Nonneuronal Control of the Differential Distribution of Myelin Along Retinal Ganglion Cell Axons in the Mouse

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Purpose. In most mammalian species, retinal ganglion cell (RGC) axons are myelinated in the optic nerve, but remain nonmyelinated in the retinal nerve fiber layer and the most proximal (i.e., retina-near) region of the nerve. Here we analyzed whether RGCs are involved in the control of this characteristic distribution of oligodendrocytes and myelin in the primary visual pathway of mice.

Methods. Neurospheres were enriched in oligodendrocyte progenitor cells (OPCs) by a short-term exposure to platelet-derived growth factor (PDGF) and grafted into the retina of young postnatal mice close to the optic disc. Immunohistochemistry was performed to study the integration and differentiation of the grafted cells, and the formation of donor-derived myelin in the normally nonmyelinated retinal nerve fiber layer and intrabulbar and most proximal retrobulbar region of the optic nerve.

Results. Intraretinal transplantations of small-sized PDGF-treated neurospheres into young postnatal mice resulted in extensive integration of the grafted cells into host retinas. A significant fraction of the donor cells differentiated into oligodendrocytes that myelinated the nerve fiber layer. Importantly, RGC axon segments within the normally nonmyelinated intrabulbar and most proximal retrobulbar region of the nerve also became myelinated in a fraction of animals.

Conclusions. This is the first report demonstrating that the normally nonmyelinated intrabulbar and retrobulbar segments of RGC axons are competent to become myelinated. Results support the view that the differential distribution of myelin and oligodendrocytes in the primary visual pathway is controlled by nonneuronal factors rather than by the RGCs themselves.

Keywords: myelination, neurosphere cultures, oligodendrocytes, retina, transplantation

Oligodendrocyte progenitor cells (OPCs) are generated in the preoptic area from where they colonize the developing optic nerve from its chiasmal toward its retinal end.[1–3] In most mammalian species, including rats and mice, OPCs stop migrating and differentiate into myelinating oligodendrocytes shortly before the optic nerve enters the retina.[4,5] In addition, the retina is incapable of generating oligodendrocytes even under culture conditions that favor oligodendrogenesis.[6,7] As a result, the retina is devoid of oligodendrocytes and remains nonmyelinated throughout life.[6,8–10] Vision is thus not impaired by a myelinated retinal nerve fiber layer that would impede light reaching the photoreceptor cells.

Abnormal myelination of the retinal nerve fiber layer has been found in various mammalian species that normally lack intraretinal myelin, including the mouse, rat, guinea pig, cat, and rhesus monkey,[11–15] and occurs sporadically also in human eyes.[16–19] Furthermore, myelination of the nerve fiber layer by Schwann cells is frequently seen in the Browman-Wyse rat, an inbred strain selected for microphthalmos,[20,21] and can be induced in normal rats by lesioning the retina via the sclera and choroid.[22] In addition, transplantation studies have demonstrated extensive myelination of the retinal nerve fiber layer following intraocular transplantations of Schwann cells, oligodendrocyte lineage cells, or neural progenitor cells into developing or adult mice or rats.[23–29] All these reports unequivocally demonstrate that the normally nonmyelinated intraretinal segments of RGC axons will become myelinated once myelinogenic cells gain access to the retina. It was therefore hypothesized that nonneuronal factors located at the retinal end of the optic nerve, rather than the RGCs themselves, control the differential distribution of oligodendrocytes and myelin in the primary visual pathway by preventing OPCs from entering the retina.[4,6,8,24]

Among the nonneuronal factors implicated in preventing intraretinal myelination is a well-developed lamina cribrosa, a distinct band of connective tissue formed by collagen fibers of the sclera.[30] In humans, retinal ganglion cell axons remain nonmyelinated proximal to the lamina cribrosa. Rabbits, in contrast, lack a well-developed lamina cribrosa and RGC axons in this species are also myelinated intraretinally.[30,31] However, rats and mice also lack a well-developed lamina cribrosa but have a nonmyelinated nerve fiber layer. Astrocytes have also been suggested to act as a barrier for migrating OPCs at the retinal end of the optic nerve. A dense astrocytic network is present proximal to the myelinated part of the optic nerve in rats and mice[4,6] and several other species that lack intraretinal

myelin, while a similar concentration of astrocytes is absent from the optic nerve of rabbits and chicken, two species with intraretinal myelination.[6,32] In the Browman-Wyse rat, however, the proximal part of the optic nerve remains nonmyelinated over an abnormally long distance, but lacks a dense astrocytic network at the transition zone between the myelinated and nonmyelinated region of the optic nerve.[20] At the molecular level, the astrocyte-derived extracellular matrix glycoprotein tenascin-C has been suggested to prevent migration of OPCs into the retina. Tenascin-C is expressed at elevated levels by astrocytes in the most proximal region of the optic nerve before the arrival of the first OPCs in this region, and has been demonstrated to inhibit OPC adhesion and migration in vitro.[4,33] Bone morphogenetic proteins (BMPs), expressed in the developing retina and at the retina-optic nerve junction, have also been implicated in preventing intraretinal myelination.[7] However, in vivo evidence for a critical role of any of these factors in controlling the differential distribution of oligodendrocytes and myelin in the primary visual pathway has not been provided.

While the available data are in line with the view that nonneuronal factors in the most proximal region of the optic nerve prevent migration of OPCs into the retina and thus intraretinal myelination, they do not exclude a neuronal control of the differential distribution of oligodendrocytes and myelin along RGC axons. In fact, axon segments of RGCs within the intrabulbar and most proximal retrobulbar region of the optic nerve may be refractory to myelination, thereby restricting oligodendrocytes and myelin to the distal part of the nerve. To address this possibility experimentally, we enriched neurosphere cultures in oligodendrocyte lineage cells by a short-term cultivation in a medium supplemented with platelet-derived growth factor (PDGF). Small-sized PDGF-treated neurospheres were grafted into the retina of young postnatal mice close to the optic disc, and the retina and the intrabulbar and most proximal retrobulbar region of the optic nerve were analyzed for the presence of donor-derived myelin.

Methods

Animals

Neural precursor cells were isolated from the cerebral cortex of 14-day-old transgenic mouse embryos ubiquitously expressing enhanced green fluorescent protein (EGFP) under control of a cytomegalovirus early enhancer/chicken β-actin promoter.[34] Intraretinal transplantation experiments were performed on 3- to 7-day-old C57BL/6J wild-type mice. Six-week-old C57BL/6J mice were used for immunohistochemical analyses of untreated retinas and optic nerves. All animal experiments were approved by the University and State of Hamburg Animal Care Committees and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Neurosphere Cultures and Oligodendrocytic Differentiation

Cerebral cortices from EGFP-transgenic mouse embryos were dissociated into single cell suspensions, and cells were plated into uncoated tissue culture flasks at a density of 200,000 cells/mL in a medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 (DMEM/F12; Life Technologies, Darmstadt, Germany) containing 2 mM glutamine, 5 mM HEPES, 3 mM sodium bicarbonate, 0.3% glucose, (all from Sigma-Aldrich, St. Louis, MO), 1% N2 and 1% B27 (both from Life Technologies), and 20 ng/mL epidermal growth factor (EGF) and 20 ng/mL fibroblast growth factor-2 (FGF-2; both from tebu-bio, Offenbach, Germany). After 7 days of culture, cells had grown to free-floating neurospheres that were further expanded in the same medium but lacking B27. After three passages, neurospheres were dissociated and cells were transferred to a medium containing 10 ng/mL FGF-2, 10 ng/mL PDGF (R&D Systems, Minneapolis, MN) and 10 μM forskolin (Sigma-Aldrich) to increase the percentage of oligodendrocyte lineage cells in the cultures. After an additional culture period of 4 days, PDGF-treated neurosphere cultures (in the following termed "PDGF-neurospheres") consisted of small cellular aggregates that were used for in vitro differentiation experiments and transplantation experiments (see below).

Quantification of Oligodendrocyte Differentiation In Vitro

For in vitro differentiation experiments, neurospheres and PDGF-neurospheres were plated onto coverslips coated with poly-D-lysine (Sigma-Aldrich) and 1% Matrigel (Becton, Dickinson and Company, Heidelberg, Germany). Oligodendrocytic differentiation was induced by cultivating the cells for 3 days in DMEM/F12 supplemented with 1% N2, 30 ng/mL 3,3′,5-triiodothyronine (T3) and 200 μM ascorbic acid (both from Sigma-Aldrich). To compare the numbers of oligodendrocytes in the differentiated cultures, cells were fixed in PBS (pH 7.3) containing 4% paraformaldehyde (PA), blocked in PBS containing 0.1% BSA and 0.3% Triton X-100 (both from Sigma-Aldrich), and incubated with rat monoclonal antibodies to myelin basic protein (MBP, 1:100; Millipore, Billerica, MA) for 4 hours at room temperature to visualize oligodendrocytes. Oligodendrocytes were additionally identified by incubating live cultures with mouse monoclonal O4 or O1 antibodies[35] (both diluted 1:50) for 45 minutes at 4°C, followed by fixation in 4% PA. Cultures were washed, incubated with Cy3-conjugated anti-rat or anti-mouse antibodies (1:200; Jackson ImmunoResearch, Inc., West Grove, PA) for 4 hours at room temperature, stained with 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride hydrate (1:2000; Sigma-Aldrich) for 5 minutes, washed again and mounted onto slides. To control the specificity of the immunocytochemical stainings, cultures were processed as described above with the only exception that incubation with primary antibodies was omitted. The percentage of O4-, O1- and MBP-positive cells was determined in five randomly selected areas of each coverslip. Between 510 and 752 cells from at least three independent experiments were analyzed for each culture condition and antigen. Statistical analysis of data was performed with the independent *t*-sample test using statistical software (SPSS 12.0; SPSS, Inc., Chicago, IL).

Intraretinal Transplantation Experiments

Three- to seven-day-old C57BL/6J mice were deeply anesthetized using isoflurane (Abbott, Wiesbaden, Germany). A glass micropipette was inserted into the vitreous space at the junction between the sclera and cornea and 1 μL of vitreous fluid was slowly removed from the eye. The same volume of a dense suspension of small-sized neurospheres in PBS was slowly injected into the vitreous cavity under visual control using a surgical microscope. To increase the integration of grafted cells into the host tissue, retinas were gently lesioned close to the optic disc with the tip of the micropipette at the time of the injection.[24,28,29] Intraretinal injections of 1 μL PBS without neurospheres into 3- ($n = 7$) or 5-day-old mice ($n = 7$) were performed as a control. Mice with grafted PDGF neurospheres were allowed to survive for 1 week ($n = 10$) or 4 to 7 weeks ($n = 128$) before eyes with attached optic nerves

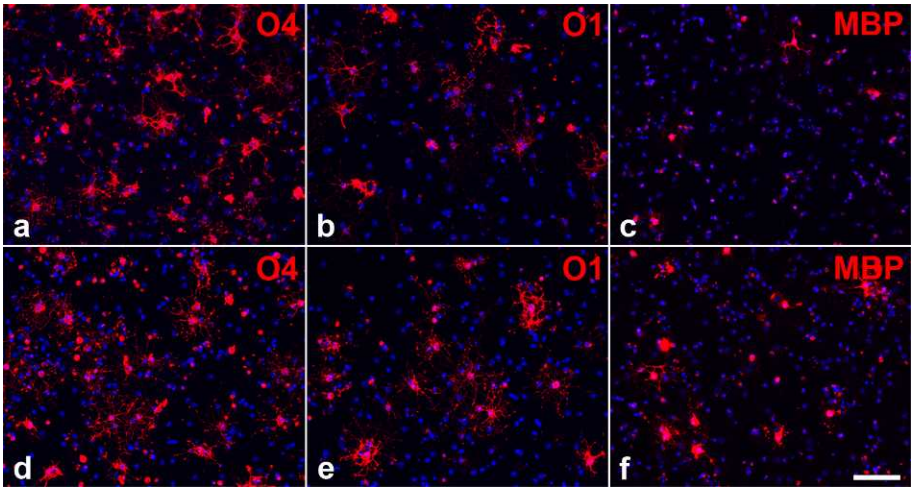

FIGURE 1. Oligodendrocyte differentiation in neurosphere and PDGF-neurosphere cultures. Neurosphere cultures were maintained for 4 days in a medium containing either FGF-2 and EGF (**a–c**) or FGF-2 and PDGF (**d–f**) as mitogens, induced to differentiate by removal of the mitogens, and analyzed for the presence of oligodendrocytes using O4 (**a**, **d**), O1 (**b**, **e**) or anti-myelin basic protein antibodies (**c**, **f**). Note that neurosphere cultures exposed to FGF-2 and PDGF contained a significantly higher percentage of oligodendrocyte lineage cells than neurosphere cultures exposed to FGF-2 and EGF. All cultures were counterstained with DAPI to visualize cell nuclei. *Scale bar* in (**f**) for (**a–f**): 50 μm.

were subjected to immunohistochemical analysis. Control animals ($n = 14$) were analyzed 4 weeks after the PBS injection.

Immunohistochemistry

Animals were killed by cervical dislocation, eyes with attached optic nerves were quickly removed, immersion-fixed in 4% PA, dehydrated in an ascending series of sucrose, embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands) and frozen. Longitudinal cryostat sections of optic nerves with attached retinas, 25 μm in thickness, were blocked in PBS containing 0.1% BSA and 0.3% Triton X-100 for 1 hour, followed by incubation with primary antibodies overnight at 4°C. The following primary antibodies were used: rabbit polyclonal antibodies to glial fibrillary acidic protein (GFAP, 1:1000; DAKO, Glostrup, Denmark), rabbit polyclonal antibodies to β-tubulin III (1:5000; Sigma-Aldrich), rat monoclonal antibodies to MBP (1:500) and goat polyclonal antibodies to myelin-associated glycoprotein (MAG, 1:100; R&D Systems). Sections were washed, incubated for 4 hours with Cy2-, Cy3- or Cy5-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, Inc.), stained with DAPI (1:2000), washed again and mounted onto slides. Selected retinas with a heavily myelinated nerve fiber layer ($n = 6$) were flat-mounted, immersion-fixed in 4% PA, blocked in PBS containing 0.1% BSA and 0.3% Triton X-100 for 1 hour, incubated with anti-MAG antibodies for 24 hours at 4°C, washed, incubated with donkey anti-goat antibodies overnight at 4°C, stained with DAPI, washed again and mounted onto slides. As a negative control, sections and flat-mounted retinas were processed as described above except that incubation with primary antibodies was omitted. Tissue sections and flat-mounted retinas were analyzed with a confocal fluorescence microscope (FluoView FV1000; Olympus America, Inc., Center Valley, PA).

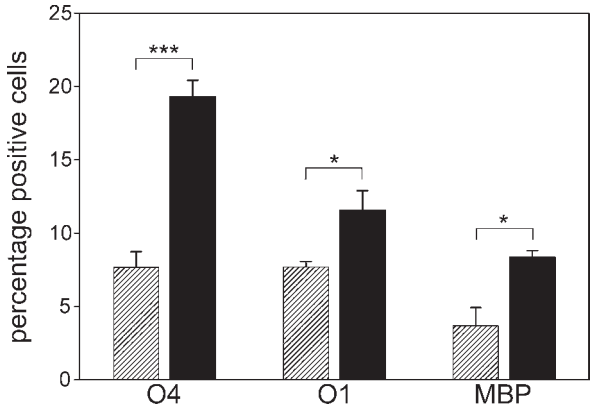

FIGURE 2. Quantification of oligodendrocyte differentiation in neurosphere and PDGF-neurosphere cultures. Neurosphere and PDGF-neurosphere cultures were induced to differentiate by removal of the mitogens, and the percentage of O4-, O1-, and MBP-positive cells was determined. Note the significantly higher percentage of oligodendrocyte cell lineage cells in differentiated PDGF-neurosphere cultures (*filled bars*) when compared with differentiated neurosphere cultures (*shaded bars*). *Bars* represent mean values (±SEM) from at least three independent experiments. $^{*}P < 0.05$. $^{***}P < 0.001$.

RESULTS

Oligodendrocytic Differentiation of Neurosphere Cultures

In previous studies, we have expanded neurosphere cultures in a medium supplemented with EGF[28] or EGF and FGF-2.[29,36,37] When these cells were intraretinally grafted into young postnatal or adult mice, a fraction of the donor cells differentiated into oligodendrocytes that myelinated the normally nonmyelinated retinal nerve fiber layer.[28,29,36,37] In the present study, we cultured the neurospheres for 4 days in a medium containing FGF-2 and PDGF ("PDGF-neurospheres") prior to transplantation to increase the percentage of oligodendrocyte lineage cells. To analyze the effect of the short-term exposure to PDGF on oligodendrocytic differentiation, neurospheres, and PDGF neurospheres were induced to differentiate for 3 days in a medium lacking the mitogens. Immunostainings with O4, O1, and anti-MBP antibodies revealed the presence of numerous oligodendrocyte lineage cells in differentiated neurosphere (Figs. 1a–c) and PDGF-neurosphere cultures (Figs. 1d–f). No labeling of cultures was detected when incubation with primary antibodies was omitted (not shown). Quantitative analyses revealed that

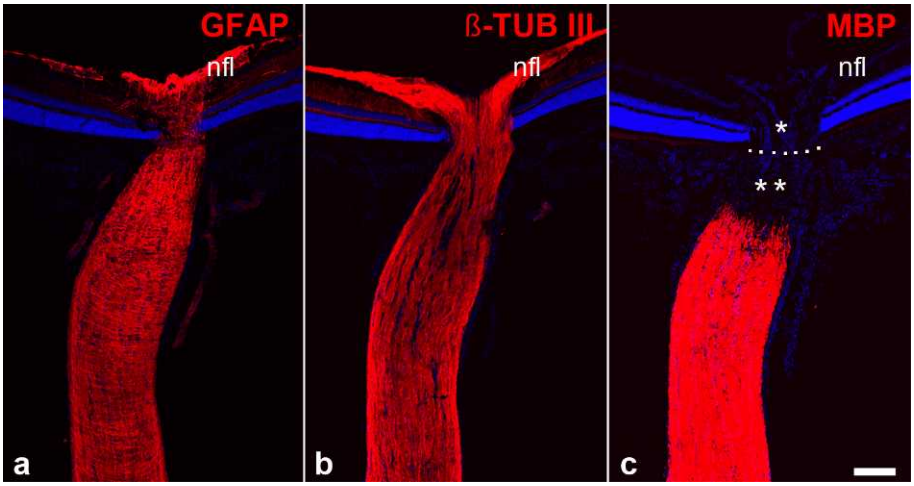

FIGURE 3. The distribution of astrocytes, retinal ganglion cell axons, and myelin in the retina and optic nerve of the adult mouse. Consecutive longitudinal sections of an adult mouse optic nerve with attached retina were stained with antibodies to (**a**) GFAP, (**b**) β-tubulin III (β-TUB III), and (**c**) MBP. GFAP-positive astrocytes (**a**) are associated with β-TUB III-positive retinal ganglion cell axons (**b**) throughout the optic nerve and in the retinal nerve fiber layer. MBP-positive myelin, in contrast, is restricted to the distal part of the optic nerve and is absent from the nerve fiber layer and the intrabulbar (*one asterisk* in [**c**]) and most proximal retrobulbar region (*two asterisks* in [**c**]) of the nerve. The *dotted line* in (**c**) indicates the boundary between the intrabulbar and retrobulbar region of the optic nerve. All sections were counterstained with DAPI to visualize cell nuclei. *Scale bar* in (**c**) for (**a**–**c**): 100 μm.

differentiated PDGF-neurosphere cultures contained a significantly higher percentage of O4-positive cells (19.3% ± 1.1% [mean ± SEM]) than differentiated neurosphere cultures (7.7% ± 1.1%; $P < 0.001$; Fig. 2). The percentage of O1-positive (11.6% ± 1.3%) and MBP-positive cells (8.4% ± 0.5%) in PDGF-neurosphere cultures was also significantly higher than in neurosphere cultures (7.7% ± 0.3% O1-positive cells, 3.7% ± 1.2% MBP-positive cells; $P < 0.05$ for both antigens; Fig. 2). The short-term exposure to PDGF thus significantly increased the percentage of oligodendrocyte lineage cells in the neurospheres cultures, and all intraretinal transplantation experiments were therefore performed with PDGF neurospheres (see below).

Oligodendrocytic Differentiation of Intraretinally Grafted PDGF-Neurosphere Cells

In adult mice, fascicles of retinal ganglion cell axons extend through the retinal nerve fiber layer toward the optic disc where they exit the retina to form the optic nerve (Fig. 3). While GFAP-positive astrocytes are associated with RGC axons along their entire length, MBP-positive myelin is restricted to the distal part of the optic nerve, but is absent from the intrabulbar and most proximal retrobulbar part of the nerve and the retinal nerve fiber layer (Fig. 3).[4,9,38]

To test the myelinogenic potential of PDGF neurospheres in vivo, we dissociated them into single cell suspensions and grafted the cells into the retina of young postnatal mice. However, analysis of animals 1 month after transplantation revealed the presence of only a limited number of integrated donor cells in only a fraction of host retinas. Furthermore, oligodendrocytes and myelin were found in only some experimental retinas (data not shown). In comparison, when small-sized PDGF neurospheres instead of single cell suspensions were grafted, we observed extensive integration of donor cells into the vast majority of host retinas. A few integrated cells were detected in the host retinas 1 week after transplantation (see Supplementary Material and Supplementary Figs. S1A, S1E). In comparison, numerous EGFP-positive cells were present in host retinas 4 to 7 weeks after transplantation (Figs. 4, 5; see Supplementary Material and Supplementary Fig. S1C). Donor cells were located in the inner retinal layers (i.e., inner plexiform layer [IPL], ganglion cell layer [GCL], and nerve fiber layer [NFL]), and had developed a complex morphology with processes extending mainly through the inner plexiform layer (Figs. 4, 5). Of note, faint EGFP-fluorescence was also associated with fascicles of RGC axons in the nerve fiber layer (Figs. 4a, 4f). At the injection sites, expression levels of GFAP were elevated in endogenous retinal astrocytes and Müller cells 1 and 4 weeks after transplantation (see Supplementary Material and Supplementary Figs. S1B, S1D).

Immunohistochemical analysis of retinas revealed small patches of MBP- or MAG-immunoreactivity in the nerve fiber layer as early as 1 week after transplantation in 4 out of 6 and 3 out of 4 animals that received PDGF-neurosphere grafts at postnatal day 3 (P3) and P6, respectively (see Supplementary Material and Supplementary Fig. S1F). Four to seven weeks after transplantation, in comparison, MBP- or MAG immunoreactivity was detectable in large areas of the nerve fiber layer in 103 (80.5%) of the totally 128 retinas with grafted PDGF neurospheres (Figs. 4, 5). Obvious differences in the extent and pattern of MAG or MBP immunoreactivity between animals analyzed at different post-transplantation intervals were not observed. Many of the EGFP-positive donor cells in these retinas expressed MAG in their cell bodies (Figs. 4c–h), demonstrating that a significant fraction of the grafted cells had differentiated into oligodendrocytes. These donor-derived oligodendrocytes extended their processes preferentially toward the nerve fiber layer (Figs. 4c–h), even when their cell bodies were located in the inner plexiform layer at some distance from the nerve fiber layer (Figs. 4c–e). In control animals that received intraretinal injections of PBS, MAG, and MBP immunoreactivity was not detected in the nerve fiber layer, but was restricted to the distal region of the optic nerve (see Supplementary Material and Supplementary Fig. S2). The grafted PDGF-neurosphere cells also differentiated into GFAP-positive astrocytes (see Supplementary Material and Supplementary Figs. S1A–D), while evidence for a neuronal differentiation of the donor cells was not observed.

MBP and MAG immunoreactivity in the nerve fiber layer of host retinas was closely associated with fascicles of RGC axons

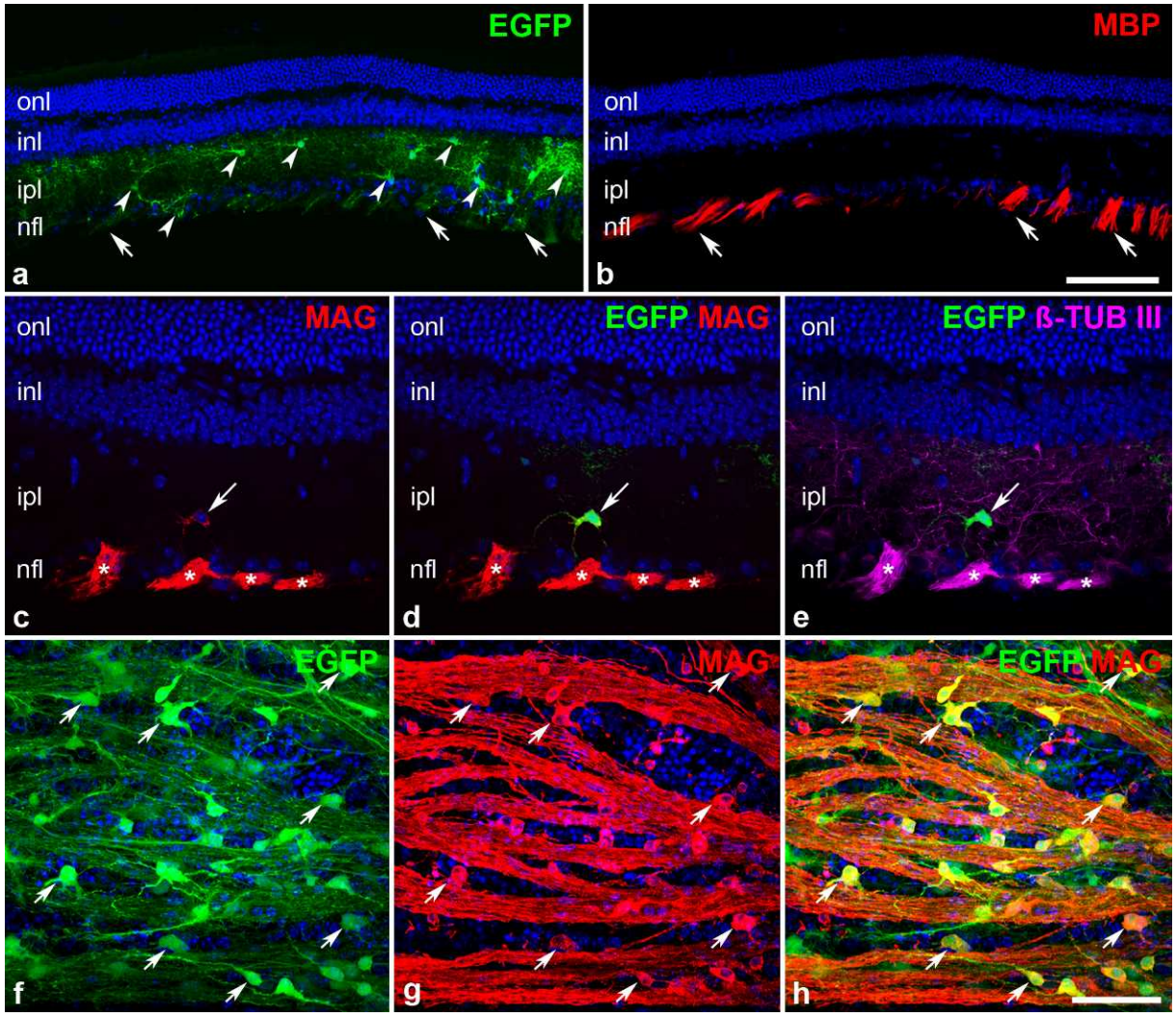

FIGURE 4. Intraretinally grafted PDGF-neurosphere cells integrate into the retina and differentiate into myelin-forming oligodendrocytes. Analysis of retinas with grafted small-sized PDGF neurospheres revealed the presence of EGFP-positive donor cells (some cell bodies are labeled with *arrowheads* in [**a**]) in the inner plexiform layer, ganglion cell layer, and nerve fiber layer. Faint EGFP-fluorescence was also associated with axon fascicles in the nerve fiber layer (**a**) where it was colocalized with MBP immunoreactivity ([**b**] some EGFP- and MBP-positive axon fascicles are marked with *arrows* in [**a**] and [**b**]), indicative for the presence of myelinated ganglion cell axons. Association of MAG immunoreactivity (**c**, **d**) with β-tubulin III-positive (**e**) fascicles of RGC axons (marked with *asterisks* in [**c**–**e**]) also suggested myelination of the nerve fiber layer. Colocalization of MAG and EGFP in the cell body (*arrow* in [**c**–**e**]) identified a donor-derived oligodendrocyte in the inner plexiform layer. Note that the cell extended processes exclusively toward the MAG-positive nerve fiber layer (**c**–**e**). Numerous EGFP-positive donor cells and EGFP-fluorescence in association with axon fascicles were also detectable in flat-mounted retinas (**f**). Colocalization of EGFP fluorescence with MAG immunoreactivity (compare [**f**] and [**g**]) identified a fraction of donor cells as myelin-forming oligodendrocytes (some labeled with *arrows* in [**f**–**h**]). (**h**) is the overlay of (**f**) and (**g**). Retinas were analyzed 6 (**a**, **b**), 5 (**c**–**e**), and 4 (**f**–**h**) weeks after transplantation of PDGF-neurospheres into 4-, 7-, and 3-day-old mice, respectively. INL, inner nuclear layer; ONL, outer nuclear layer. *Scale bar* in (**b**) for (**a**, **b**): 100 μm; in (**h**) for (**c**–**h**): 50 μm.

(Figs. 4, 5). Double immunostainings with antibodies to β-tubulin III confirmed the close association of both myelin-specific markers with RGC axons (Figs. 4c–e), and strongly suggested myelination of RGC axons by donor-derived oligodendrocytes. Myelination of retinal cell types other than RGCs was not observed. Electron microscopic analysis confirmed the presence of numerous RGC axons in the nerve fiber layer that were surrounded by morphologically normal central nervous system myelin sheaths (see Supplementary Material and Supplementary Fig. S3).[28,36] Ultrastructural evidence for myelination of RGC axons by Schwann cells (i.e., the presence of myelinating glial cells that had established 1:1 ratios with axons or of myelin sheaths that were covered by a basal lamina) was not detected.

Myelination of the Intrabulbar and Proximal Retrobulbar Region of the Optic Nerve

In 69 (67%) of the 103 retinas with a myelinated nerve fiber layer, myelin extended close to the optic disc. To analyze these eyes for the presence of donor-derived myelin in the normally nonmyelinated intrabulbar and most proximal retrobulbar part of the optic nerve, longitudinal sections of optic nerves with attached retinas were stained with anti-MBP or anti-MAG antibodies. In 17 (24.6%) of the 69 animals with a myelinated nerve fiber layer at the optic disc, myelinated RGC axons extended into the normally nonmyelinated intrabulbar part of the optic nerve, but not into the most proximal retrobulbar region of the nerve, resulting in a myelin-free gap between donor-derived myelin in the retina and endogenous myelin in

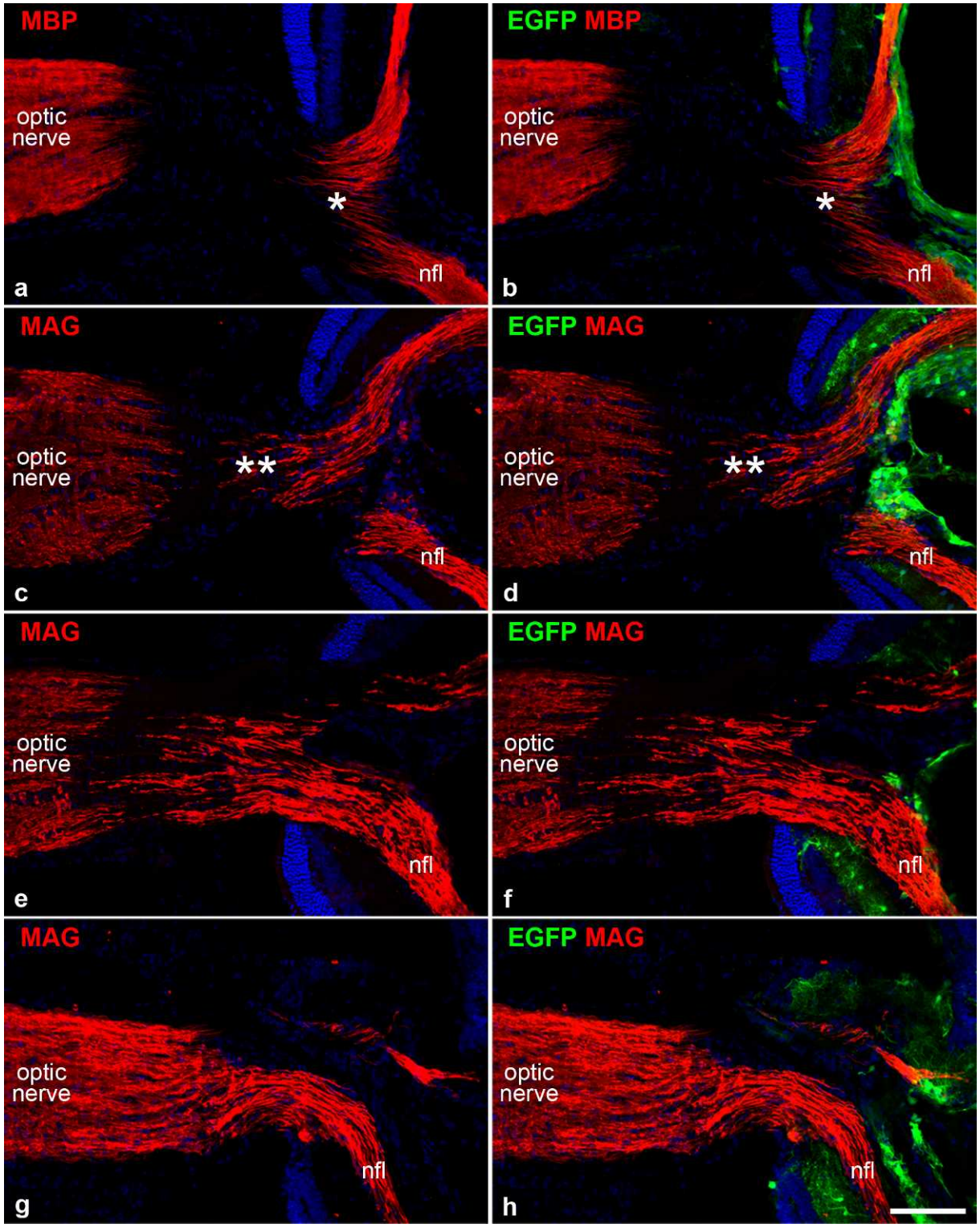

FIGURE 5. Myelination of the normally nonmyelinated nerve fiber layer and intrabulbar and proximal retrobulbar region of the optic nerve. Longitudinal sections of optic nerves with attached retinas were stained with antibodies to myelin basic protein (**a**, **b**) or myelin-associated glycoprotein (**c**–**h**) to visualize myelinated retinal ganglion cell axons. Representative examples of retinas with a myelinated nerve fiber layer (**a**–**h**) and numerous EGFP-positive donor cells (**b**, **d**, **f**, **h**) at the optic disc are shown. In some animals, donor-derived myelin in the nerve fiber layer extended into the normally nonmyelinated intrabulbar region of the optic nerve (*asterisk* in [**a**, **b**]). In other animals, a few myelinated ganglion cell axons were present in the most proximal retrobulbar region of the optic nerve (*two asterisks* in [**c**, **d**]). In a fraction of mice, donor-derived myelin in the retina was in direct continuity with the endogenous myelin in the optic nerve (**e**–**h**). Animals were analyzed 4 (**a**, **b**), 5 (**c**–**f**), and 7 (**g**, **h**) weeks after transplantation of PDGF neurospheres into (**a**, **b**) 4-, (**c**–**f**) 7-, and (**g**, **h**) 3-day-old mice. *Scale bar* in (**h**) for (**a**–**h**): 100 μm.

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the optic nerve (Figs. 5a, 5b). In another 12 (17.4%) animals, myelinated RGC axons extended into the normally nonmyelinated retrobulbar region of the nerve, leaving only a narrow myelin-free gap between the donor-derived myelin and the endogenous myelin in the optic nerve (Figs. 5c, 5d). Finally, in totally 22 (31.9%) of the 69 eyes, the donor-derived myelin in the retinal nerve fiber layer was in direct continuity with the endogenous myelin in the optic nerve (Figs. 5e–h). In 14 eyes, only a few myelinated RGC axons extended from the retina up to the normally myelinated distal part of the optic nerve. In eight animals, however, the normally nonmyelinated intrabulbar and retrobulbar regions of the nerve were massively myelinated, making it difficult to define the transition zone between the donor-derived myelin and the endogenous myelin in the optic nerve (Figs. 5g, 5h).

Differences in the pattern of myelination between animals that received PDGF-neurosphere grafts at different developmental ages were not apparent. From the total 60 and 43 animals that received injections of PDGF-neurospheres at P3 or P4 and P5 to P7, intraretinal myelination was observed in 44 and 25 eyes, a myelinated nerve fiber layer close to the optic disc in 35 and 16 eyes, myelinated axons extending into the intrabulbar part of the optic nerve in 9 and 8 eyes, myelinated axons extending into the retrobulbar part of the optic nerve in 9 and 3 eyes, and myelinated axons extending up to the endogenous myelin in the optic nerve in 17 and 5 eyes, respectively.

Discussion

Abnormal myelination of the normally non-myelinated retinal nerve fiber layer by Schwann cells or oligodendrocytes has been observed in various species, including humans,[11–17,20–22] and also occurs after intraocular transplantations of myelinogenic cells.[23–26,28,29] Based on these observations, it has been suggested that nonneuronal factors located at the retinal end of the optic nerve prevent migration of oligodendrocyte progenitor cells into the retina, and as a consequence intraretinal myelination.[4,6,8,24] However, it is not known whether RGC axon segments within the normally nonmyelinated intrabulbar and retrobulbar regions of the optic nerve also become myelinated under pathological or experimental conditions. In fact, these axon segments may be refractory to myelination and may thereby restrict oligodendrocytes and myelin to the distal region of the nerve. To experimentally address the possibility that the differential distribution of oligodendrocytes and myelin in the primary visual pathway is under neuronal control, we extended our previous work[24,28] and grafted neurosphere cells into the developing mouse retina close to the optic disc.

Transplantation experiments were performed with small-sized neurospheres that were cultivated for a short time period in a medium supplemented with PDGF and FGF-2 to increase the percentage of oligodendrocyte lineage cells.[39,40] Compared with neurospheres expanded in the presence of EGF[28] or FGF-2 and EGF,[36,37] and to PDGF-neurospheres that were dissociated into single cell suspensions prior to transplantation (the present study), we observed a markedly increased capability of the grafted cells to integrate into host retinas and to differentiate into myelinating oligodendrocytes. In fact, we observed the first myelinated axons in the nerve fiber layer as early as 1 week after transplantation, and widespread myelination of the nerve fiber layer in approximately 80% of the experimental animals 4 to 7 weeks after transplantation. We suggest that this high myelinogenic potential of grafted PDGF neurospheres is the result of both, an increased number of oligodendrocyte lineage cells present in the PDGF-treated neurosphere cultures and a better survival of cells when grafted as small-sized spheres as opposed to single-cell suspensions.

Differentiation of a significant fraction of the intraretinally grafted PDGF-neurosphere cells into mature oligodendrocytes was confirmed by the colocalization of EGFP fluorescence and MAG immunoreactivity in their cell bodies and processes. EGFP fluorescence was also associated with myelinated RGC axons, although it decreased to very low levels as a result of myelin compaction. These observations together with the fact that we never observed intraretinal myelination in animals that received injections of the vehicle only, strongly suggested that the intraretinal myelin was donor-derived. Furthermore, ultrastructural studies revealed that RGC axons were surrounded exclusively by central nervous system myelin sheaths, thus excluding the possibility that infiltrating Schwann cells had myelinated the nerve fiber layer of the host retinas.

In totally 69 of the 103 eyes with a myelinated nerve fiber layer, myelinated RGC axons extended close to the optic disc. Further analysis of the proximal optic nerves revealed the presence of myelinated RGC axons in the intrabulbar region of the nerves in 51 animals. In 34 of these eyes, myelinated RGC axons extended further into the retrobulbar region of the nerves. Of note, in 22 of the 34 animals, myelinated RGC axons extended from the nerve fiber layer throughout the entire length of the intrabulbar and proximal retrobulbar region of the optic nerve to the endogenous myelin in the distal nerve. In 14 eyes, only a few myelinated RGC axons extended from the nerve fiber layer up to the normally myelinated distal region of the nerve. In eight eyes, however, myelination of the entire proximal part of the nerve was extensive, precluding a clear identification of the normal transition zone between the myelinated and nonmyelinated region of the optic nerve. Results thus unequivocally demonstrate that the entire length of retinal ganglion cell axons is competent to become myelinated, and further support the view that the differential distribution of oligodendrocytes and myelin in the primary visual pathway is controlled by a nonneuronal barrier for migratory oligodendrocyte progenitor cells at the retinal end of the nerve, rather than by the RGCs themselves.

We attempted to clarify whether myelination of the intrabulbar and most retrobulbar region of the optic nerve correlated with the presence of oligodendrocyte cell bodies in these regions of the nerve. Although we occasionally found EGFP-positive cell bodies and cell bodies containing proteolipid protein-specific transcripts in the experimentally myelinated proximal region of a few nerves, such cells were not detectable in the vast majority of animals. Given that a single oligodendrocyte is capable of myelinating a large number or RGC axons in the mouse optic nerve,[41,42] it is possible that the myelin in the retinal end of the nerve had been formed by only a few oligodendrocytes that have escaped our analysis. In fact, due to the technical problems associated with the histological analysis of the small proximal region of the nerve, we were only rarely able to evaluate a complete series of optic nerve sections from one eye. We thus cannot exclude the possibility that OPCs in the retina, similar to OPCs in the optic nerve, are prevented from entering the most proximal region of the optic nerve, and that the intrabulbar and most proximal retrobulbar regions of the nerve had been myelinated by oligodendrocytes that are located with their cell bodies in the retina close to the optic disc.

One might argue that the grafted oligodendrocyte lineage cells had access to the most proximal end of the optic nerve before the hypothetical barrier for oligodendrocytes had formed, and were therefore capable of myelinating the normally nonmyelinated intrabulbar and retrobulbar segments of RGC axons. Myelination of the proximal part of

the optic nerve was indeed frequently observed after intraretinal transplantations of PDGF-neurospheres into 3- and 4-day-old animals. However, myelination of the intra- and proximal retrobulbar region of the optic nerve was also found in eyes that received PDGF-neurosphere grafts between postnatal day 5 and 7, developmental ages at which endogenous oligodendrocyte progenitor cells have already reached the prospective myelin border at the retinal end of the nerve.[4,5] These observations thus imply that the intrabulbar and proximal retrobulbar region of the optic nerve can be myelinated by oligodendrocytes from the retinal side at developmental ages when endogenous oligodendrocytes in the optic nerve are prevented from myelinating this region of the nerve. A barrier with such properties is most easily explained with the presence of one or more factors that are expressed as a gradient at the retinal end of the optic nerve and either allow or prevent myelination of RGC axons, depending on the slope oligodendrocyte lineage cells encounter this gradient.

In summary, we have shown that intraretinal transplantations of PDGF-treated, small-sized neurospheres into young postnatal mice resulted in extensive myelination of the retinal nerve fiber layer. The normally nonmyelinated intrabulbar and most proximal retrobulbar regions of the optic nerve became also myelinated in a fraction of animals. Results thus demonstrate that the entire proximal segments of RGC axons are competent to become myelinated, and further support the view that nonneuronal factors located at the retinal end of the optic nerve prevent oligodendrocytes from myelinating the most proximal segment of the optic nerve and the retina.

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