated (Fig. 6 B) optic nerves, oligodendrocytes were present along the entire length of the nerve at P7. However, quantification of MBP-positive cells at P7 demonstrated a 50% reduction in the density of oligodendrocytes

(Fig. 6 C) and a total absence of myelination following P0 retinal ablation. These studies establish that OPCs can colonize regions of the optic nerve and produce oligodendrocytes in the absence of viable axons.

Discussion

A prevailing notion suggests that retinal ganglion cell axons play a critical role in several aspects of the development of optic nerve oligodendrocytes (Barres et al., 1992, 1993; Barres and Raff, 1993, 1994; Raff et al., 1993). In this study, we demonstrate that axonal transection inhibited myelin formation, altered the three-dimensional organization of oligodendrocytes, and reduced the number and length of oligodendrocyte processes. However, the temporal and spatial appearance and density of optic nerve OPCs and oligodendrocytes were similar in control and transected optic nerves. These data demonstrated that oligodendrogenesis occurs in the absence of viable retinal ganglion cell axons and argues against direct axonal regu-

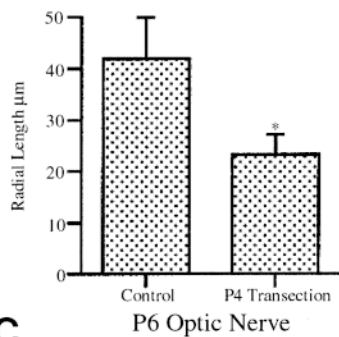
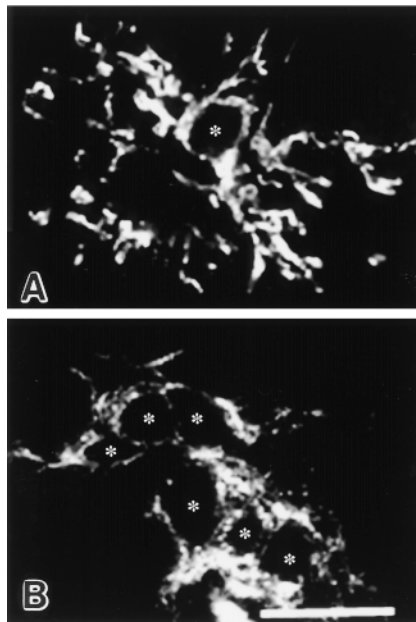


Figure 4. Axonal transection reduces the length and number of premyelinating oligodendrocyte processes. The morphology of premyelinating oligodendrocytes was investigated by confocal microscopy at P6. Premyelinating oligodendrocytes in control sections extended multiple processes that branched extensively (A). Premyelinating oligodendrocytes in transected nerves often occurred in clusters (B) and extend few, short processes. The radial extent of processes was significantly ($*P < 0.0001$) reduced in transected nerves (C). A and B are 1.0- μm -thick optic sections. Asterisks in A and B indicate oligodendrocyte nuclei. Bar, 20 μm .

lations of optic nerve oligodendrogenesis. Because axon-depleted nerves fail to grow radially, they do contain fewer OPCs and oligodendrocytes, indicating that axons indirectly influence oligodendrocyte lineage cell number by increasing optic nerve volume.

Oligodendrocyte Lineage following Optic Nerve Transection

This and previous studies that supported direct axonal regulation of oligodendrocyte lineage development (Barres et al., 1992, 1993; Barres and Raff, 1993) both used optic nerve transection paradigms. However, they diverged in the methods used to identify and quantify OPCs and oligodendrocytes and in the postnatal age of optic nerve transection. Previous studies often used indirect immuno-

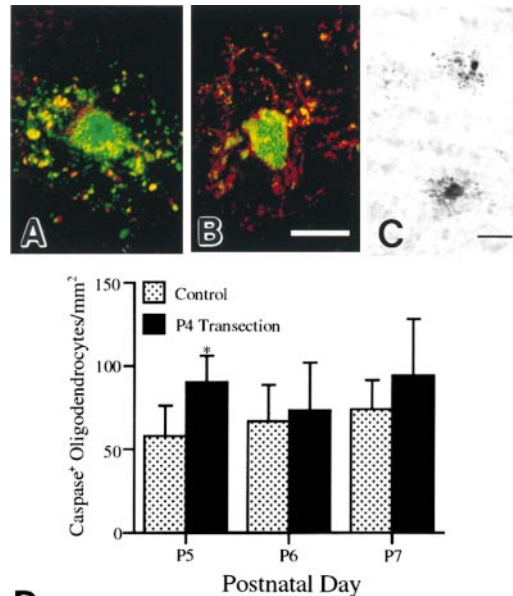


Figure 5. Oligodendrocyte PCD is similar in control and transected optic nerves. Sections of control and transected optic nerves were double-labeled with MBP (red) and caspase-3 (green) antibodies and examined by confocal microscopy (A and B). Caspase-3 immunoreactivity was enriched in the cell body of MBP-positive cells. Most (A) but not all (B) MBP caspase-3-positive cells had fragmented MBP-stained processes. Compared with control nerves, caspase-3-positive cells/mm² of tissue (C) were increased in transected nerves at P5 ($*P < 0.01$), but unchanged at P6 and P7 (D). Bars: 10 μm (A and B); 20 μm (C).

cytochemical techniques (i.e., delivery of antibody *in vivo* by intraventricular transplantation of hybridoma cells) to identify and quantify OPCs and oligodendrocytes, and concluded that loss of viable axons caused decreased OPC proliferation (Barres and Raff, 1993) and increased death of oligodendrocytes (Barres et al., 1993). This study used recently developed (Trapp et al., 1997) immunocytochemical methods to identify and quantify OPCs and oligodendrocytes in fixed sections of control and transected optic nerves. These methods label OPCs and premyelinating oligodendrocytes in a predictable temporal and spatial pattern during brain (Trapp et al., 1997) and optic nerve development, and they establish in this report that the percentage of BrdU-labeled OPCs and dying oligodendrocytes are similar in control and transected optic nerves. The OPC proliferation data may be consistent between the studies, and the differences reported may reflect the denominators used to express the data (total BrdU-positive OPCs in Barres et al., 1993, and Barres and Raff, 1993; percentage of total OPCs in this study). In electron micrographs, all axons were fragmented and in advanced stages of degeneration by 14 h after transection and retinal ablation. Therefore, our studies established that oligodendrocyte production *in vivo* can occur in the absence of viable axons and neuronal electrical activity. Small amounts of axonal debris remained at 24 h after transection, and could have some effect on oligodendrocyte lineage. A similar time course of axonal degeneration was re-

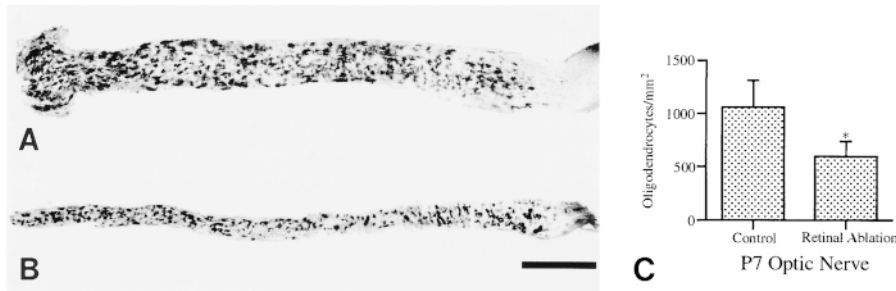


Figure 6. OPCs colonize the entire optic nerve and produce oligodendrocytes in the absence of viable axons. Retinas of rats were surgically ablated at P0, and the distribution and density of MBP-positive oligodendrocytes were determined at P7. Compared with normal P7 nerves (A), the loss of viable axons and neuronal electrical activity at P0 had no effect on the distribution of oligodendrocytes in P7 nerves (B). However, the density of oligodendrocytes per mm² of tissue (C) was significantly reduced (* $P < 0.001$). Bar, 500 μm .

ported in optic nerves transected during the second and third postnatal week (Barres et al., 1992, 1993). Therefore, it is unlikely that axonal debris accounts for significant differences between this and previous studies.

A major objective of this study was to investigate the role of viable axons on OPC colonization of optic nerve and their subsequent differentiation into oligodendrocytes. Past studies supporting axonal regulation of oligodendrocyte lineage analyzed optic nerves transected during the second and third postnatal week. It is possible that oligodendrocyte lineage is differentially regulated during different stages of optic nerve development. However, previous immunocytochemical studies (Kidd et al., 1990) detected normal numbers of oligodendrocytes 5, 10, 20, and 40 d after P21 optic nerve transection. In addition, myelin protein mRNA in these P21-transected optic nerves decreased gradually and depending on the mRNA, representing between 25 and 60% of mRNA levels in control nerves at 40 d after transection. Similarly, two additional studies (McPhilemy et al., 1990; Ludwin, 1990) failed to detect a decrease in oligodendrocytes after P18 or older optic nerve transection. These data indicate that oligodendrocytes do not die after P18 optic nerve transection and/or that new oligodendrocytes are generated. Both events are inconsistent with the hypothesis that viable axons directly regulate oligodendrocyte lineage.

The Migration and Proliferation of Optic Nerve OPCs Do Not Depend on Viable Axons

The distribution and morphology of OPCs in the intact developing optic nerve and the pattern of oligodendrogenesis in explants isolated from P0 and P4 optic nerve were consistent with previous analyses, suggesting that OPCs enter the chiasmal end of the rat optic nerve just before birth and reach confluency along the nerve by P9 (Small et al., 1987). Elongated NG2-positive cells were enriched at the migratory front of the OPCs and had a morphology very similar to the migratory OPCs in the chick optic nerve (Ono et al., 1997). These cells were invariably oriented parallel with the optic axons and their process often terminated in growth cone-like structures. It seems likely that with maturation, some elongate cells develop into stellate cells, since the density of the stellate cells increased significantly in older nerves. The cellular substrates used by migrating OPCs are unknown. While axons

are a candidate for such a substrate (Ono et al., 1997), our P0 retinal ablation study establishes that OPCs can migrate the length of the optic nerve in the absence of viable retinal axons, and migrate after P2 in the absence of detectable axonal fragments. Other potential substrates in the transected nerves include astrocytes and longitudinally oriented blood vessels. Indeed, NG2 cell bodies and the processes of NG2-positive cells frequently terminated near or associated with blood vessels. OPC migration on the extracellular matrix of astrocytes or endothelial cells would support the *in vitro* finding that specific integrin expression may regulate OPC migration (Milner et al., 1996).

Previous studies suggested that OPC proliferation in the developing optic nerve was driven through release of the mitogen PDGF that was dependent on axonal electrical activity (Barres and Raff, 1993). By contrast, this study clearly establishes that viable axons and electrical activity are not essential for the mitosis of OPCs and for expansion of oligodendrocyte lineage cells in the optic nerve. Our data are consistent with the normal myelination in P9 optic nerves following intraocular injection of tetrodotoxin every 2 d starting at P0 (Colello et al., 1995). As suggested previously, our data support mitogenic stimulation of OPC proliferation by factors released from astrocytes and/or endothelial cells. Astrocytes are a major source of PDGF (Raff et al., 1988), and conditioned medium from purified cultured type I astrocytes is a strong mitogen for purified OPCs (Noble and Murray, 1984).

The Differentiation of Oligodendrocytes Is Not Dependent on Optic Axons

In the normal rat optic nerve, *in situ* hybridization studies of myelin protein mRNA (Colello et al., 1995) and the distribution of MBP (Fig. 3) describe a chiasmal-to-retinal gradient in the developmental appearance of oligodendrocytes. However, myelination proceeds in a retinal-to-chiasmal gradient (Skoff et al., 1980; Colello et al., 1995), suggesting that oligodendrocyte maturation and the induction of myelination are independently regulated. Oligodendrocytes mature to the MBP-positive stage but fail to initiate myelination in the axon-free environment. Thus, the early stages of oligodendrocyte development can occur independently of direct axonal signals, but myelination is critically dependent on axons. Axonal control of myelination is further supported by the differences we observed in the

apparent life span of premyelinating oligodendrocytes at the retinal and chiasmal ends of the normal optic nerve. Oligodendrocytes in the chiasmal end of the optic nerve do not myelinate for at least 2–3 d after their initial detection by MBP antibodies, whereas oligodendrocytes at the retinal end of the nerve can myelinate within 24 h or less.

It has been proposed that survival of premyelinating oligodendrocytes is determined by a competition for axonally derived trophic signals, and cells that do not receive such signals within 2–3 d initiate a suicide program (Barres et al., 1992, 1993; Barres and Raff, 1993, 1994; Trapp et al., 1997). The distribution of dying premyelinating oligodendrocytes along the length of the developing normal optic nerve suggests that there is not a defined interval between generation of premyelinating cells and the induction of PCD. Rather, it may be that oligodendrocyte PCD in normal optic nerves is induced by positive death signals or failed development. P4 axonal transection caused a reduction of oligodendrocyte density and a slight increase in the percentage of oligodendrocytes undergoing PCD at P5. However, 2 and 3 d after P4 optic nerve transection, the density of oligodendrocytes and percentage of oligodendrocytes undergoing PCD in control and transected nerves were similar. These data argue against the hypothesis that oligodendrocyte survival is primarily dictated by limiting quantities of axonally derived survival factors.

Regulation of Oligodendrocyte Numbers and Myelination in Optic Nerve

The number of optic nerve myelin internodes formed is precisely matched to the number of axons requiring myelination. How such matching is achieved is unclear. One hypothesis is that the number of axons directly regulates the number of oligodendrocytes (Barres and Raff, 1994). Consistent with this notion, when the number of axons is increased in the optic nerve, oligodendrocyte numbers increased proportionally (Burne et al., 1996). Our data suggest that this simple correlation is incomplete and support an alternative mechanism. In axon-free transected nerves, OPC and oligodendrocyte densities are similar to control nerves, but their total numbers are decreased because the nerve fails to grow radially due to loss of axons and failed myelination. Since oligodendrocyte lineage cell density is regulated by a homotypic density-dependent inhibition of OPC proliferation in vitro (Zhang and Miller, 1996), one possible mechanism operating in developing optic nerves is that the OPCs proliferate until they reach a critical density. Increasing or decreasing the size of the nerve through addition or loss of axons will change the potential area, and thus the number of OPCs and oligodendrocytes, but does not necessarily articulate direct axonal regulation of oligodendrocyte number.

Retinal axons are required for the generation of the optic nerve during early development and they may directly regulate type I astrocyte colonization of the optic nerve, as astrocytes fail to develop in the optic stalk of mice that do not form retinas (Robb et al., 1978). This study indicates that endothelial cells or the astrocytes which bundle groups of developing axons provide sufficient substrates for colonization of optic nerves by OPCs, for proliferation of OPCs, and for production of oligodendrocytes. Endo-

thelial cells have received little attention as a regulator of oligodendrocyte lineage. Closure of the blood–brain barrier mimics the in vitro conditions (no serum) that drives oligodendrocyte production from O2A cells (Raff et al., 1983). Astrocyte–endothelial interactions may regulate oligodendrocyte lineage by modulating astrocyte expression of mitotic, chemotactic, and survival factors and by restricting inhibitory serum factors following blood–brain barrier closure. If axons indirectly regulate oligodendrocyte numbers via type I astrocytes, our data demonstrate that this axonal influence occurs before P1, and viable axons need not be present at the time of OPC migration or oligodendrocyte production.

The faithful match between myelin production and axonal length argues for axonal control of myelination. This study seriously questions direct axonal regulation of oligodendrocyte number during early stages of optic nerve development, and raises the alternative possibility that axons regulate the number of myelin internodes formed by individual oligodendrocytes. In support of this hypothesis, premyelinating oligodendrocytes in the corpus callosum and optic nerve extend many processes and form 30–40 thin, short myelin internodes (Trapp et al., 1997). Premyelinating oligodendrocytes in the region of cerebral cortex and spinal cord extend fewer, longer processes and form few, thick long myelin internodes. Therefore, developing axons may directly regulate premyelinating oligodendrocyte process number, and in turn, myelin internode number, thereby assuring an appropriate match between oligodendrocyte number and myelination. This matching may reflect differences in the electrical activity of developing fiber tracks, or molecules specific to or present at varying concentrations on different populations of developing axons.

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