The Chronology of Oligodendrocyte Differentiation in the Rat Optic Nerve: Evidence for a Signaling Step Initiating Myelination in the CNS

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In order to determine the signals that initiate axon myelination in the CNS, we have chronicled the differentiation of oligodendrocytes in the rat optic nerve and related this to the time course and spatial gradient seen for optic axon myelination. By using markers specific to the varying stages of oligodendrocyte differentiation we found that oligodendrocyte progenitor cells, present throughout the length of the nerve at postnatal day 2, mature into GC+ oligodendrocytes in a chiasm to eye progression. This gradient along the nerve of oligodendrocyte differentiation continues with oligodendrocytes near the chiasm expressing the genes and encoded proteins to MBP and PLP 3 d before oligodendrocytes near the eye. Although oligodendrocyte differentiation and maturation occurs in a chiasm to eye gradient along the nerve, optic axon segments near the eye are ensheathed with myelin before segments near the chiasm. This suggests that the myelination of optic axons is initiated by a signaling step that is independent of oligodendrocyte differentiation and is stronger near the eye than the chiasm region of the nerve. By examining proposed axonal signals, we found that the onset of myelination is independent of the electrical activity of an axon but can be correlated to the size of an axon.

[Key words: myelination, oligodendrocyte, retinofugal pathway, optic axons, myelin basic protein, proteolipid protein]

The myelination of axons by oligodendrocytes is of vital importance to the function of the CNS. By insulating an axon with a myelin sheath, the oligodendrocyte greatly enhances the speed at which action potentials propagate along the axon. In the absence of this sheath, as seen in demyelinating diseases, impulse conduction is impeded resulting in severe sensory and motor deficits (Lisak, 1986; Compston et al., 1991).

Although much is known about the structure and function of the proteins that make up the myelin sheath (Campagnoni, 1988;

Mikoshiba et al., 1991) and when, during oligodendrocyte differentiation, they become expressed (Bologa-Sandru et al., 1981; Hartman et al., 1982; Pfeiffer, 1984), it is not clear what initiates oligodendrocytes to ensheath axons. Through *in vitro* studies, it has been shown that oligodendrocytes can express myelin-associated proteins (Mirsky et al., 1980; Zeller et al., 1985; Dubois-Dalcq et al., 1986) and elaborate myelin membrane (Bradel and Prince, 1983) in the absence of neurons. These results suggest that the onset of myelination may be independent of a neuronal signal and simply the consequence of a constitutive pathway of oligodendrocyte differentiation and maturation. However, the specificity of myelin formation to axons (Lubetzki et al., 1993) and its occurrence during a precise period of fiber pathway maturation (Morell, 1984) suggest that axons exert an important regulatory function in the initiation of this event.

In an attempt to determine whether the onset of myelination is solely the consequence of oligodendrocyte maturation or dependent on an axonal signal, oligodendrocyte differentiation was chronicled in the rat optic nerve and related to the time course of optic axon myelination. In the rat optic nerve, the ensheathment of axons has been described to occur in a rostro-caudal gradient, that is, from eye to chiasm (Skoff, 1978; Skoff et al., 1980). This suggests that either oligodendrocyte differentiation occurs in a similarly graded fashion along the nerve prior to myelination, or that if it occurs in a dissimilar manner, an additional graded signal, which is strongest near the eye, must initiate myelination.

We now show that within the developing rat optic nerve, oligodendrocyte differentiation, as shown by the expression of two major myelin genes and their encoded proteins, occurs in a distinct chiasm-to-eye gradient along the nerve. Myelin formation, however, occurs many days later in a reverse gradient (eye-to-chiasm) suggesting that oligodendrocyte differentiation cannot be the sole criterion for initiating myelination. Finally, we found that myelin formation is independent of the electrical activity of an axon but its onset is correlated to the diameter of an axon.

Materials and Methods

Lewis albino rats were used throughout this study. In order to determine the precise postnatal age of each pup, pregnant rats were examined every 8–10 hr and the day of birth was counted as postnatal day (P) 0.

Riboprobe synthesis. A 0.5 kb XhoII restriction fragment derived from the coding region of a rat myelin basic protein (MBP) cDNA clone (Pott, unpublished result) was subcloned into the BamHI site of p-BKSIIplus (Stratagene). A 0.8 kb BamHI/PstI restriction fragment from the coding region of a rat Proteolipid protein (PLP) cDNA clone (Milner et al., 1985) was subcloned into BamHI/PstI cleaved pBKSIIplus. The constructs were linearized with NotI, and Digoxigenin or Fluorescein

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labeled antisense run-off transcripts were synthesized from the vector's T3 promoter using the Boehringer Mannheim RNA labeling kit according to instruction.

In situ hybridization. Rat pups from each of the following age groups, P2, 3, 4, 5, and 6, from four litters, were ether-anesthetized, decapitated and the optic nerves and chiasm dissected free. For each animal, the nerves were left attached to the chiasm and frozen for cutting on a cryostat. Longitudinal sections (15 μ m) were collected and fixed onto organosilane-coated slides with 4% paraformaldehyde. The sections were subsequently washed with phosphate-buffered saline, acetylated with 0.1 M triethanolamine and 0.25% acetic anhydride and prehybridized for 3 hr at room temperature in 50% formamide, 5 $\times$ SSPE, 5 $\times$ Denhardt's mix, 250 μ g/ml yeast RNA, and 100 μ g/ml herring sperm DNA. Hybridization was done overnight at 70°C in the same solution containing 100 ng/ml of the MBP or PLP antisense riboprobes. After two 30 min washes in 0.2 $\times$ SSPE at 68°C the hybridized probe was detected with an alkaline phosphatase coupled anti-digoxigenin or anti-fluorescein antibody. The phosphatase reaction with the color reagents NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) was run for 1–3 hr in the dark. After the reaction had been stopped with 10 mM Tris-Cl and 20 mM EDTA, pH 7.8, the sections were washed in dH₂O, air dried and photographed with a Zeiss microscope.

MBP and PLP immunostaining. Optic nerves from rat pups ($n = 4$) aged P2–6 were longitudinally cut with a cryostat, collected on gelatinized slides, fixed with 4% paraformaldehyde and immunostained for MBP and PLP as previously described (Colello et al., 1994). Briefly, sections were ethanol/acetic acid treated, rehydrated, blocked with 5% serum albumin, and incubated overnight with either a 1:500 diluted mouse monoclonal antibody against MBP (Boehringer Mannheim, Germany) or 1:1000 diluted mouse monoclonal antibody against PLP (gift of Dr. K. Nave, Heidelberg, Germany). The primary antibody was detected with a biotinylated anti-mouse secondary antibody (Vector) which was subsequently localized using the avidin–biotin–peroxidase complex (Vector, Burlingame, CA).

Electron microscopy. Rat pups aged P6–9 were ether-anesthetized and intracardially perfused with 100 ml of a solution of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (PO₄), pH 7.4. Upon decapitation and removal of the lower jaw, the optic nerves were cut free from the eye at the eye cup/optic stalk junction and cut free from the diencephalon at the chiasmatic region. The dissected optic nerves were postfixed overnight in primary fixative, rinsed in 0.1 PO₄, immersed for 2 hr in 2% osmium tetroxide, dehydrated, block stained in uranyl acetate, and embedded in epon/araldite resin. This procedure was similarly repeated on the optic nerves of P9 rat pups given intracocular injections of tetrodotoxin as described below.

The total number of myelinated axons along the optic nerve of P7 and P9 rat pups ($n = 3$ for each age group) was determined from transverse thin-sections (90 nm) of EM embedded nerves taken every 0.5 mm along the nerve. The entire cross-sectional area of the optic nerve was examined for myelinated axons (at 5000 $\times$) and any axon surrounded by at least two myelin lamellae was counted as a myelinating fiber.

The diameter of the axon population within P6 rat optic nerves ($n = 3$) was determined at two locations: 500 μ m and 3.5 mm from the eye cup in a similar method as that previously described (Colello et al., 1994). Briefly, 16 photomicrographs (5000 $\times$) were taken systematically along the areal extent of the optic nerve at these levels from transverse thin sections. Measurements were taken of all the axons underlying the intersection of a 10 by 10 grid placed over each photomicrograph of the nerve using a digitizing tablet and SIGMASCAN software.

The axon diameter when oligodendrocytes begin ensheathment was determined by measuring 100 axons in a P8 optic nerve prepared for EM. Only axons showing three-quarters to one whole turn of myelin were measured. Three P8 optic nerves were used in this analysis.

Intraocular tetrodotoxin injections. As previously described (O'Leary et al., 1986), newborn rats ($n = 3$) were ice-anesthetized and intraocularly injected with 0.4 μ l of a 1 mg/ml (3.1×10^{-3} M) solution of TTX (Sigma) using a 1 μ l Hamilton syringe. Three control animals were intraocularly injected with an equal amount of citrate buffer, the solution used to dissolve the TTX. This injection procedure was repeated on alternate days (animals were ether-anesthetized beyond P4) with the dosage increasing to 0.8 μ l by P8. The efficacy of the TTX injection was confirmed for animals aged P6 and older by the subse-

quent loss of pupillary response (Radel et al., 1992). The results are shown as means $\pm$ SEM.

Cell culture. Optic nerves from eight P2 rat pups were dissected into three equal segments (eye, middle, chiasm) and segments of each group were pooled in DMEM plus 10% FCS. Each pool was dissociated in 250 μ l Hanks' solution containing 1% collagenase and 0.2% trypsin for 30 min at 37°C. Dissociated cells were plated onto poly-L-lysine coated coverslips in DMEM/10% FCS for 30 min. The medium was replaced with serum-free defined DMEM (Small et al., 1987) and the cultured cells incubated for 4 hr in a 5% CO₂ incubator. Cells identified as progenitors (A2B5+) were immunostained in the following manner: cells attached to coverslips were prefixed in phosphate-buffered 2% paraformaldehyde for 10 min at room temperature (rt). Primary antibody was applied as an undiluted hybridoma supernatant for 30 min at rt, and then visualized with a secondary goat anti-mouse RITC (Jackson, Immunoresearch). In a separate group of eight P2 rat pups, optic nerves were again dissected into three equal segments, dissociated and plated in the manner above. Cells were fixed and subsequently identified as differentiated oligodendrocytes using anti-O1 primary as an undiluted hybridoma supernatant and visualized with a secondary goat anti-mouse RITC. Cells for each segment were plated onto three coverslips and 1000 cells per coverslip were assayed for fluorescent labeling from numerous fields.

Results

In order to understand the sequence of events leading to the myelination of optic axons, the differentiation of oligodendrocytes was chronicled for the rat optic nerve. In particular, mature, premyelinating oligodendrocytes were identified within the optic nerve using riboprobes to the mRNAs for two myelin proteins, myelin basic protein (MBP) and proteolipid protein (PLP). Earlier stages of oligodendrocyte differentiation were identified using the following antibodies: A2B5, an antibody that recognizes cell surface gangliosides found on O2-A oligodendrocyte progenitor cells (Eisenbarth et al., 1979; Abney et al., 1983) and O1, an antibody that recognizes galactocerebroside expressed by early differentiated oligodendrocytes (Schachner et al., 1981). Fully mature oligodendrocytes were identified with anti-MBP and anti-PLP, antibodies recognizing myelin basic protein (Sternberger et al., 1978) and proteolipid protein (Agrawal et al., 1977), respectively. Finally, axons within the optic nerve were examined at the ultramicroscopic level to determine when myelination begins.

Expression of mRNAs for myelin proteins

To determine the progress of oligodendrocyte differentiation in the optic nerve prior to myelination, cryostat sections of nerve from the postnatal days (P) 2–6 were hybridized with riboprobes to MBP or PLP mRNAs.

The most striking observation in these nerves was that oligodendrocytes expressing MBP- and PLP mRNA were distributed in a strikingly graded fashion along the nerve (Fig. 1). Beginning at P3, MBP mRNA-positive cells were found restricted to the chiasmatic end of the nerve. During P4, cells expressing MBP mRNA were found throughout the intracranial half of the nerve with no positive cells located nearer the eye. This staining pattern continues through P5 with more cells in the midnerve becoming MBP mRNA positive. Interestingly, optic nerves examined between P5 and P6 contained a population of cells lying on the intraorbital side of the optic foramen that began expressing MBP and PLP mRNA before cells lying in regions of the nerve within the optic foramen (Fig. 1, arrow-head).

At P6, MBP mRNA expression was found in cells throughout the length of the nerve, except in the region of the lamina cribrosa (an area devoid of oligodendrocytes, Hernandez et al.,

Figure 1. Longitudinal cryostat sections (15 μ m) of Lewis rat optic nerves at varying stages of development hybridized with a digoxigenin-labeled myelin basic protein (MBP) antisense riboprobe. The far right section, taken from a P5 rat optic nerve, was hybridized with a proteolipid protein (PLP) antisense riboprobe. Notice the clear chiasm-to-eye gradient of cells expressing MBP-mRNA (from P3–P6) and PLP-mRNA (P5). Interestingly, cells within the intracranial segment of the nerve (arrowhead) begin to express these genes before cells lying adjacent to the optic foramen (arrow). Scale bar, 1 mm.

1989). Furthermore, at this stage fewer positive cells were observed in a region of the nerve adjacent to the optic foramen (Fig. 1, arrow). The mRNA of proteolipid protein, a lipoprotein of myelin (Agrawal et al., 1977), was also found to be expressed at approximately the same time and in a similarly graded fashion as MBP mRNA (Fig. 1).

Expression of myelin basic protein and proteolipid protein

To determine if MBP and PLP are expressed in a similarly graded fashion as their mRNA, nerves were immunostained for these proteins. Prior to P4, MBP and PLP were undetectable in any region of the nerve (Fig. 2). At P4, however, cells expressing MBP and PLP were distributed in a clearly graded fashion, similar to the mRNA at P3, with staining restricted to the intracranial nerve segment (Fig. 2). This staining pattern continues through P5 and P6 with more cells in the midnerve becoming positively stained. By P7 (not shown), MBP staining was found throughout the nerve, excluding the region of the lamina cribrosa. Proteolipid protein showed a similar extent of staining in the nerve as MBP for all the ages examined (Fig. 2, far right).

Extent of progenitor migration and oligodendrocyte differentiation within the postnatal optic nerve

As the cells destined to become optic nerve oligodendrocytes, the O-2A progenitors (Raff et al., 1983), migrate into the nerve from the chiasm end (Small et al., 1987), one explanation for the graded expression of MBP and PLP mRNA within the nerve is that these cells have yet to migrate the full length of the nerve prior to P6.

To determine the extent of O2-A progenitor migration, cells from three segments of P2 optic nerves were isolated and stained with A2B5 antibody, a marker specific to O2-A progenitor cells,

and O1 antibody, a marker specific to early differentiated oligodendrocytes (Table 1). The results from these experiments show that, prior to the expression of MBP mRNA in the nerve, O-2A progenitor cells, and to a lesser extent O1 positive cells, are found throughout the length of the nerve. The numbers of A2B5 labeled cells in each segment of the P2 nerve, decreasing in number from the chiasm to the eye, is in agreement with the findings of Small et al., (1987) for the Sprague–Dawley rat. Further, the decreasing numbers of O1-labeled cells from the chiasm to eye suggest that progenitors near the chiasm are the first to differentiate into oligodendrocytes.

Axon ensheathment within the optic nerve

The presence of a chiasm to eye gradient in the numbers of early differentiated oligodendrocytes, followed by the transcription and translation of myelin proteins in a similarly graded fashion, suggests that oligodendrocytes near the chiasm are further advanced through differentiation than oligodendrocytes near the eye. Axon segments within the chiasmatic region of the nerve should myelinate before segments near the eye if the onset of myelination follows, in succession, oligodendrocyte differentiation and maturation, and is not the result of an additional signaling step.

To determine when and where myelination is initiated, and whether this event occurs in a graded fashion similar to MBP expression, we looked for the presence of myelinating axons during the developmental period when MBP was expressed.

At the electron microscopic (EM) level, we found that myelination begins at about P7, with compact myelin being found predominantly around axons in the intraorbital segment of the nerve (Fig. 3a). In each of the three nerves examined there is a clear gradient, along their length, in the number of myelinating

Figure 2. Longitudinal cryostat sections (15 μ m) of Lewis rat optic nerves at varying stages of development immunostained for MBP. The far right section, taken from a P5 rat optic nerve, was immunostained for PLP. Notice the clear chiasm-to-eye gradient of cells expression both MBP and PLP protein. Scale bar, 1 mm.

fibers. Surprisingly, this gradient was inverse to the gradient of oligodendrocyte differentiation: there were nearly 10-fold more axons myelinated near the eye as compared with axons at the chiasm end of the nerve. Although each nerve examined showed the presence of "peaks and troughs" of numbers of myelinating axons along its length, an unmistakable gradient of axon ensheathment was found in all cases. This gradient of myelination was maintained in P9 optic nerves (Fig. 3*b*) with nearly fourfold more axons near the eye being myelinated than axons near the chiasm. Again, the presence of "peaks and troughs" in the number of myelinated axons was observed for all three P9 nerves.

By comparing the time course of axon myelination with that of myelin gene expression and translation, for oligodendrocytes at both ends of the optic nerve, it becomes clear that these events do not emerge, one after the other, in the same timely fashion for oligodendrocytes in every part of the nerve (Fig. 4). Rather, oligodendrocytes lying near the chiasm have a longer delay between protein production (MBP and PLP) and myelination than those near the eye.

Evaluating proposed axon signals for initiating myelination

The fact that axon ensheathment occurs many days after, and in a reverse gradient to, oligodendrocyte differentiation supports

the notion that axon-associated signals initiate myelination. From activity studies it has been suggested that the electrical state of an axon may play a role in triggering myelination (Gyllenstein et al., 1963; Tauber et al., 1980). Alternatively, measurements of axons at the EM level support the notion that a minimum diameter of an axon is needed in order to initiate myelination (Duncan, 1934; Peters and Vaughn, 1970; Voyvodic, 1989).

In order to determine whether the physiological state of a neuron and its axon plays a role in initiating myelination in the optic nerve, electrical activity was blocked throughout the first nine postnatal days and optic nerve myelination assessed. Results from a group of three P9 rat pups given intraocular injections of either tetrodotoxin (TTX) or a control buffer beginning at birth showed that the number of axons becoming myelinated in nerves without electrical activity was similar to the numbers found in normal, active nerves (Fig. 5). These results suggest that optic axon myelination is independent of normal electrical activity.

In order to determine whether the physical size of an axon, its diameter, can be correlated to when myelination is initiated, optic axons were measured at two locations within three P6 optic nerves prior to myelination. These locations, the retinal and chiasm ends of the optic nerve, represent the parts of the nerve that are first and last to myelinate, respectively.

By determining the diameters of the axon population near the eye and the chiasm prior to myelination, we found that axons are noticeably larger near the eye (Fig. 6). This quantification becomes more meaningful when compared to our measurements on the average axon diameter when myelination commences (Fig. 6, dashed line). Prior to myelination, nearly 14% of the axon population near the eye is greater than or equal to the average diameter when axons become ensheathed. Only 2% of the axon population near the chiasm are of that size. These re-

Table 1. Number of progenitor cells and differentiated oligodendrocytes within the optic nerve segments of P2 Lewis rats (per 1000 cells)

Segment	A2B5+	01+
Eye	101 $\pm$ 10	77 $\pm$ 2
Middle	222 $\pm$ 12	154 $\pm$ 5
Chiasm	548 $\pm$ 46	358 $\pm$ 11

Values are the mean number of labeled cells $\pm$ SEM.

Figure 3. Total counts of myelinating axons along the optic nerve of three P7 (*a*) and three P9 (*b*) Lewis rat pups. Myelinating axons numbers were determined at the electron microscopic (EM) level from transverse sections taken every 0.5 mm along the nerve. An axon surrounded by at least two myelin lamellae was counted as a myelinating fiber.

Figure 5. The number of myelinated axons in the optic nerve of three P9 Lewis rats given intraocular injections of either tetrodotoxin (TTX) or a control buffer (normal) on alternate days beginning with the day of birth. Counts were taken in region of the nerve half-way between the eye and the chiasm. These results are shown as means $\pm$ SEM.

sults are consistent with the notion that the onset of myelination is correlated to the size of an axon.

Discussion

From the results above, it can be seen that oligodendrocytes will run their normal course of maturation but require additional signals to establish the axonal/glia interactions that underlie their myelinating function. The notion that oligodendrocyte differentiation and axon myelination are linked by an intermediate signaling step is demonstrated by the inverse gradient by which they occur. Interestingly, oligodendrocytes near the chiasm express MBP and PLP up to 4 d before they elaborate myelin, whereas oligodendrocytes near the eye make myelin within 24 hr of protein production. These results suggest that oligodendrocyte progenitor cells located near the eye advance through the stages of differentiation and myelin production at a faster rate than similar cells located near the chiasm. The presence of a graded signal in the nerve, which drives oligodendrocytes to maturity, would account for these differences.

Figure 4. Time course of MBP mRNA and protein expression for oligodendrocytes lying near the orbital or chiasmatic end of the optic nerve in relation to the onset of axon myelination in these regions. All events continue beyond postnatal day 8.

Figure 6. Histogram showing the diameters (μm) of the axon population within the P6 rat optic nerve at 500 μm (*a*) and 3.5 mm (*b*) from the eye cup. These results were obtained from three animals and shown as means $\pm$ SEM. The dashed line indicates the average axon diameter when oligodendrocytes begin ensheathment.

From the results described in this study, taken with those from previous studies, a model of optic nerve myelination begins to emerge: oligodendrocyte progenitor cells enter the optic nerve prenatally from the chiasmatic end of the nerve (Small et al., 1987). The factor(s) that influences their directional migration into the nerve are unknown but they may be related to some property of the recently arriving optic axons. Indeed, rat retinal ganglion cells are physiologically active at the time of progenitor cell migration (Galli and Maffei, 1988). Activity-induced growth factor release by already present Type I astrocytes (Barres et al., 1993) could establish a molecular gradient for progenitor cell migration. Alternatively, the presence of favorable substrate proteins found on the surface of early axons (see review, Hynes and Lander, 1992) could act to guide progenitor cells into the nerve. Since oligodendrocyte progenitor cells are found throughout the length of the nerve by E17 (Small et al., 1987) and the first to differentiate are found nearest the chiasm (above results), this would suggest that the optic nerve oligodendrocyte population is set up by an early "wave" of mitotic cells moving towards the orbit, with postmitotic cells (differentiated oligodendrocytes) dropping out of this population to take up positions, one after the other, in a chiasm-to-eye arrangement. Upon differentiation, oligodendrocytes begin to express myelin protein genes at a time similar to that observed for oligodendrocytes grown *in vitro* in the absence of neurons (Zeller et al., 1985; Dubois-Dalcq et al., 1987). This suggests the presence of an intrinsic program for myelin gene expression, which runs independent of neuronal influences. The subsequent translation of myelin genes into protein occurs with the same time delay for

oligodendrocytes located in any part of the nerve, suggesting that this event may also be part of an intrinsic oligodendrocyte maturation program (see also Mirsky et al., 1980). However, the event of myelination appears to be regulated by an extrinsic signal and not part of this intrinsic oligodendrocyte program. This is evident by the difference in time delay between myelin protein production and myelination for oligodendrocytes lying near the chiasm as opposed to oligodendrocytes lying near the eye. This suggests the presence, at approximately P6–7, of an as of yet unknown myelin initiation signal, which is graded along the nerve and strongest near the eye. This signal, by, presumably, hastening the maturation of oligodendrocytes near the eye, enables these cells to myelinate first.

In an attempt to determine the nature of this signal we have evaluated the role of two previously proposed signals, the electrical state of the axon (Gyllenstein et al., 1963; Tauber et al., 1980) and its size (Duncan, 1934; Peter and Vaughn, 1970), in initiating myelination. We found that when electrical activity was blocked with intraocular injections of TTX, the onset of myelination proceeds normally. This observation argues against the hypothesis that the physiological state of an axon initiates myelination. Although this result appears, at first, to be contrary to the findings made in dark-rearing and early eye-opening experiments (Gyllenstein et al., 1963; Tauber et al., 1980), the differences can be addressed by examining the effect of electrical activity on oligodendrocyte progenitor cells. In the presence of electrical activity, progenitor cells are stimulated to proliferate, thereby increasing the number of oligodendrocytes that develop in the optic nerve (Barres and Raff, 1993). As retinal ganglion

cells become electrically active from E16 onward (Galli and Maffei, 1988), progenitor cells present in the nerve at this time would begin to proliferate. Our first TTX injection (at P0) would block electrical activity in the nerve and prevent further progenitor cell proliferation. Nevertheless, the progenitor cells already in the nerve would continue to differentiate in the absence of axonal activity (as described above) and would be able to ensheath axons at the time when a myelin-initiation signal is presented. An examination of activity-blocked nerves past the age described in our study may show that myelination levels are lower than in normal, active nerves due to a lower number of oligodendrocytes being present. Indeed, the number of myelinated axons present in the optic nerve of dark-reared mice past P20 is reduced in number from that found in normally reared mice (Gyllenstein et al., 1963). By contrast, early eye opening experiments have been shown to increase the level of myelin protein expression within the optic nerve (Tauber et al., 1980), presumably by increasing the degree of electrical activity and, therefore, the number of progenitor cells.

By measuring the diameter of optic axons in nerves prior to myelination, we found that a proportion of axons near the eye were larger than any axons found near the chiasm. As axon segments near the eye myelinate before segments near the chiasm, these results are in agreement with the notion that a minimum axon diameter is required to initiate myelination (Duncan, 1934; Peters and Vaughn, 1970). This would be similar to what has been found for PNS axons, where the axon caliber appears to be the crucial determinant of whether an axon becomes myelinated (Voyvodic, 1989).

Although it is possible that the diameter of an axon, alone, can initiate myelination, it is more likely that size reflects a level of maturation of the axon when new factors, which initiate myelination, are being expressed. Indeed, ultrastructural studies suggest fibers undergo membrane remodeling prior to myelination (Hildebrand and Waxman, 1984). Interestingly, in oligodendrocytes a signaling pathway involving myelin-associated glycoprotein, MAG, and Fyn tyrosine kinase has recently been established (Umemori et al., 1994). This pathway, which is activated during the initial stages of myelination, may be involved in transducing the axonal signal to the glial cell. Moreover, Notterpek and Rome (1994) have recently isolated a monoclonal antibody directed against the axolemma, which repeatedly blocks myelination in cerebellar cultures. Although the antigen has not been fully characterized, their results suggest an involvement of axons in CNS myelination. Finally, it is possible that increasing the size of an axon may dilute an already present inhibitory signal for myelination below the threshold for functional activity. Interestingly, the cell surface protein, M6, which inhibits neurite outgrowth in culture, loses its expression in the mouse optic nerve in an eye-to-chiasm gradient prior to axon myelination (Lund et al., 1986). Whether or not this protein also inhibits optic axon myelination remains to be addressed.

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