Adaptive Plasticity of *Xenopus* Glial Cells In Vitro and After CNS Fiber Tract Lesions In Vivo

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ABSTRACT *Xenopus* oligodendrocytes and aspects of their differentiation were analyzed in vitro and in vivo using cell- and stage-specific antibodies. Undifferentiated oligodendrocytes were derived from optic nerves or spinal cords. They divided in vitro, were of elongated shape, were glial fibrillary acidic protein and O4 positive, transiently exhibited several antigens including HNK-1 and L1, and promoted axon growth as do Schwann cells. With forskolin they differentiated and, much like myelin-forming oligodendrocytes in the intact optic nerve and spinal cord, they expressed sets of advanced myelin markers. These advanced myelin markers disappeared from the regenerating optic nerve 4 weeks after lesion. The optic nerve instead was populated by cells with radial processes and somata in the center of the nerve; among them were cells and processes that were O4 positive and that are suspected to represent undifferentiated oligodendrocytes. Where processes of these cells reached to the retinal axons in the nerve's periphery, advanced myelin markers typical of differentiated oligodendrocytes reappeared 8 weeks after lesion. These glial changes did not occur in the absence of retinal axons. Thus, the apparent capability of *Xenopus* oligodendrocytes to adapt to the transient absence, reappearance, and regenerative state of the axons enables them to contribute to central nervous system fiber tract repair. This occurs in the lesioned optic nerve but not in the spinal cord, where no such glial changes were observed and where axons fail to regenerate. © 1996 Wiley-Liss, Inc.

INTRODUCTION

The glial cell contribution to success or failure of axon regeneration has been recognized in a variety of species and systems. Axon regeneration is successful in the vertebrate peripheral nervous system (PNS) where regrowth of axons is promoted by Schwann cells (Bunge and Hopkins, 1990) but fails in the central nervous system (CNS) of mammals and birds where oligodendrocytes and reactive astrocytes impair axon regrowth (Reier and Houle, 1988; Schwab et al., 1993). The growth-inhibiting influence of CNS glia is also present in the spinal cord of the frog *Xenopus* (Lang et al., 1995) and correlates—as in mammals—with lack of axon regrowth, but is absent from the *Xenopus* visual system (Lang et al., 1995) and from the fish CNS (Bastmeyer et al., 1991; Wanner et al., 1995). Accordingly, in both

the CNS of fish and the visual system of amphibians, lesioned axons regenerate successfully (Gaze, 1970). In the case of fish, oligodendrocytes apparently adapt to the state of the axons (Ankerhold et al., 1995) much like Schwann cells in mammalian PNS. Schwann cells and fish oligodendrocytes are capable of dedifferentiating upon axon injury, of providing growth-supportive molecules, and of subsequently redifferentiating for axon remyelination (Bastmeyer et al., 1993, 1994; Bunge, 1993; Schwab et al., 1996; Wolburg, 1981). The expression of proteins involved in myelination is induced by axon contact, and this expression can, in the

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case of Schwann cells, be simulated by agents (e.g., forskolin) that elevate the intracellular concentration of cAMP (Lemke and Chao, 1988; Morgan et al., 1991). As regenerated axons in vivo become remyelinated, the lesioned systems can recover their proper functions. Such an adaptation to the state of the system is not observed in oligodendrocytes of mammals. Oligodendrocytes in the adult mammalian CNS appear to be less responsive to the state of the axons (Raff and Miller, 1984) and fail to undergo the range of changes observed in Schwann cells and fish oligodendrocytes in response to axon lesion. Thus, repair of lesioned fiber tracts including axon regeneration and remyelination is paralleled by and may therefore depend on timely responses of glial cells as exemplified by Schwann cells and fish oligodendrocytes.

Since retinal axons do regenerate in frogs and restore vision, glia in optic nerves of frogs are therefore expected to respond to deprivation from axons, to assist axonal regrowth, and to be capable of remyelination. That *Xenopus* glial cells indeed undergo changes when the optic nerve is severed has in fact been demonstrated earlier (Bohn and Reier, 1985; Reier and Webster, 1974). The somata of *Xenopus* glial cells cluster in the center of the nerve and, reminiscent of radial or ependymoglia, extend long centropерipherally oriented processes to the outer perimeter. The regenerating axons take abnormal routes in that they course through the periphery of the nerve and orthogonally to the glial processes. The regenerated axons later become remyelinated, but apparently by glial cells whose somata retained their central position in the nerve. This indicates the presence of oligodendrocytes, but the fate of oligodendrocytes and their contribution to the glial organization and axon regeneration in the lesioned nerve remained unclear.

In the present study oligodendrocytes from the frog CNS were raised in culture to characterize their properties when deprived of axons and during differentiation with a battery of cell- and stage-specific antibodies. Oligodendrocytes resemble Schwann cells in several respects, including their axon growth-supportive properties. With application of the relevant markers to the lesioned optic nerve we have identified elongated glial cells with centropерipheral oriented processes as undifferentiated oligodendrocytes in situ. At early stages of axon regeneration, they are labeled by O4 antibody but do not express advanced oligodendrocyte/myelin-specific molecules. At later stages, however, advanced oligodendrocyte/myelin markers reappear in association with the regenerated axons in the peripheral portion of the optic nerve, indicating that frog optic nerve oligodendrocytes differentiate to remyelinate the axons. They are thus able to adapt to the state of the retinal axons. These changes were not observed in the case of oligodendrocytes in the spinal cord, where axon regeneration does not occur (Beattie et al., 1990; Forehand and Farel, 1982; Lang et al., 1995).

MATERIALS AND METHODS

Surgery

Xenopus laevis tadpoles and frogs were bred and raised at the animal research unit of the University of Konstanz. All surgery was in compliance with animal welfare legislation. Prior to operations, young frogs (6 months old) were anesthetized in MS 222 (Sigma) and kept on ice for the duration of the surgical procedure to minimize bleeding. For optic nerve transections, the optic nerve was approached through a small incision in the roof of the mouth, the surrounding muscle tissue and blood vessels were gently teased away, and the nerve was cut with iridectomy scissors. Care was taken not to injure the blood vessels supplying the retina. The spinal cord was transected in frogs (6 months old) by exposing the vertebral column between vertebrae 3 and 4 and suturing the wound.

Glial Cell Cultures

Glial cell cultures were obtained from optic nerves, spinal cord, or sciatic nerves of tadpoles (stage 51–60; Niewkoop and Faber, 1956) or 6 month old frogs. In a few animals, a conditioning lesion was made in the optic nerve 2 weeks prior to preparation of the glia cultures (Agranoff et al., 1967). The glial cells obtained from lesioned optic nerves closely resembled those of normal nerves. No difference was found between glial cells obtained from frogs or tadpoles. In frogs, axons were already myelinated (Lang et al., 1995; Maier and Miller, 1995), whereas most of those in tadpoles were not.

The animals were terminally anesthetized with an overdose of MS 222. Optic nerves, spinal cord, and sciatic nerves were dissected in sterile Leibovitz medium (L15, Gibco). Care was taken to remove connective tissue and meninges. The nerves and cord were cut into small fragments on a McIlwain tissue chopper, transferred to L15 containing 20% fetal calf serum (FCS), and allowed to settle. The supernatant was discarded. Nerve fragments were then taken up in culture medium, consisting of DMEM/F12 (1:1, Gibco) supplemented with 5% FCS, 0.4% methyl cellulose, 15 mM HEPES, 50 µg/ml transferrin, 5 µg/ml bovine insulin, 100 µM putrescine, 30 nM sodium selenite, 20 nM progesterone (all from Sigma), and 50 µg/ml gentamycin (Gibco). The nerve fragments were placed on polylysine/laminin-coated glass coverslips (500 µl suspension/coverslip) and held in place by a second, uncoated coverslip, separated from the tissue by stainless steel spacers. The sandwich cultures were kept in plastic Petri dishes in a humidified chamber at 22°C in an incubator. Glia cells emigrate from these fragments and multiply. The culture medium was partially exchanged every 3–4 d, and cultures were maintained over several weeks.

Retinoic acid, thyroid hormone (T3), or forskolin (all from Sigma) were added to cultures at concentrations of 1–10 µM, 3–30 nM, and 10–100 µM, respectively, to determine their influence on glial cell differentiation.

The cells were grown in the presence of these agents for at least 7 d. Some cultures were analyzed between 2 and 7 d after forskolin was withdrawn from the medium.

CNS- and PNS-derived glial cells were grown in the presence of 10 μ M bromodeoxyuridine (BrdU) for 72 h (Nowakowski et al., 1983) and then fixed and processed for immunocytochemistry as described below. This duration of BrdU treatment allows us to gain an impression of the rate of divisions of precursors (which was high) and, more importantly, also allowed us to show the absence of BrdU incorporation from differentiating (round) oligodendrocytes.

Axon Outgrowth Assay on Glial Cells

Optic nerve, spinal cord, or sciatic nerve explants (prepared as above) were distributed over polylysine-coated 96-well dishes (Nunc). After 7 d in culture, the dishes were shaken vigorously for 2 min and rinsed twice with DMEM/F12 medium. The original explants were dislodged, leaving behind glial cells that had emigrated from the explants. Low-density glial cell carpets were thus obtained, which facilitated counting of axons in coculture assays.

Retinal explants were prepared from tadpoles and frogs that had not received a conditioning lesion of their optic nerves. Retinae were isolated in L15, freed of the pigment epithelium, and flattened with suction on nylon filters. The retinae were cut into squares of $200 \times 200 \mu$ m, rinsed twice in L15 containing 20% FCS, and resuspended in DMEM/F12 culture medium with the above supplements. Retinal explants were added to the wells containing the glial cells or, in control experiments, placed into polylysine-coated wells without cells. These cultures were kept for 48 h at 22°C and fixed in 5% glutaraldehyde in 0.1 phosphate buffer. The number of axons that had emerged from each retinal explant were counted using phase contrast optics (Lang et al., 1995).

Immunostaining of Glial Cells

Glial cells were subjected to immunostaining procedures as described previously for mammalian or fish glial cells (Bastmeyer et al., 1991; 1994). Monoclonal antibodies (MAbs) A2B5 recognizing O-2A progenitor cells in mammals (Raff et al., 1983; Raff and Miller, 1984; dilution 1:2), 6D2 against fish myelin proteins IP1/IP2 (Jeserich and Waehneldt, 1986; Jeserich and Rauen, 1990; dilution 1:5), 192-IgG against the low-affinity nerve growth factor (NGF) receptor (gift of D. Lindholm; dilution 1:2), O4 as well as O1 (Sommer and Schachner, 1981; dilution 1:5), and anti-galactocerebroside (GalC; Ranscht et al., 1982; dilution 1:20) were applied to live cells. Fixed cells were exposed to MAbs against the peripheral myelin protein Po (gift of J. Archelos; dilution 1:500), the frog-specific myelin marker

"Olig"-antigen (Steen et al., 1989; dilution 1:10), glial fibrillary acidic protein (GFAP; clone G-A-5; Debus et al., 1983; dilution 1:500) and to phosphorylated neurofilaments (SMI-31; Sternberger-Meyer Immunochemicals; dilution 1:500). Fixed cells were also exposed to MAb HNK-1/L2 (Keilhauer et al., 1985; dilution 1:10) and MAb 6C2 against myelin basic protein (MBP; gift of C. Linington; dilution 1:5) to rabbit antisera against fibronectin (FN; Telios; dilution 1:500), the myelin-associated glycoprotein (MAG; provided by M. Schachner; dilution 1:200), proteolipid protein (PLP; gift of C. Linington; dilution 1:400), the astrocyte and Schwann cell marker protein S-100 (Dakopatts; dilution 1:100), and to an antiserum against the cell adhesion molecule L1 (gift of F. Rathjen; dilution 1:1000). The specificity of antibodies against the mammalian myelin proteins MBP, PLP, MAG, and Po and as well as MAb 6D2 against the fish myelin proteins IP1 and IP2, was tested on immunoblots with *Xenopus* CNS- and PNS-derived myelin and on tissue sections through the *Xenopus* nervous system (Lang et al., 1995). All substances except 6D2 against IP1 and IP2 (see Results) recognized proteins of the appropriate sizes and selectively stained myelin of the CNS and PNS, respectively.

Cells were fixed in methanol (5 min, -20°C) and 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) containing 10% sucrose, rinsed in PBS (3×5 min), and incubated with the primary antibodies [diluted in PBS containing 1% bovine serum albumin (BSA)] in a humidified chamber (2h; 37°C). Secondary antibodies were goat anti-mouse conjugated with tetramethylrhodamine isothiocyanate (TRITC) for fluorescein (FITC; dilution 1:200), donkey anti-rat-carboxymethylindocyanine (Cy-3; preadsorbed against mouse proteins; dilution 1:1,000), or goat anti-rabbit-TRITC or FITC (dilution 1:200; all from Dianova), which were used in suitable combinations for double immunofluorescence. The cultures were rinsed ($3 \times$ in PBS), incubated briefly (5 min) with 0.5 $\mu\text{g}/\text{ml}$ diamidinophenylindole (DAPI; Sigma) in PBS to visualize cell nuclei, rinsed in PBS, and mounted in Mowiol (Hoechst), containing n-propylgalate (Sigma) as an anti-fading agent. For staining of living glial cells, the cultures were incubated with the relevant antibodies diluted in L15 for 1 h at 4°C prior to fixation and exposure to secondary antibodies.

In some cases the Vectastain ABC kit (Vector Laboratories) was employed to detect immunoreactivity. Immunostaining was performed according to the manufacturer's instructions. To block non-specific antibody binding, the fixed glial cultures were incubated in 5% normal serum in PBS prior to addition of primary antibodies. In controls, primary antibodies were omitted. Staining was visualized using a Vector aminoethylcarbazole (AEC) substrate kit.

BrdU-treated glial cultures were subjected to a staining procedure allowing the simultaneous visualizing of O4 and BrdU immunoreactivity (Bastmeyer et al., 1994; Nowakowski et al., 1989). After treatment of live cells

Fig. 1. In vitro characterization of *Xenopus* glial cells derived from optic nerve and spinal cord. **a:** MAb 6D2 labels elongated undifferentiated oligodendrocytes but not the round differentiated oligodendrocytes (arrowheads). **b:** The elongated undifferentiated cells (in contrast to their round and differentiated counterparts in the center) are S-

100 positive. **c:** The elongated undifferentiated cells and the round and spread-out differentiated oligodendrocytes are both stained by O4. **d:** Elongated undifferentiated and differentiated oligodendrocytes (see Fig. 4) express GFAP. Scale bar = 100 μ m.

with MAb O4 (IgM), cultures were fixed in methanol and 4% PFA, washed in PBS, and incubated in goat anti-mouse-FITC secondary antibodies. After postfixation (2 min) in PFA, the cultures were exposed to 2 N HCl (10 min) and rinsed repeatedly in 100 mM borate buffer and PBS containing 1% BSA and 0.025% Triton X-100. MAb against BrdU (IgG; Becton-Dickinson) was added to the cultures at a dilution of 1:100 in PBS/BSA/Triton X-100 (2 h; 37°C), which were then exposed to a TRITC-conjugated IgG-specific goat anti-mouse antibody (1:200; Dianova) for 2 h at 37°C. Cultures were counterstained with DAPI and mounted as described above.

Immunostaining of Cryosections

Normal and transected optic nerves were immersed in TissueTek (Miles Inc.) and frozen in liquid nitrogen. Cryostat sections (10 μ m) were air dried for 1–2 h on

Vectabond-treated slides (Vector Laboratories). For immunohistochemical detection of myelin proteins (with antibodies to MAG, MBP, PLP, and "Olig"-antigen) sections were fixed in Clark's solution (95% ethanol, 5% acetic acid) for 25 min at 4°C. Sections to be stained with MAbs O4, O1, and anti-GalC were fixed in methanol (–20°C) over 1 min. Those to be stained with anti FN, GFAP, S-100, and SMI-31 were fixed for 5 min.

To prevent nonspecific binding, sections were blocked for 30 min with PBS containing 1% BSA. Incubation with primary antibodies was for 2 h at 37°C or overnight at 4°C as described above. Secondary antibodies were Cy-3-conjugated donkey anti-mouse IgG or IgM, donkey anti-rat, or donkey anti-rabbit (all from Dianova) to which sections were exposed for 2 h (37°C) at a dilution of 1:1,000. Sections were counterstained with DAPI and coverslipped under Mowiol containing n-propylgalate.

In some cases, cryosections were processed for immu-

Fig. 2. Elongated undifferentiated oligodendrocytes (**b**, phase contrast) are labeled by the HNK-1 antibody (**a**), carry the cell adhesion molecule L1 (**c**), and are O4 positive (**d**). O4-positive differentiated oligodendrocytes are HNK-1 and L1 negative (a,c). Scale bar = 100 μ m.

Fig. 3. Undifferentiated oligodendrocytes (phase contrast in **a**) incorporate BrDU (**b**), but highly branched oligodendrocytes (**a**), whose DAPI labeled nuclei are marked by arrowheads (a, b, and c) do not. Scale bar = 100 μ m.

Fig. 4. Highly branched oligodendrocytes (phase contrast in **c**) express GFAP (**a**) and MBP (**b**) in their membrane sheets between the GFAP-positive veins. Scale bars = 50 μ m. Anti-MAG (**d**) and anti-PLP (**e**) immunoreactivities of differentiated oligodendrocytes are visualized by the vectastain ABC kit. The densely stained irregular structures represent PLP-labeled myelin debris. Scale bar = 200 μ m.

nostaining according to the Vectastain ABC standard protocol. Immunoreactivity was visualized using a Vector diaminobenzidine (DAB) substrate kit.

RESULTS

Characterization of Undifferentiated and Differentiating *Xenopus* Oligodendrocytes In Vitro

To identify oligodendrocytes at various stages of differentiation after lesioning of the optic nerve and spinal cord, and to evaluate their properties, they were first characterized in vitro. Whether derived from already myelinated tracts of frogs or from tracts of tadpoles at premyelination stages (Lang et al., 1995; Maier and Miller, 1995), glial cultures from optic nerves and spinal cord at 14 d in vitro contained predominantly undifferentiated oligodendrocytes and a few differentiating oligodendrocytes interspersed between them. GFAP-

positive astrocytes, which were negative for oligodendrocyte/myelin-specific markers, formed clusters and eventually detached from the substrate.

Undifferentiated oligodendrocytes exhibited an elongated bipolar shape reminiscent of axon-deprived Schwann cells. They were O4 positive and contained GFAP-positive fibrils (Fig. 1) but did not express O1/Gal-C, which was, however, present on round differentiating oligodendrocytes (Lang et al., 1995). They shared further molecular components with Schwann cells (see Fig. 5), which were lost on differentiation. These were S-100 protein (Fig. 1) (Mirsky and Jessen, 1990), the HNK-1/L2 epitope, the cell adhesion molecule L1 (Fig. 2) (Keilhauer et al., 1985; Künemund et al., 1988), and a protein recognized by MAb 6D2 (Fig. 1), which differs from the fish myelin proteins IP1/IP2 of 27 and 29 kd, respectively (Jeserich and Waehneldt, 1986; Jeserich and Rauen, 1990), against which 6D2 was made. In immunoblots with *Xenopus* myelin 6D2 recognized a band of approximately 100 kd. The identity of this frog

Fig. 5 *Xenopus* Schwann cells are labeled by O4 (a) and express GFAP (b) and low-affinity NGF receptor (c, shown by the typical punctate staining). Phase contrast (d) of (c). After exposure to forskolin, Schwann cells acquire a morphology reminiscent of oligodendrocytes and express Gal-C (e) and Po (f, demonstrated by the Vectastain ABC kit). Scale bar = 200 μ m.

myelin protein is presently unknown but is of interest since it appears to be developmentally regulated. In contrast to mammalian glia precursors of the O₂A lineage (Raff et al., 1983), undifferentiated *Xenopus* oligodendrocytes were A2B5 negative. While most of the undifferentiated oligodendrocytes incorporated BrdU even after 14 d in vitro, O1/Gal-C-positive, round oligodendrocytes did not (Fig. 3). During their differentiation into O1/Gal-C-expressing cells, which was induced by forskolin (Lang et al., 1995), oligodendrocytes lost HNK-1/L2, L1, and 6D2 immunoreactivity and later acquired the myelin markers characteristic of mature oligodendrocytes—MBP, PLP, MAG (Fig. 4), and the “Olig” antigen (Steen et al., 1989). They retained GFAP (Fig. 4), which is unusual for differentiated oligodendrocytes (Raff et al., 1983). GFAP was restricted to the thickened

veins of the cell, while myelin marker proteins were found in the membraneous sheets between them.

Xenopus Schwann cells, being O4 and GFAP positive, also acquired O1/Gal-C (Fig. 5) and MAG on exposure to forskolin (Lemke and Chao, 1988; Morgan et al., 1991) but expressed Po instead of PLP and carried low-affinity NGF receptors (Fig. 5). This clearly distinguishes Schwann cells from *Xenopus* oligodendrocytes.

Both undifferentiated *Xenopus* oligodendrocytes and Schwann cells were able to support axon outgrowth when they were cocultured with retinal explants (Fig. 6). When derived from frogs that had not received a conditioning optic nerve lesion, explants showed little or no outgrowth on polylysine. (Eighteen explants had 0 axons, five had less than 20, and one explant more than 20 axons.) Substantial outgrowth from retinal ex-

Fig. 6. Retina mini-explants on polylysine (a) after 2 d in vitro fail to extend axons, but axons do extend (b) in the presence of CNS-derived glial precursor cells (arrowheads). Videoprints; vertical bars 100 μ m.

plants is only seen on polylysine when the optic nerve has received a conditioning lesion several days prior to retina explantation (Agranoff et al., 1967). However, when cocultured with Schwann cells or with optic nerve- or spinal cord-derived oligodendrocytes, all explants extended axons, and the majority had more than 20 axons. (On glial precursor cells of the CNS, 16 explants of 23 had more than 20 axons. Of 23 explants on sciatic nerve-derived Schwann cells, 15 gave rise to more than 20 axons). These axons expressed HNK-1 and L1 molecules, which could conceivably participate in axon-glia interactions (Bastmeyer et al., 1993; Keilhauer et al., 1995; Kühnemund et al., 1988).

On forskolin-induced differentiation and the concurrent loss of the developmentally regulated molecules, oligodendrocytes also lost their axon growth-stimulating properties (Lang et al., 1995). Retinoic acid and thyroid hormone (T3)—were ineffective in driving oligodendrocytes maturation. When cultures were subse-

quently deprived of forskolin and glial cells left therein for up to 7 d, oligodendrocytes remained in their differentiated state and did not revert to precursor stages. Thus withdrawal of the agent that induced oligodendrocyte differentiation is not sufficient for dedifferentiation.

Xenopus Glial Cells After Lesion of the Optic Nerve and Spinal Cord

Based on this in vitro characterization glial cell features in optic nerves and spinal cords of normal and lesioned frogs were analyzed. Special attention was paid to the optic nerve because it has been demonstrated that its glial cells undergo marked changes in morphology and spatial arrangement on lesion (Bohn and Reier, 1985; Reier and Webster, 1974).

In normal optic nerves of frogs (Fig. 7), retinal axons identified by the neurofilament marker SMI-31 occupied the entire cross section of the nerve (Reier and Webster, 1974) and were surrounded by structures immunoreactive to O4, GalC, MBP, PLP, and "Olig" representing myelin sheaths (Lang et al., 1995). GFAP staining was associated with distinct cellular processes lying between the paths of the axons. Glial cell bodies identified by the nuclear stain DAPI were scattered throughout the nerve. This spatial organization of markers indicative of axons, myelin, oligodendrocytes, and astrocytes (Maier and Miller, 1995) was in perfect agreement with an earlier light and electron microscopic description of these nerve components (Reier and Webster, 1974). Optic nerves 2 weeks after lesion were filled with myelin debris that stained with all myelin-specific antibodies including O4. Therefore, it was difficult to visualize and characterize the O4- and GFAP-positive glial cells with radial processes that had emerged intermittently (see below).

Four weeks after optic nerve transection (Fig. 8), the myelin markers GalC, MBP, PLP, and "Olig" were absent from the nerve (except for minor amounts of debris). This allowed us to recognize the abundance of O4- and GFAP-positive, radially oriented glia processes that emanated from somata in the center of the optic nerve. Consistent with the report of Reier and Webster (1974), DAPI-labeled glial cell somata were clustered in the center, and glial processes reminiscent of radial glia extended in a centropерipheral orientation. Cells and processes were GFAP positive but in adjacent sections radial processes were intensively stained by O4. It cannot be determined unequivocally from light microscopical analyses whether both these markers are coexistent in the same cells and processes. Regenerating and SMI-31-positive retinal axons were seen in the periphery of the nerve (Bohn and Reier, 1985; Reier and Webster, 1974). The core of the nerve contained hardly any axons, but more glial cell somata than normal nerves. Exposure of sections to antibodies HNK-1/L2, 6D2, anti-L1, and anti-S100 gave a diffuse and homogenous stain

Fig. 7. Consecutive longitudinal sections of a normal *Xenopus* optic nerve, with retinal axons revealed by the neurofilament marker SMI-35 (a). GFAP-positive glial cell processes are distributed across the nerve (b). The nerve displays the oligodendrocyte/myelin markers O4 (c), Gal-C (d), and PLP (e). Glial cell nuclei revealed by DAPI stain (f) are seen throughout the nerve (whose borders are marked by white arrows) and in neighboring tissue. The retina is to the left. Scale bar = 100 μ m.

across the nerve so that it was not possible to determine whether axons and/or glial cells carried the markers.

Eight weeks after optic nerve section (Fig. 9), when most axons have re-formed connections in the optic tectum (Taylor and Gaze, 1985) and myelin sheets have re-formed (Bohn and Reier, 1985; Reier and Webster, 1974), the myelin markers GalC, MBP, PLP, and "Olig" were present. These markers appeared in the periphery of the nerve in those regions where SMI-31-positive axons were located. No staining was obtained with anti-

Po in the lesioned (or normal) nerve, at either 2, 4, or 8 weeks, indicating that there was no Schwann cell invasion. Most glial cell somata, identified by nuclear DAPI stain, were still at the center of the nerve and were rare in among the axons, the position most common for myelin-forming oligodendrocytes. The nerve core remained mostly free of advanced myelin markers. Radial processes emanating from glial cells that were positive for O4 and GFAP persisted.

However, when retinal axons were prevented from

Fig. 8. Consecutive longitudinal sections of the optic nerve 4 weeks after lesion. Regenerating retinal axons labeled by SMI-35 (a) course preferentially in the peripheral portion of the nerve, and only a few pass through its central portion, where glial cell nuclei are clustered (f). Glial cells labeled by GFAP (b) and O4 (c) form centropерipherally oriented processes in the nerve's peripheral portion. Gal-C (d) and MBP (e) are absent from the nerve at this stage. Nuclear DAPI staining (f) Scale bar = 100 μ m.

invading the nerve (by removal of the eye 8 weeks prior to examination), the distribution of O4-positive cells in the optic nerve was different from that of GFAP-positive cells, and no advanced oligodendrocyte and myelin marker was present. Moreover, the glial cell somata were not clustered, and centropерipherally oriented processes were not observed (Fig. 10). Thus, the re-organization of glia cells including formation of centropерipheral processes and the acquisition of oligodendrocyte/myelin-specific markers apparently requires the presence of regenerating retinal axons.

These findings show the loss of advanced myelin markers, probably due to myelin breakdown, phagocytosis, and dedifferentiation or death of mature oligodendrocytes during retinal axon regeneration. They further demonstrate the emergence of O4-positive cells with radially oriented processes that either coexpress GFAP or coexist with morphologically similar cells expressing GFAP. O4-positive glial cells appear to represent undifferentiated oligodendrocytes, which, in the presence of axons, can apparently differentiate to oligodendrocytes expressing advanced myelin markers.

Fig. 9. Consecutive, oblique-longitudinal sections at 8 weeks after lesion. The regenerated axons, identified by SMI-35 (a) remain primarily in the nerve's peripheral portion. Glial cells labeled by GFAP (b) and O4 (c) persist throughout, and the radial orientation of their processes is visible in the obliquely cut sections. Sections deeper into

the nerve reveal the presence of Gal-C (d) and MBP (e) predominantly in the periphery of the nerve, where most of the regenerated axons are located. DAPI stain (f) of glial nuclei demonstrating their persistence in the nerve's center. Scale bar = 100 μ m.

The central position of most glial cell somata and the persistence of O4-positive radial processes suggest that the undifferentiated oligodendrocytes differentiate and express myelin markers where they are in contact with the axons.

No such changes were observed after transection of the spinal cord in frogs, 4 or 8 weeks after lesion. Four weeks after spinal cord transection, the lesion site was readily identified as a cell-free gap between the proximal and distal cord (Fig. 11). Descending axons in the

ventral half of the cord were visualized by SMI-31 and they ended near the lesion site. Myelin around the descending axons (Lang et al., 1995) appeared to persist, as demonstrated by the presence of Gal-C, MBP, "Olig", and PLP in the ventral cord in association with the axons (Fig. 11). O4 staining was co-extensive with these markers. The pattern of GFAP expression was distinct from that of O4 (Maier and Miller, 1995). At the lesion, a narrow rim of GFAP- and fibronectin-positive cells formed at the cut ends of the cord. This occurred at 4

Fig. 10. Longitudinal sections through an optic nerve, 8 weeks after removal of the eye; phase contrast (a) Glia cells labeled by O4 (b) and GFAP (c) do not exhibit a preferred orientation of their processes, and Gal-C (d) and MBP (e) are absent (except for some Gal-C-positive debris). Scale bar = 100 μ m.

weeks and was unchanged 8 weeks after lesion, except that the gap between the cut ends gradually filled up with GFAP- and fibronectin-positive cells. Thus, in the *Xenopus* spinal cord where lesioned axons fail to regenerate (Beattie et al., 1990; Forehand and Farel, 1982; Lang et al., 1995), myelin markers in the vicinity of the cut axons persist. O4-expressing glia precursors and radial processes, both of which are present in the optic nerve, are not seen at the lesion or elsewhere in the cord.

DISCUSSION

This study provides evidence for an abundance of undifferentiated oligodendrocytes during retinal axon regeneration following optic nerve transection in frogs. The *in vitro* and *in vivo* behavior of these cells suggests that *Xenopus* glial cells of the oligodendrocyte lineage are highly susceptible to the absence, presence, and regenerative state of the retinal axons and are induced to differentiate into cells that express myelin markers. This adaptive plasticity may be advantageous for CNS fiber tract repair. Indirect evidence in support of this view is the persistence of myelin markers and absence of abundant undifferentiated oligodendrocytes after lesion in the spinal cord where axons fail to regenerate (Beattie et al., 1990; Lang et al., 1995).

The *in vitro* properties of *Xenopus* oligodendrocytes show their ability to respond to deprivation of axons. Their phenotype in culture is that of undifferentiated cells that divide over weeks. This sets them apart from mammalian oligodendrocytes (Raff and Miller, 1984), which otherwise have many of the same myelin marker molecules (Pfeiffer et al., 1993). In this adaptive plasticity, *Xenopus* oligodendrocytes resemble Schwann cells (Bunge, 1993), although they differ in other respects (Lemke and Chao, 1988; Morgan et al., 1991). However, both undifferentiated oligodendrocytes and Schwann cells continue to divide in culture, transiently express similar molecules, support axonal growth, and require external cues to differentiate. Related characteristics have been found in goldfish oligodendrocytes (Bastmeyer et al., 1993, 1994; Jeserich and Rauen, 1990) and may be helpful or prerequisite for axon regeneration and subsequent remyelination.

These properties were similar whether oligodendrocytes in culture were derived from the *Xenopus* optic nerve or spinal cord, and whether oligodendrocytes had been engaged in axon myelination or not. Those obtained from frogs must be derived from dedifferentiating oligodendrocytes or from *in vivo* resident precursors, but which of these alternatives applies is unknown. Likewise, available data do not allow us to decide whether individual oligodendrocytes that were engaged in myelination dedifferentiate on lesioning of the optic nerve and redifferentiate to remyelinating cells. However, cells of the oligodendrocyte lineage of both sources, optic nerve and spinal cord, apparently possess the ability to adapt to lesions in the system of origin, as evidenced by their *in vitro* properties. Only in the optic

Fig. 11. Consecutive longitudinal sections through the spinal cord 4 weeks after spinal cord transection. The gap between the proximal (right) and distal (left) cord is indicated by an arrowhead. Descending axons (a) labeled by SMI-35 fail to cross the gap. Anti-GFAP (b) and -fibronectin (c) positivity is seen in a narrow zone bordering the cut

cord. Myelin markers O4 (d), Gal-C (e), and MBP (f) are present mainly in the proximal stump of the cord and predominantly in those regions where large numbers of myelinated axons are present. Scale bar = 200 μ m.

nerve do they display the lesion-induced repair response. That regenerating retinal axons indeed become remyelinated by oligodendrocytes was known from previous investigations (Bohn and Reier, 1983; Reier and Webster, 1974), but their intermittent fate after optic nerve lesion and during axon re-elongation was unresolved. Our present data reveals an abundance of O4-positive undifferentiated oligodendrocytes in the regenerating optic nerve. These cells are present during axon regeneration and are apparently in among the pool of glia cells that display the unusual spatial organization so typical of and unique to the *Xenopus* injured optic nerve (Bohn and Reier, 1983; Reier and Webster, 1974). Whether these O4-positive cells support axon regrowth as do their presumed counterparts in vitro cannot be determined at present. However, that they are responsive to the presence and regenerative state of the axons and therefore are susceptible to cues from their environment is suggested by two further results. First, the rearrangement of optic nerve glia was not observed

after eye removal, which deprives the nerve of axons. Second, we found expression of advanced myelin markers in the peripheral portion of the optic nerve where elongated processes of the glial cells are near regenerating retinal axons. This speaks for the view that O4-positive oligodendrocyte precursor-like cells are induced to express advanced myelin markers (for axon remyelination) through sustained contact with regenerating axons, which meanwhile have re-formed connections with their targets (Taylor and Gaze, 1985). In their dependence on axons, oligodendrocytes in vivo resemble O4 oligodendrocyte precursors in vitro, which require specific stimuli for expression of advanced myelin markers like Schwann cells (Bunge, 1993) and fish oligodendrocytes (Bastmeyer et al., 1994).

In contrast to the latter, most *Xenopus* glial cells producing myelin marker molecules and enwrapping axons apparently retain their somata in the optic nerve's center, instead of moving into the axon territories. This is deduced from DAPI-labelled nuclei found predomi-

nantly in the center of the nerve rather than among the axons, and by related observations of Reier and Webster (1974), who identified oligodendrocyte somata ultrastructurally within the centrally located pool of glia. This configuration in turn implies that myelin-forming cells may reach the axons by means of their elongated processes and express myelin markers locally in these peripheral structures. This would resolve the question of where the oligodendrocytes remained and how they gained access to the axons.

Because of their morphology and content of fibrils most of the glial cells with long processes were thought to be astrocytes (Reier and Webster, 1974). Our data show that GFAP-positive and O4-positive cells and processes occur in the very same spatiotemporal pattern. Hence, both markers may coexist within one cell, as was in fact observed *in vitro*. However, Reier and Webster (1974) also reported other features specific to astrocytes, such as desmosomes, glial cell processes partitioning bundles of axons, and the presence of a glia limitans. Thus, astrocytes are definitely present along with oligodendrocytic cells. In culture, both macroglial types can be distinguished since astrocytes are GFAP positive but negative for O4 and all oligodendrocyte specific markers. They are also distinguishable in the normal optic nerve and in the spinal cord (Maier and Miller, 1995). However, since oligodendrocytes possess GFAP in addition to O4 and advanced myelin markers, we cannot discern the *in vivo* astrocytes with the present set of markers. This in turn implies, however, that the lesion-induced glia reorganization extends to astrocytic cells as well.

That both types of glia respond quite differently to fiber tract lesions in other parts of the *Xenopus* CNS is demonstrated in the case of the spinal cord. The distribution of cellular markers indicative of axons, oligodendrocytes, fibronectin, and GFAP in the lesioned spinal cord is consistent with the view that myelin around the proximal portion of axons persists, that axons fail to regrow (Beattie et al., 1990; Lang et al., 1995), and that a sort of glial scar made up of astrocytic and mesenchymal cells (Reier and Houlé, 1988) develops near the lesion. No signs of oligodendrocyte dedifferentiation were noted. This contrasts with the apparent ability of these glial cells to display the phenotype of undifferentiated oligodendrocytes *in vitro*. This finding means that *in vivo* conditions prevent their change.

Whether the failure of axon regeneration—due to the presence of neurite growth inhibitors associated with spinal cord oligodendrocytes (Lang et al., 1995)—is the primary cause for the absence of oligodendrocyte changes or whether other signals participate in these events remains to be explored.

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