Original Paper

Developmental Neuroscience

Dev Neurosci 2001;23:327–337

Received: June 21, 2001
Accepted: June 25, 2001

Growth Factor Control of CNS Myelination

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Key Words

Leukemia inhibitory factor · Myelination · Neuregulin · Oligodendrocyte development · Platelet-derived growth factor · T_3

Abstract

The molecular signals required for initiating myelination and maintenance of the myelin internode are not known. Several growth factor families have been implicated in promoting oligodendrocyte survival or differentiation and may have consequences on formation of myelin. We developed a reliable assay for detecting ensheathment of neurites by oligodendrocytes in spinal cord explants. This system was used to assay the effect of selected growth factors on myelin internode formation. We examined the influence on myelination of the polypeptide growth factors neuregulin (NRG), platelet-derived growth factor (PDGF), leukemia inhibitory factor (LIF), and the thyroid hormone T_3. We found that NRG, PDGF, and T_3 treatments enhanced myelination while LIF treatment inhibited it. We furthermore found that the most potent combination of factors to enhance myelination was NRG and T_3. Our results demonstrate that the role of growth factors on CNS myelination can be reliably studied in a controlled in vitro environment and that the impact of individual or combinations of growth factors on myelination cannot be predicted by their known effects on oligodendrocyte survival, proliferation, or differentiation.

Introduction

Myelin formation by oligodendrocytes is an essential component of normal development in the vertebrate central nervous system (CNS). The development of myelinating oligodendrocytes depends on a number of distinct molecular signals. In the spinal cord, the initial induction of oligodendrocyte precursor cells (OPCs) in the ventral ventricular zone requires sonic hedgehog derived from the floor plate and notochord [Noll and Miller, 1993; Ono et al., 1995; Orentas et al., 1999; Pringle et al., 1996; Soula et al., 2001; Timsit et al., 1995; Warf et al., 1991; Yu et al., 1994]. Once specified, OPCs migrate outward from the ventral ventricular zone and proliferate in response to platelet-derived growth factor-AA (PDGF-AA) and basic fibroblast growth factor [Gard and Pfeiffer, 1990; McKinnon et al., 1990; Noble et al., 1988; Raff et al., 1988]. Early-stage OPCs are mAb A2B5 immunoreactive and bipolar [Raff et al., 1983], highly motile [Armstrong et al.,

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1991; Noble et al., 1988; Osterhout et al., 1997; Simpson and Armstrong, 1999; Small et al., 1987], and highly proliferative [Noble et al., 1988]. These A2B5+ OPCs mature into proligodendroblasts which are less motile [Armstrong, 1998; Warrington et al., 1993], mAb O4 immunoreactive [Gard and Pfeiffer, 1990], have more processes, and proliferate to a lesser extent only in response to PDGF-AA [Gard and Pfeiffer, 1990]. Further differentiation results in cells that express galactocerebroside detected by mAb O1 immunoreactivity [Raff et al., 1978b; Sommer and Schachner, 1981], have numerous highly branched processes [Hardy and Friedrich, 1996], and are no longer capable of dividing [Gard and Pfeiffer, 1990; Hart et al., 1989a]. Fully mature oligodendrocytes express myelin genes including proteolipid protein [Dubois-Dalcq et al., 1986] and myelin basic protein (MBP) [Sternberger et al., 1978] and will go on to ensheath axons and form myelin internodes [for review of myelination see Colman et al., 1996].

The molecular control of myelin formation and the signaling systems involved are still poorly understood. For example, the molecular cues that direct oligodendrocyte processes to axons and how the number of myelin segments formed by oligodendrocytes is accurately matched to the number and lengths of axons requiring myelination are unknown. Certain cell surface receptors involved in cell adhesion, such as integrins and cadherins, are likely important for development and integrity of the myelin sheath. Integrin signaling has been shown to enhance axon-mediated oligodendrocyte survival [Frost et al., 1999], and disruption of integrin-mediated adhesion has been shown to lead to decreased expression of proteolipid protein and MBP in oligodendrocytes [Malek-Hedayat and Rome, 1994]. The oligodendrocyte lineage, from early OPCs through mature oligodendrocytes, has been found to express N-cadherin in culture [Payne et al., 1996]. Additionally, adherence of OPCs to N-cadherin was found to promote rapid maturation and production of myelin [Payne et al., 1996]. Certain growth factors acting through cytokine and single membrane-spanning receptor tyrosine kinases are known to have potent effects on oligodendrocyte survival, proliferation, and size [Volpe, 2001] and are also likely to play a role in the formation and maintenance of the myelin sheath.

The present study examines the potential role played in controlling CNS myelination of three polypeptide growth factors and one hormone: neuregulin (NRG), leukemia inhibitory factor (LIF), PDGF-AA, and triiodothyronine (T_3). We show that myelination by spinal cord oligodendrocytes is enhanced when treated with NRG, PDGF, or T_3 and inhibited when treated with LIF. Furthermore, we demonstrate that combined NRG and T_3 treatment synergistically enhances spinal cord myelination.

Materials and Methods

Antibodies and Growth Factors

The following primary antibodies were used: rabbit polyclonal IgG against neurofilament (NF) 200 kD (Sigma), O1 mAb (ATCC), mouse mAb IgG against MBP (SMI 99, Sternberger Monoclonals), and rabbit anti-cow S100 (DAKO). The following growth factors and hormone were used: recombinant human NRG-β1 (EGF domain; R&D Systems), recombinant human PDGF-AA (Sigma), recombinant murine LIF (Life Technologies) and T_3 (Sigma).

Spinal Cord Explants

Spinal cords were dissected out between the thoracic and lumbar regions of E14.5 mouse embryos and cut transversely into 1-mm fragments for explant culture onto polylysine and laminin (Collaborative Biomedical Products)-coated coverslips in DMEM (Life Technologies) containing 2% FBS. Half of the culture media were replaced with fresh media every 3 days.

Immunocytochemistry and Fluorescent Microscopy

Spinal cord explants were fixed with methanol for 10 min on ice at the desired time points. The explants were then incubated with the antibodies against MBP, NF 200 kD, or S100 in 2% NGS and 2% Triton X-100 in PBS at 4°C overnight. Incubation with the O1 mAb was done for 15 min at 37°C prior to methanol fixation. After incubation with primary antibodies, the explants were then washed with PBS and incubated with the appropriate secondary anitbodies (Jackson ImmunoResearch). Immunofluorescent images were obtained using a Nikon Eclipse 660 Microscope and SPOT RT Software v3.2.4 (Diagnostic Instruments).

Results

To study myelination by spinal cord oligodendrocytes, we developed a reliable in vitro assay in which the time course of myelination parallels in vivo myelination. Spinal cords dissected from the thoracic to lumbar regions of E14.5 mouse embryos were cultured on polylysine- and laminin-coated coverslips in DMEM containing 2% FBS. Explants were fixed at 3 days, and 1, 2, and 4 weeks and then labeled for surface galactocerebroside and MBP. This staining shows that O1+ cells first appear at 3 days in culture, corresponding to E17.5 in vivo (fig. 1a). We observe an increase in O1+ cells at 1 week, again at 2 weeks (P8 in vivo), and a marked increase at 4 weeks in culture (P22 in vivo; fig. 1a). MBP+ cells first appear at 1 week (P1 in vivo), increase at 2 weeks (P8 in vivo), and are relatively abundant at 4 weeks in culture (P22 in vivo; fig. 1a).

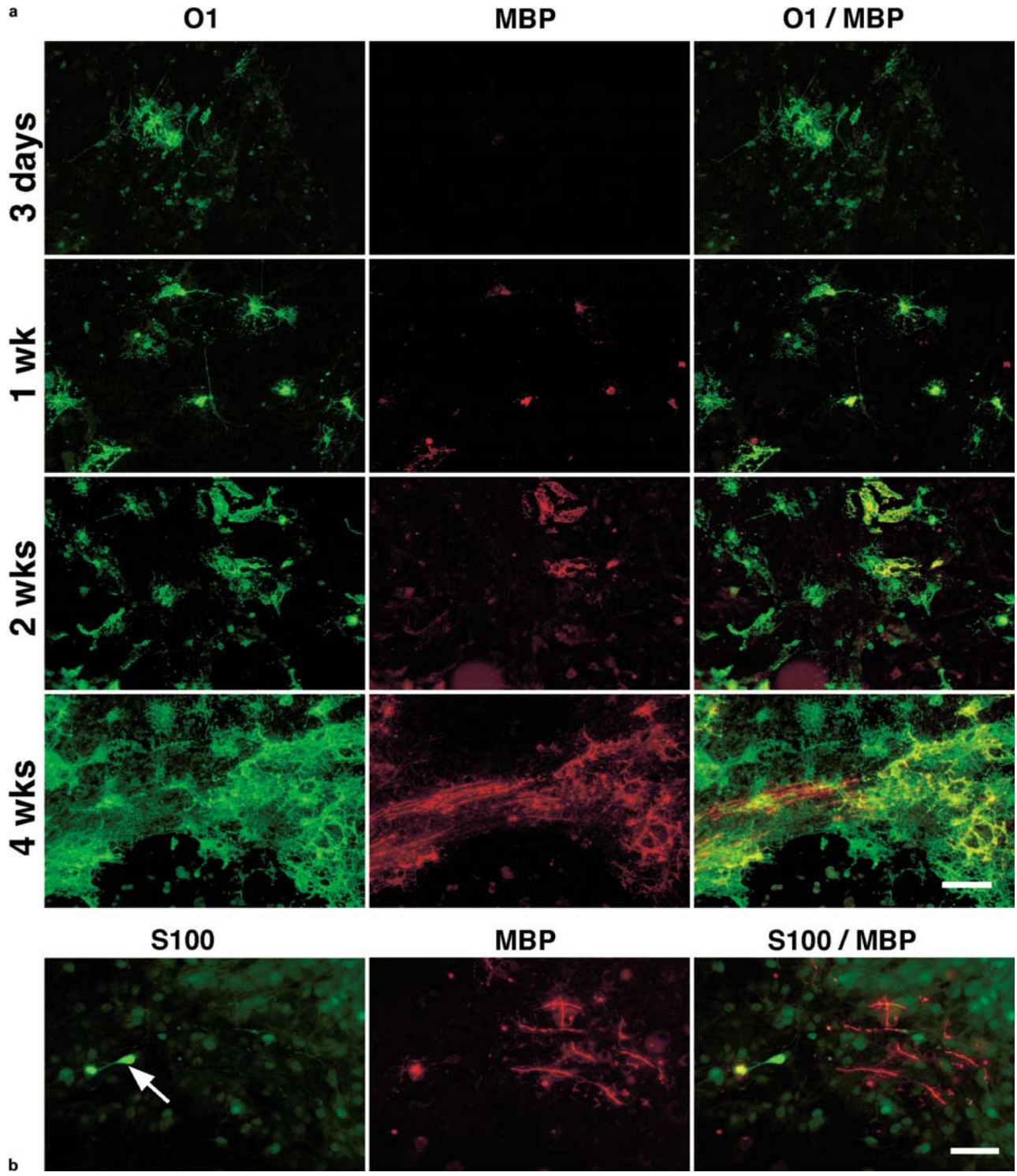


Fig. 1. Time course of myelination by spinal cord oligodendrocytes. **a** Spinal cord explants from E14.5 mouse embryos were cultured for 3 days, and 1, 2 and 4 weeks and then labeled for galactocerebroside and MBP. Scale bar = 100 μm. **b** 4-week explant cultures were also labeled for MBP and S100 to confirm that myelinating cells are oligodendrocytes and not Schwann cells. Arrow indicates an S100+ cell not involved in ensheathment of neurites. Scale bar = 50 μm.

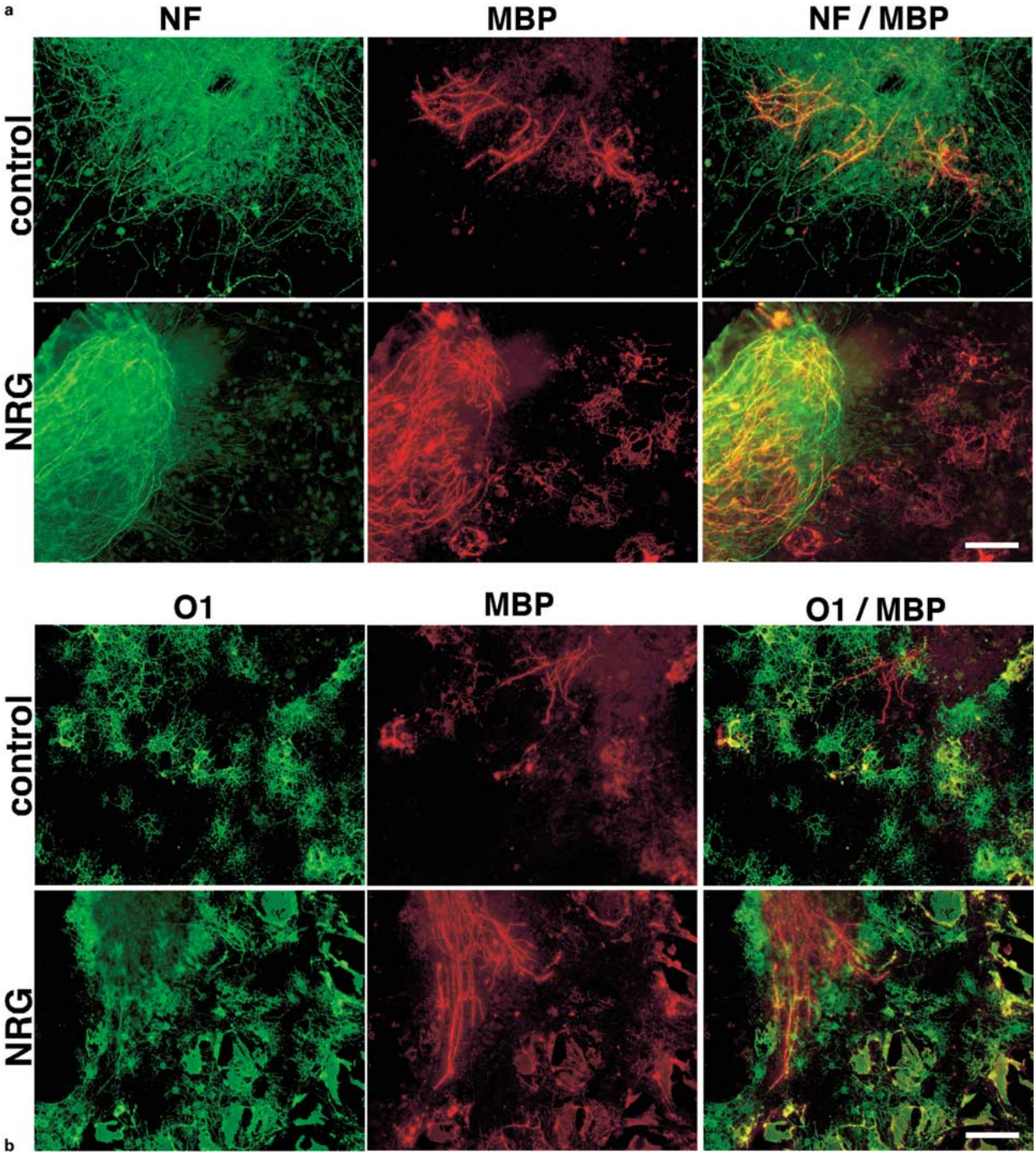


Fig. 2. NRG enhances myelination by spinal cord oligodendrocytes. Spinal cord explants from E14.5 mouse embryos were cultured for 4 weeks in the presence and absence of 1 n*M* NRG and then labeled for NF and MBP (**a**) or galactocerebroside and MBP (**b**). Scale bar = 100 μm.

To be certain the myelinating cells in our explant cultures were oligodendrocytes, we used an antibody against S100, an antigen expressed by Schwann cells and astrocytes but not oligodendrocytes [Brennan et al., 2000; Ghandour et al., 1981]. The majority of MBP+ cells were S100–, and none of the few cells that were both MBP+ and S100+ were involved in the ensheathment of neurites (fig. 1b). This indicates that very few Schwann cells were present in our embryonic spinal cord explants and confirms that myelinating cells were oligodendrocytes.

Since NRG is likely a candidate for being an axonal-derived signaling molecule essential for myelin formation [Cannella et al., 1998; Vartanian et al., 1994, 1999, 1997], we examined the role of NRG in regulating myelin formation in spinal cord explants. Spinal cord explants from E14.5 embryos were cultured as described above except for the addition of 1 n*M* NRG to the culture media. These explants and controls were fixed at 4 weeks and stained for galactocerebroside, NF 200 kD, and MBP. This staining reveals that 1 n*M* NRG treatment produced a marked increase in the number of MBP+ cells and caused oligodendrocytes to extend broad, sheet-like processes (fig. 2b). Furthermore, merged images of NF and MBP staining show that NRG treatment induced formation of numerous structures that are consistent with myelin internodes (fig. 2a).

To test if these effects were unique to NRG, we treated explants with three other molecules known to be important in controlling oligodendrocyte survival, proliferation, and development: LIF, PDGF, and T_3 [Mayer et al., 1994; Fruttiger et al., 1999; Noguchi et al., 1985]. As described above, explants from E14.5 embryos were used and treated with 1 n*M* NRG, 200 U/ml LIF, 1 ng/ml PDGF-AA, or 30 n*M* T_3. These explants and controls were fixed at 4 weeks and stained for NF 200 kD and MBP. We observed that LIF treatment dramatically increased the number of MBP+ cells per explant (fig. 3a, c) but reduced ensheathment of neurites compared to controls (fig. 3a, b). PDGF treatment increased the number of MBP+ cells and additionally increased the number of structures morphologically resembling myelin internodes (fig. 3a–c). T_3 treatment also increased the number of MBP+ cells and greatly increased the formation of structures resembling myelin internodes (fig. 3a–c).

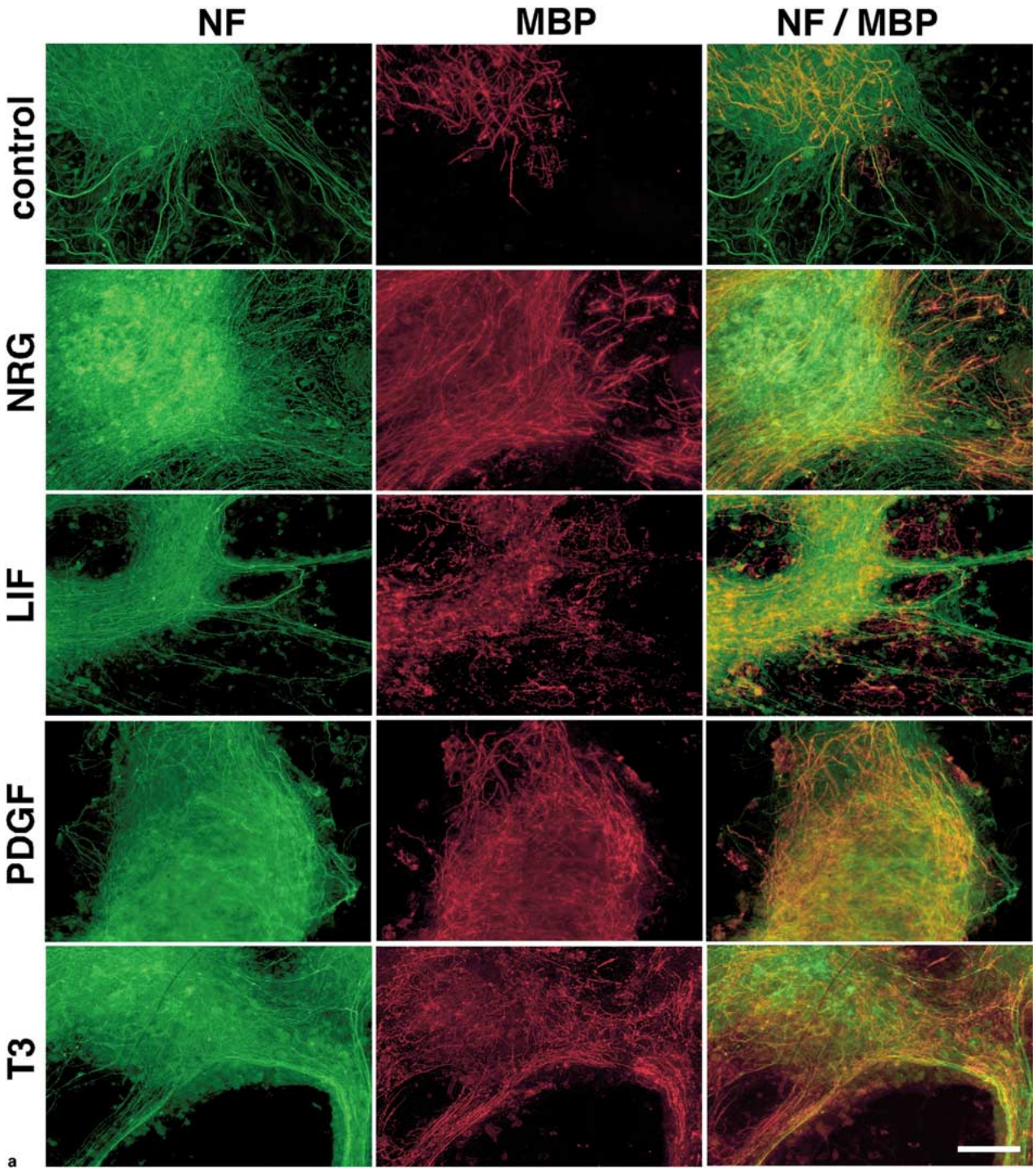
It was interesting that LIF reduced the number of myelin internodes formed while increasing the number of MBP+ cells. On closer examination, LIF treatment produced MBP+ cells that were less developed with fewer sheet-like processes and shorter branches (fig. 3a). In contrast, PDGF, and T_3 treatments produced cells with larger branches and more differentiated shapes similar to NRG treatment (fig. 3a). These data indicate that PDGF and T_3 might also play a significant role in regulating myelin formation by spinal cord oligodendrocytes.

It is likely that several signaling molecules collectively control myelination. Thus, we studied possible synergistic effects of growth factors on oligodendrocyte development and the formation of myelin internodes. In this study, 1 n*M* NRG treatment was studied in conjunction with 200 U/ml LIF, 1 ng/ml PDGF-AA, or 30 n*M* T_3 treatment on spinal cord explants from E14.5 embryos as above. After 4 weeks, explants were fixed and stained for NF 200 kD and MBP. Combining LIF with NRG treatment greatly increased the number of MBP+ cells but appeared to inhibit myelination as very few myelin internodes were observed (fig. 4). Combining PDGF with NRG treatment vastly increased the number of MBP+ cells but also seemed to inhibit myelination (fig. 4). However, a combined NRG and T_3 treatment produced large increases in the number of MBP+ cells and induced the formation of more myelin internodes than NRG or T_3 treatment alone (fig. 4).

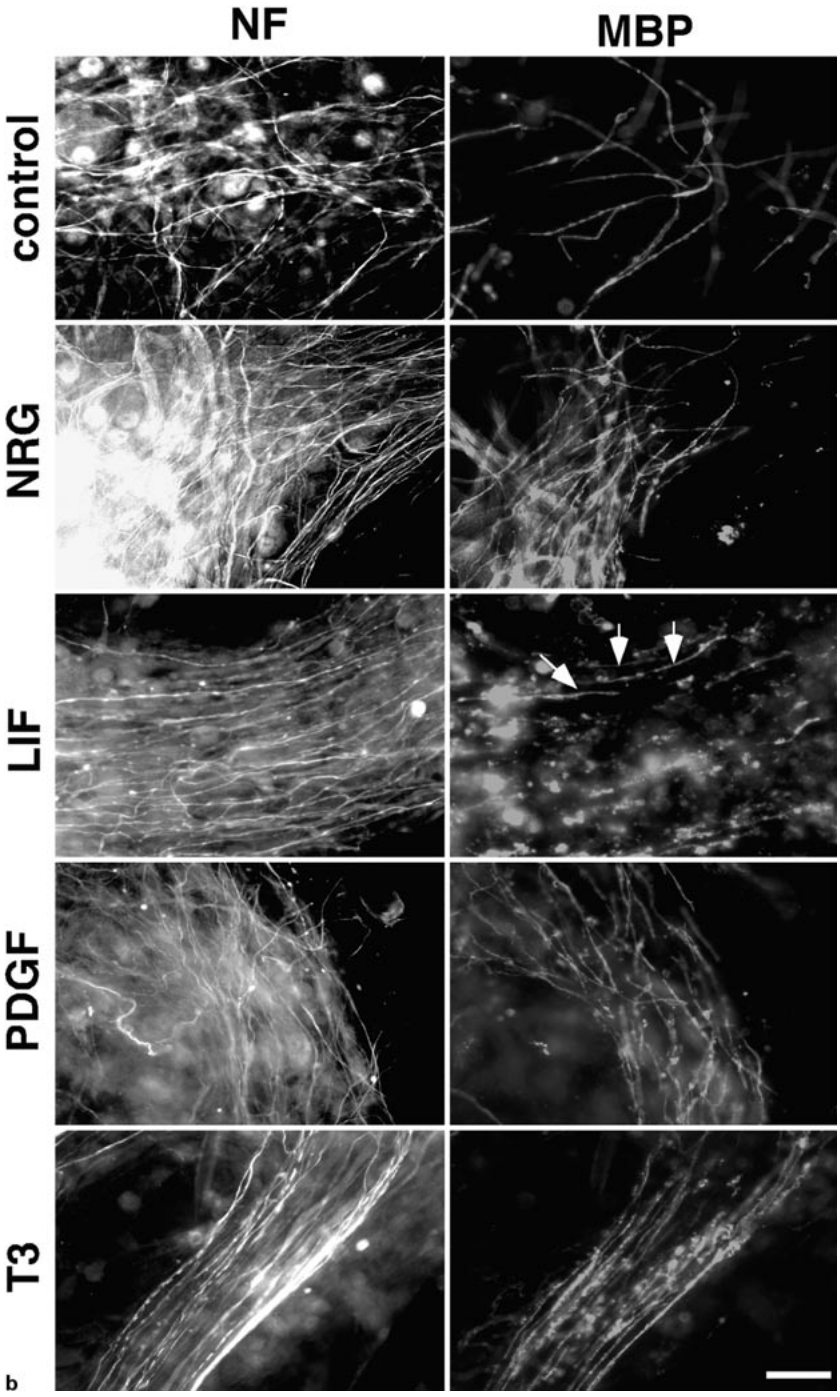
Discussion

In a proposed model of oligodendrocyte development, newly differentiated oligodendrocytes stop being responsive to astrocyte-derived survival signals such as PDGF and have 2–3 days to contact the unmyelinated region of an axon which provides new signals required for survival [Barres and Raff, 1999]. Much evidence has been collected to support this model. One study has shown that if the number of axons is experimentally increased, the number of oligodendrocytes that survives increases proportionally [Burne et al., 1996]. Another study has shown that oligodendrocytes that are successful in contacting axons preferentially survive over oligodendrocytes that are not [Trapp et al., 1997]. The above model is also confirmed by an experiment in which early oligodendrocyte numbers in mice are increased by transgenic overexpression of PDGF but fall to normal numbers less than 1 week after birth [Calver et al., 1998]. This model, of course, does not exclude additional non-axonal signals necessary for myelin formation.

One likely candidate for an axonal-derived signaling molecule that regulates oligodendrocyte survival, development, and myelin formation is NRG-1. The NRGs are a large family of proteins related to epidermal growth factor which occur in multiple isoforms, some soluble and



some membrane bound. The first isoform identified was found to promote proliferation of astrocytes and Schwann cells in vitro and was called glial growth factor [Brockes et al., 1980; Goodearl et al., 1993; Marchionni et al., 1993; Raff et al., 1978a]. Since this work, NRGs have been found to be expressed on most axons [Dong et al., 1995; Jo et al., 1995; Loeb et al., 1998; Sandrock et al., 1995; Trachtenberg and Thompson, 1996] and to be important in oligodendrocyte development as well. In vitro experiments have shown that NRG promotes the proliferation



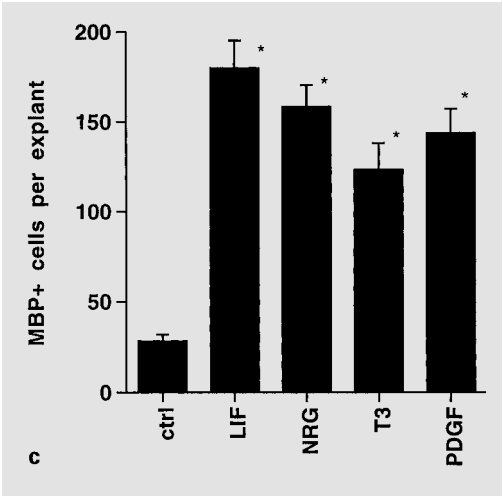


Fig. 3. Effect of growth factors on myelination by spinal cord oligodendrocytes. **a** Spinal cord explants from E14.5 mouse embryos were cultured for 4 weeks in the presence and absence of 1 n*M* NRG, 200 U/ml LIF, 1 ng/ml PDGF-AA, or 30 n*M* T_3 and then labeled for NF and MBP. Scale bar = 100 µm. **b** Higher magnification images were also used to assess the formation of myelin internodes. Arrows indicate the few myelin internodes present in LIF-treated explants in contrast to the numerous internodes present in NRG, PDGF, and T_3 treated explants. Scale bar = 30 µm. **c** In addition, the number of MBP+ cells in these explants was counted, and ANOVA was performed showing statistical differences between control and treatment groups, * $p < 0.0001$.

of OPCs [Canoll et al., 1996, 1999; Shi et al., 1998]. In long-term cultures of OPCs, NRG transduces a potent survival signal [Fernandez et al., 2000; Flores et al., 2000] mediated through the Akt pathway [Flores et al., 2000]. Additionally, in vivo inhibitors of NRG increase normal oligodendrocyte cell death in the developing optic nerve while adding exogenous NRG decreases it [Fernandez et al., 2000]. Moreover, addition of exogenous NRG blocks oligodendrocyte death normally induced by optic nerve transection [Fernandez et al., 2000]. NRG has also been

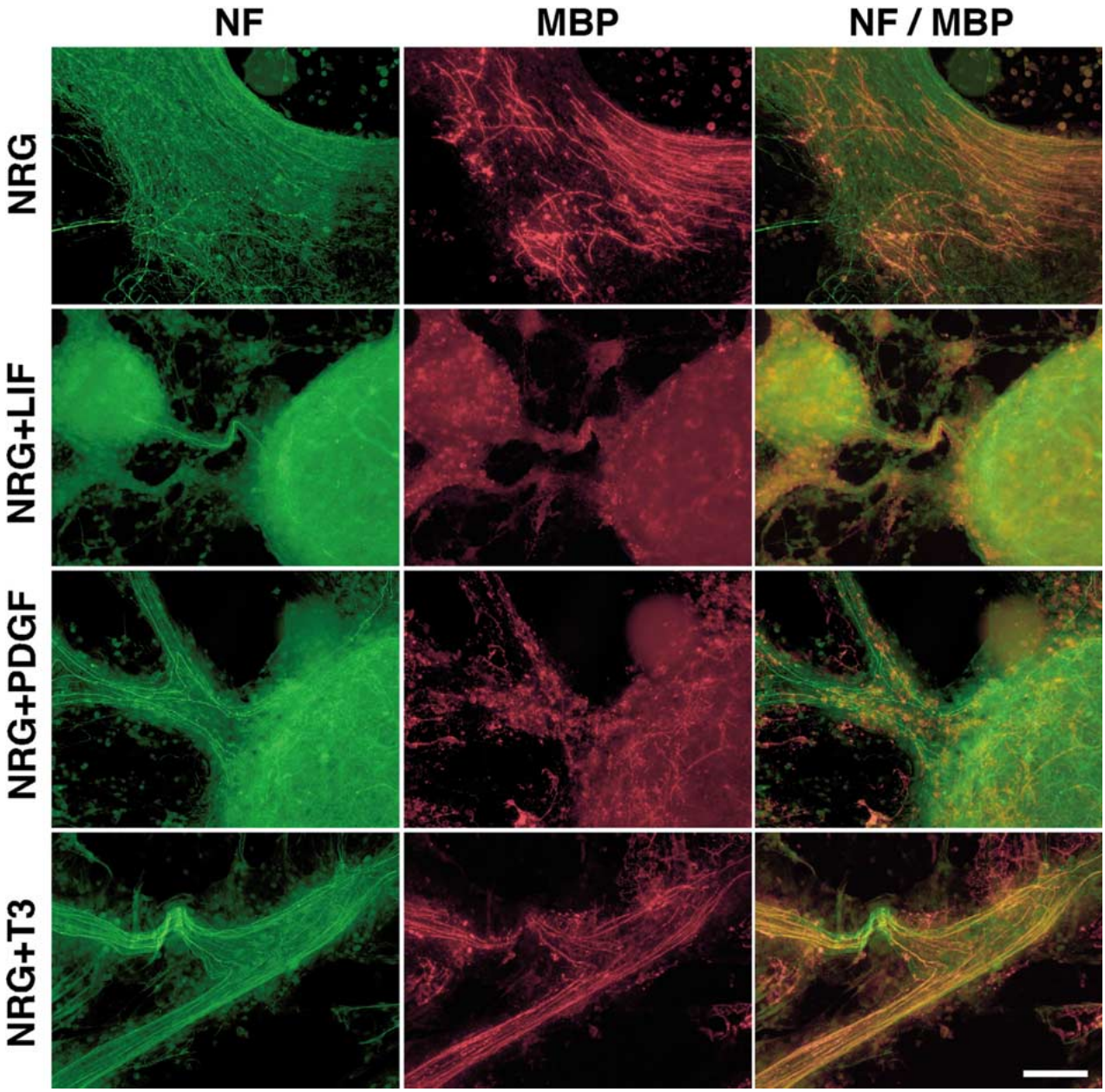


Fig. 4. Synergistic effect of NRG plus T_3 on myelination by spinal cord oligodendrocytes. Spinal cord explants from E14.5 mouse embryos were cultured for 4 weeks in the presence of 1 n*M* NRG and 200 U/ml LIF, 1 ng/ml PDGF-AA, or 30 n*M* T_3 and then labeled for NF and MBP. Scale bar = 100 μm.

implicated in the early development of the oligodendrocyte lineage. Early ventral structures such as the ventral ventricular zone and the floor plate of the spinal cord [Vartanian et al., 1999] and the subventricular zone of the forebrain [Corfas et al., 1995; Vartanian et al., 1994] express NRG at the time that OPCs initially appear. The oligodendrocyte lineage fails to develop, at least to the O4+/O1– stage, in spinal cord explants from wild-type mice when NRG signaling is blocked [Vartanian et al., 1999]. Also, O4+/O1– oligodendrocytes fail to develop in spinal cord explants from mice lacking NRG-1 (NRG-1–/–), but their development can be rescued by addition of recombinant NRG [Vartanian et al., 1999]. The role of NRG in regulating the late stages of oligodendrocyte

development is not yet established, but the present study demonstrates that soluble NRG enhances myelination by spinal cord oligodendrocytes.

While strong evidence shows that axons play a major role in regulating survival of newly differentiated oligodendrocytes and controlling subsequent myelination, other non-axonal signals have also been implicated to have a role. One such molecule is the amino-acid-derived hormone T_3 produced by the thyroid. Hypothyroidism has long been known to lead to delayed myelinogenesis and hypomyelination [Noguchi and Sugisaki, 1984; Rodriguez-Pena, 1999]. Recent work has shown that T_3 blocks proliferation of OPCs and induces their differentiation [Baas et al., 1997]. Studies have determined that three T_3 receptor isoforms are expressed in the oligodendrocyte lineage: α_1, α_2, and β_1 [Fiero-Renoy et al., 1995]. Interestingly, T_3 receptors α_1 and α_2 are expressed in both OPCs and differentiated oligodendrocytes, but the T_3 receptor β_1 is only expressed in differentiated oligodendrocytes [Baas et al., 1998; Carre et al., 1998]. This suggests that T_3 through the T_3 receptor β_1 might regulate oligodendrocyte differentiation and myelination [Rodriguez-Pena, 1999]. The present study shows that T_3 promotes the differentiation of and enhances myelin formation by spinal cord oligodendrocytes.

Other molecules thought to possibly be important in controlling late stages of oligodendrocyte development and myelin formation are LIF and PDGF-AA [Mayer et al., 1994; Fruttiger et al., 1999]. LIF is a 20-kD protein originally identified by its ability to induce macrophage differentiation of the murine myeloid leukemic cell line, M1 [Hilton et al., 1988]. Embryonic stem cells, the totipotent cell lines derived from preimplantation embryos, are maintained in the presence of LIF which prevents their differentiation [Williams et al., 1988]. Astrocytes and most neurons of the CNS express LIF at low levels in the adult brain [Lemke et al., 1996], and astrocyte production and secretion of LIF is vastly increased during inflammation or infection [Banner et al., 1997]. In contrast, little is known about expression of LIF in the CNS during early development. However, in vitro experiments have shown that LIF plays an important role in the generation and survival of newly formed oligodendrocytes through binding the gp130 cytokine receptor [Barres et al., 1993; Mayer et al., 1994]. Furthermore, interferon-γ-induced apoptosis of oligodendrocytes is blocked by LIF [Vartanian et al., 1995]. The present study demonstrates that LIF promotes survival of spinal cord oligodendrocytes increasing cell numbers but inhibits myelination by preventing differentiation and inhibiting the formation of sheet-like processes.

The role of PDGF in the early development of oligodendrocytes has been well characterized. OPCs predominantly express the PDGF-α receptor which binds with high affinity to PDGF A-chain produced by astrocytes [Hart et al., 1989b; McKinnon et al., 1990; Pringle et al., 1992; Yeh et al., 1993]. Both in vitro and in vivo experiments have shown that OPCs migrate, survive, and divide in response to astrocyte-derived PDGF [Fruttiger et al., 1999; Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988]. However, terminally differentiated oligodendrocytes lose responsiveness to PDGF as a mitogen and survival factor [Hart et al., 1989b; McKinnon et al., 1990]. Despite all of this work, little is known about the role of PDGF in regulating the late stages of oligodendrocyte development and myelin formation. The present study shows that PDGF promotes proliferation of spinal cord oligodendrocytes increasing MBP+ cell numbers which results in more myelin formation.

One of the most interesting aspects of the present study was the combination of NRG with PDGF, LIF, and T_3 treatments. NRG and PDGF treatments alone increased the formation of myelin internodes, yet in conjunction they decreased formation. NRG and PDGF are both known to be potent mitogens for OPCs [Canoll et al., 1996; Noble et al., 1988]. It is likely that a combined dose of these mitogens might keep OPCs in the explants proliferating, preventing their differentiation and subsequent myelination. Our unpublished data show that supramaximal doses of 10 and 100 ng/ml PDGF-AA inhibit myelination by spinal cord oligodendrocytes. It has been demonstrated that supramaximal doses of NRG inhibit myelination by Schwann cells [Zanazzi et al., 2001], but whether this is the case for oligodendrocytes remains to be determined.

Our results demonstrate that NRG and T_3 powerfully control CNS myelination. Future research will investigate other molecules possibly important in controlling CNS myelination and the mechanisms by which oligodendrocytes extend broad sheet-like processes or giant lamellipodia prior to ensheathing nearby axons.

Acknowledgments

This work was supported by grants from the National Institute of Neurologic Disorders and Stroke (NS02028; T.V.) and the United States National Multiple Sclerosis Society (RG2912-A-1; T.V. and FA1311-A-1; S.K.P.).

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