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Similarities and Differences Between Fish Oligodendrocytes and Schwann Cells In Vitro

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ABSTRACT In light of the striking differences between oligodendrocytes of the optic nerve/tract of adult goldfish and their mammalian counterparts, a further characterization of goldfish oligodendrocytes was performed. A comparison with Schwann cells was included because fish optic nerve/tract-derived oligodendrocytes bear remarkable similarities to this type of glial cell. Fish optic nerve/tract-derived oligodendrocytes that had differentiated into 04 and 6D2-positive cells and thus expressed early myelin marker molecules were found to incorporate BrdU and to divide in vitro over a period of weeks. For the induction of more advanced markers of myelinogenesis such as the CNS-specific myelin protein 36K, oligodendrocytes required extensive contact with axons. Other agents, such as fetal calf or carp serum, substrate components, or forscolin failed, however, to induce 36K expression. 04/6D2-positive oligodendrocytes could be distinguished from fish 6D2-positive Schwann cells derived from cranial nerves by their antigenic phenotype: Schwann cells but not oligodendrocytes exhibited the low affinity NGF receptor. While both cell types carry the cell adhesion molecules NCAM, E 587 antigen, and the L2/HNK-1 epitope, only Schwann cells possess a further adhesion molecule, Neurolin.

INTRODUCTION

The influence of myelin-forming glial cells on axotomized neurons in the vertebrate nervous system is quite diverse. Schwann cells of the vertebrate peripheral nervous system (PNS) support neurite extension and are capable of remyelinating regenerated axons (reviewed in Wood et al., 1990). Oligodendrocytes and myelin of the mammalian central nervous system (CNS), however, carry neurite growth inhibitors that prevent axonal regrowth (reviewed in Schwab, 1990). In contrast to mammals, myelin of the teleost CNS lacks such inhibitors (Bastmeyer et al., 1991; Caroni and Schwab, 1988). Moreover, oligodendrocytes derived from the regenerating fish optic nerve/tract acquire a state in which they favor the growth of regenerating (fish and rat retinal) axons along their surfaces (Bastmeyer et al., 1993) much as PNS-derived Schwann cells do (Hopkins and Bunge, 1991; Morrissey et al., 1991). Accordingly, retinal ganglion cell (RGC) axons in warmblooded vertebrates fail to regenerate after optic nerve injury, while those in fish re-elongate successfully and restore vision (reviewed in Gaze, 1970). Along with restoration of function, the regenerated axons in the fish visual system become completely remyelinated in a manner that is indistinguishable from the normal situation (Wolburg, 1981; Wolburg and Bouzehouane, 1986).

Hence, with regard to their positive interaction with injured and regenerating axons, fish optic nerve/tract oligodendrocytes resemble mammalian Schwann cells rather than mammalian oligodendrocytes. This similarity extends to the major fish myelin proteins, IP1 and IP2, which are shared by CNS and PNS myelin-forming cells (Jeserich and Waehneldt, 1986a,b). IP1 and IP2 are structurally related to Po, the major PNS

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myelin protein of mammals (Jeserich and Waehneldt, 1986a,b; Schliess and Stoffel, 1991). In terms of morphology, however, the myelin-forming glia cells of the fish CNS differs markedly from those in the PNS. Characteristic features of fish PNS myelin-forming glia defining them as Schwann cells are the ultrastructure of the PNS myelin sheath (Jeserich and Waehneldt, 1986a), the occurrence of a basal lamina, and the investment of each axonal segment by an individual Schwann cell (Scherer and Easter, 1983).

Fish CNS myelin exhibits the same structural features as mammalian CNS myelin. Differentiated oligodendrocytes of the fish CNS carry a protein of 36KD molecular weight (protein "36K") that is absent from the myelin of fish PNS (Jeserich and Waehneldt, 1986b) and does not occur in higher vertebrates (Waehneldt et al., 1986).

During development this protein is expressed late by oligodendrocytes in vitro and is not maintained by cells that had been deprived of axonal contact in vitro (Jeserich and Rauen, 1990; Jeserich and Stratmann, 1992). The same holds true for the expression of other marker molecules of fish CNS myelin, such as the glycolipid Gal-C and IP1 (Jeserich and Rauen, 1990, Jeserich and Stratmann, 1992). This is in contrast to mammalian oligodendrocytes which develop spontaneously and in the absence of axonal contact from dividing A2B5-positive progenitor cells (Raff, 1989) into mature oligodendrocytes, stably expressing all major myelin markers (Gebicke-Härter et al., 1984). Concomitantly, they lose the A2B5 epitope, acquire a multipolar morphology, and stop dividing (Raff et al., 1983). This gave rise to the assumption that fish oligodendrocytes, like mammalian Schwann cells (Mirski and Jessen, 1990), may require axonal contact and/or additional stimuli for the continued expression of myelin markers and the maintenance of a differentiated phenotype (Jeserich and Rauen, 1990; Jeserich and Stratmann, 1992).

A direct test of this assumption for oligodendrocytes in vitro and a comparison with and distinction from Schwann cells was therefore desired. This was necessary in particular for oligodendrocytes of the adult goldfish optic nerve/tract in vitro because these oligodendrocytes exhibit a morphology that is highly reminiscent of Schwann cells. Moreover, like PNS glia they carry adhesion molecules and promote axonal growth (Bastmeyer et al., 1993). Furthermore, like Schwann cells they increase in number for weeks in culture (Bastmeyer et al., 1991).

In view of their positive influence on CNS fiber tract repair, we have extended here the characterization of oligodendrocytes derived from adult fish optic nerves/tracts and defined for this cell type further differences from mammalian oligodendrocytes and Schwann cells. Within roughly 1 week in vitro, fish optic nerve/tract-derived oligodendrocytes (some of which have GFAP-positive fibrils) exhibit the early myelin marker 04 and concurrently 6D2 immunoreactivity (Bastmeyer et al., 1989, 1991). Although they lack more mature myelin markers, they do acquire spontaneously at least one of the typical fish myelogenic proteins, IP2 (Jeserich and Waehneldt, 1986; Bastmeyer et al., 1991), and enwrap rat axons in long-term co-cultures (Bastmeyer et al., 1993). We therefore consider them as, and term them, oligodendrocytes, although they have not completed their differentiation. Here, we have examined the overall proliferation pattern of O4/6D2-positive oligodendrocytes. We tested in particular whether oligodendrocytes having reached this stage of differentiation would, like Schwann cells, be capable of undergoing cell division or whether the multiplication of these oligodendrocytes in vitro resulted from a more undifferentiated in vitro precursor. Furthermore, we searched for conditions under which oligodendrocytes would express the advanced myelin marker 36K protein and subjected them to stimuli that are known to induce differentiation of mammalian Schwann cells (Mirsky and Jessen, 1990). Finally, to enable us to distinguish fish optic nerve/tract derived oligodendrocytes from fish Schwann cells, we isolated glial cells from the adult fish PNS. Both cell types were subjected to a variety of glial cell and stage-specific antibodies as well as to antibodies against cell adhesion molecules.

MATERIALS AND METHODS

Cell Culture of CNS Glial Cells

CNS glial cells were obtained from adult goldfish as described earlier (Bastmeyer et al., 1989). In brief, small pieces of regenerating optic nerves and optic tracts (2 weeks after optic nerve section) were attached to a nylon filter (Hybond, Amersham), cut into 200 μm wide segments, and explanted onto polylysine/laminin-coated coverslips from which glial cells emigrate. In contrast to earlier experiments, we routinely kept the glial cells in F-12 medium (Ham's F-12 medium (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (FCS) and 0.4% methyl cellulose) at 28°C. In control experiments, the cells were grown in L15 [Leibowitz medium (Gibco, Grand Island, NY), supplemented with 10% FCS and 0.4% methyl cellulose] at 20°C or 28°C. In F-12 medium and at 28°C, the glial cell carpet grew faster and to greater density than in L15 at 20°C or 28°C. The medium was exchanged every 3 days and the cells survived for at least 3–4 months. In other experiments, F-12 medium was supplemented with various concentrations of FCS (1%, 5%, and 20%), or FCS was replaced by 10% carp serum. To test the influence of the substrate on glia cell development, we used polylysine-, fibronectin-, or Concanavalin A (ConA)-coated coverslips in some experiments.

Cell Culture of PNS Glial Cells

PNS glial cells from the fifth to tenth cranial nerves were obtained by incubating small pieces of cranial nerves at 28°C for 20 min in L15 with 0.03% collagenase (Worthington Seromed, Berlin) and 0.1% dispase (Boehringer,

Mannheim). The enzyme mixture was replaced by L15 with 20% FCS and the nerve pieces centrifuged at 200g for 5 min. The nerve pieces were then resuspended in F-12 medium and plated onto polylysine/laminin-coated coverslips. An uncoated coverslip was placed on top and these "sandwich preparations" were incubated for several days or weeks.

Dissociated Glial Cell Cultures

Small pieces from regenerating optic nerve/tracts of adult goldfish were treated with collagenase/dispase as described above, dispersed by repeated tituration through a glass pipette and plated on polylysine/laminin-coated coverslips. In some experiments, we used coverslips with a findergrid (Millipore) to relocate identified cells.

Goldfish Retinal Explants

Goldfish retinal explants were prepared as previously described (Vielmetter and Stuermer, 1989). In brief, as for the glial cell cultures, the optic nerves of adult goldfish (4–5 cm long) were cut under MS-222 anesthesia; 14 to 17 days later the retina was isolated and attached to a nylon filter (Hybond, Amersham). Retina and filter were cut into 300 μm wide segments and explanted, ganglion cell layer down, onto the goldfish glial cells. Small metal blocks were placed onto the end of the segments to keep the retina in contact with the substrate. The co-cultures were kept in F-12 or L15 at 28°C for 3–4 weeks. In some experiments ascorbic acid (50 μg/ml) was added to the medium.

Antibodies

The following polyclonal sera or monoclonal antibodies (Mab) were used: α-goldfish glial fibrillary acidic protein (GFAP) (polyclonal serum, Nona et al., 1989; kindly provided by S. Nona), α-bovine GFAP (polyclonal serum, Dakopatts), α-Fibronectin (polyclonal serum, Telios), α-36K myelin protein (polyclonal serum, Jeserich and Waehneldt, 1986b), A_2B_5, O1, O4, L2/HNK-1 (Mabs, kindly provided by M. Schachner), α-GalC (Mab, Boehringer Mannheim), α-NGF-receptor (Mab 192-IgG, kindly provided by D. Lindholm), 6D2 (Mab against the fish myelin proteins IP1/2, Jeserich and Waehneldt, 1987), α-BrdU (Mab, Boehringer Mannheim), E587 (Mab aganst an L1-like molecule in goldfish, Vielmetter et al., 1991), D3 (Mab against NCAM 140/180, Schlosshauer, 1989; Bastmeyer et al., 1990), and E21 (Mab against Neurolin, Paschke et al., 1992). In one set of co-culture experiments the neurofilament antibody SMI 31 (Sternberger-Meyer Immunochemicals) was used to visualize retinal axons in contact with glial cells. All secondary antibodies were obtained from Dianova and used at a dilution of 1:200 in PBS.

Immunostaining of Cryosections

The brains and cranial nerves of adult goldfish were dissected in phosphate-buffered saline (PBS), immersed in Tissue-Tec, and frozen in liquid nitrogen. Frozen sections (10–15 μm) were cut on a Reichert-Jung cryostat, collected on slides, and air dried at room temperature for 1–2 h. Sections containing the PNS/CNS boundary of the fifth to tenth cranial nerves were fixed in methanol (−20°C, 5 min) and rinsed in PBS. They were then simultaneously exposed to Mab 6D2 (1:10 in PBS) and the 36K antiserum (1:100 in PBS) for 1 h at room temperature. After three washes in PBS they were incubated simultaneously in fluoresceine (FITC)-coupled goat anti-rabbit antibodies and rhodamine isothiocyanate (RITC)-coupled goat anti-mouse antibodies for 1 h at room temperature, washed again in PBS, and embeded in Mowiol. Sections were viewed with a Zeiss Axiophot using the appropriate filter sets.

Immunostaining of Glial Cells

For detection of intracellular antigens cells were permeabilized with methanol (at −20°C, 5 min) and fixed in paraformaldehyde (2% in PBS at room temperature, for 5 min). After three rinses in PBS (5 min each) cultures were treated with primary antibodies, washed in PBS, and incubated with FITC- or RITC coupled secondary antibodies (1 h at 37°C each). For double labeling of cells, primary antibodies were applied simultaneously after fixation. The cells were routinely stained with DAPI (0.005 μg/ml in PBS for 5 min) to visualize cell nuclei and mounted in Mowiol containing n-propylgallate to reduce fading.

For visualization of extracellular antigens, the living cells were exposed to the relevant antibodies for 30 min. These antibodies were used as hybridoma supernatants diluted 1:2 in L15 (A_2B_5, O4, O1, 192-IgG) or purified IgG (α-GalC, 50 μg/ml) in L15. After two short rinses in L15 the cultures were fixed and secondary antibodies were added. For simultaneous detection of extra- and intra-cellular antigens, antibodies against the extracellular antigens were used as above and antibodies against the intracellular epitopes were applied after fixation.

BrdU Incorporation Assay

The extent of CNS glial cell proliferation was determined by a bromodeoxyuridine (BrdU) incorporation assay (Nowakowski et al., 1989). Glial cultures of various days in vitro (div) received 10 μM BrdU (Sigma Chemical Co., St. Louis, MO, in L15, for 12 h at 28°C). For simultaneous detection of BrdU and GFAP the cultures were, after fixation as above, treated with 1N HCl (10 min) to make the DNA accessible for the BrdU antibody, and rinsed in $Na_2B_4O_7$ (100 mM, 5 min each). After a brief rinse in PBS the cultures were simultaneously exposed to rabbit GFAP antiserum (1:1,000 in

PBS) and anti-BrdU antibody (1:20 in PBS) for 1 h at room temperature. Following three washes in PBS, the cultures were treated with FITC-coupled goat anti-rabbit and RITC-coupled goat anti-mouse antibodies, as described above. For simultaneous detection of BrdU and O4 antigen the living cultures were first treated with 04 antibody and fixed as described. They were then exposed to RITC coupled goat-anti-mouse antibodies for 1 h and rinsed in PBS. After fixation in formaldehyde and the above treatment with HCl and anti-BrdU antibodies, a FITC-coupled IgG-specific goat anti-mouse antibody was used as secondary antibody to avoid cross-reaction with 04 antibodies.

RESULTS

Extensive Proliferation of Fish Oligodendrocytes

The two major cell types in fish optic nerve/tract-derived glial cultures maintained in F12 at 28°C were from 7 days in vitro onwards polygonal shaped and GFAP-positive astrocytes (Fig. 1A–C, D–F) (resembling mammalian astrocytes type I) and elongated cells with long processes and membraneous protrusions (Fig. 1G,H). The latter were O4- and 6D2-positive (Fig. 1F,H) and thus identified as oligodendrocytes (Bastmeyer et al., 1991), of which a few exhibited GFAP. By one week in culture the number of 6D2/O4 positive oligodendrocytes was higher than under conditions used earlier. However, the proportion of 04/6D2-positive oligodendrocytes that contained GFAP-positive fibrils was smaller than in L15 at 22°C (Bastmeyer et al., 1989, 1991) and was in older cultures always less than 10%. This suggests that the present culture conditions accelerated the appearance of 04/6D2 on and the loss of GFAP from oligodendrocytes. No other changes of the principal glial cell antigenic profile described in earlier studies were noted (Bastmeyer et al., 1989, 1991; Hoppe et al., 1991 and below).

Unlike mammalian oligodendrocytes, O4/6D2-positive fish oligodendrocytes failed to maintain a differentiated phenotype in culture and ceased expression of Gal-C and the advanced myelin marker 36K (Table 1, but see below). However, fish oligodendrocytes were obviously capable of proliferating as their number increased dramatically over weeks in culture, which is reminiscent of axon-deprived mammalian Schwann cells (Morrisey et al., 1991).

To obtain an impression of the overall proliferation pattern in optic nerve/tract-derived glial cultures, from 1 week onwards and at 2, 3, 4, and 12 weeks, such glial cultures were treated with BrdU for 12 h. For technical reasons oligodendrocytes were identified by 04 in these experiments. The antibody A2B5 was used to examine whether cells selectively incorporating BrdU would exhibit this marker, since A2B5 defines a glia precursor stage and is lost from glial cells upon differentiation into astrocytes and non dividing oligodendrocytes (Raff et al., 1983; Jeserich and Stratmann, 1992). Surprisingly, all cells in the cultures, i.e., O4-positive oligodendrocytes and GFAP-positive astrocytes, were always A2B5-positive (Fig. 1C), independent of whether they incorporated BrdU or not. In fact, A2B5 also stained all PNS-derived glial cells, including Schwann cells and fibroblasts (Table 1). The observation that none of the cells lost A2B5 within the periods of study suggests that A2B5 staining was not applicable to define any specific stage of differentiation in the cultures considered here. This differs from findings on A2B5-expression and loss in glial cells of fish embryos (Jeserich and Stratmann, 1992).

After the addition of BrdU to optic nerve/tract-derived glial cells, 50–70% of all cells were BrdU-positive in 1 week old cultures. In double-labeling experiments with either anti-BrdU and O4, or anti-BrdU and GFAP antibodies the vast majority of BrdU-positive cells in the 1 week old cultures, were O4-positive oligodendrocytes (Fig. 2A–D). In 3–4 weeks old cultures, oligodendrocytes had assembled to form a network-like carpet (Bastmeyer et al., 1993). Astrocytes occurred mainly in separate clusters within the oligodendrocyte carpet. Most of the BrdU-labeled cells in these older cultures were astrocytes in these clusters (Fig. 2E,F). BrdU-positive oligodendrocytes were absent from these clusters and were less frequent than in 1- and 2-week-old cultures. Consistent with GFAP/O4 and GFAP/6D2 double-labeling experiments (described above) there were a few cells of the typical oligodendrocyte morphology which contained GFAP-positive fibrils (Fig. 3A,B). In cultures of all ages they were randomly distributed between GFAP-negative oligodendrocytes. Some but not all of such GFAP-containing oligodendrocytes had incorporated BrdU (Fig. 3C,D). These observations suggest that O4-positive (and some GFAP-containing) fish oligodendrocytes undergo DNA synthesis over weeks, much like axon-deprived Schwann cells.

This was tested by plating glial cells from freshly dissociated optic nerves and tracts onto coverslips with a finder grid and following individual cells and their progeny over time. Individual astrocytes and individual oligodendrocytes were identified by their morphology and their progeny by morphology and O4- and GFAP antibodies. Eleven individual cells, five of bi- or multipolar shape (oligodendrocytes), and six of polygonal shape (astrocytes), and their progeny were followed in daily intervals over 7 days. Bi- or multi-polar cells divided and gave rise to daughter cells of the very same morphology which were O4-positive. Among these less than 10% co-expressed GFAP. Polygonal cells divided to produce polygonal cells which were GFAP positive and always 04 negative.

Induction of 36K Protein Expression in Oligodendrocytes

Under standard culture conditions (Methods) oligodendrocytes did not exhibit anti-Gal-C or anti-36K immunoreactivity, indicating that unlike mammalian oligodendrocytes they require additional stimuli for further

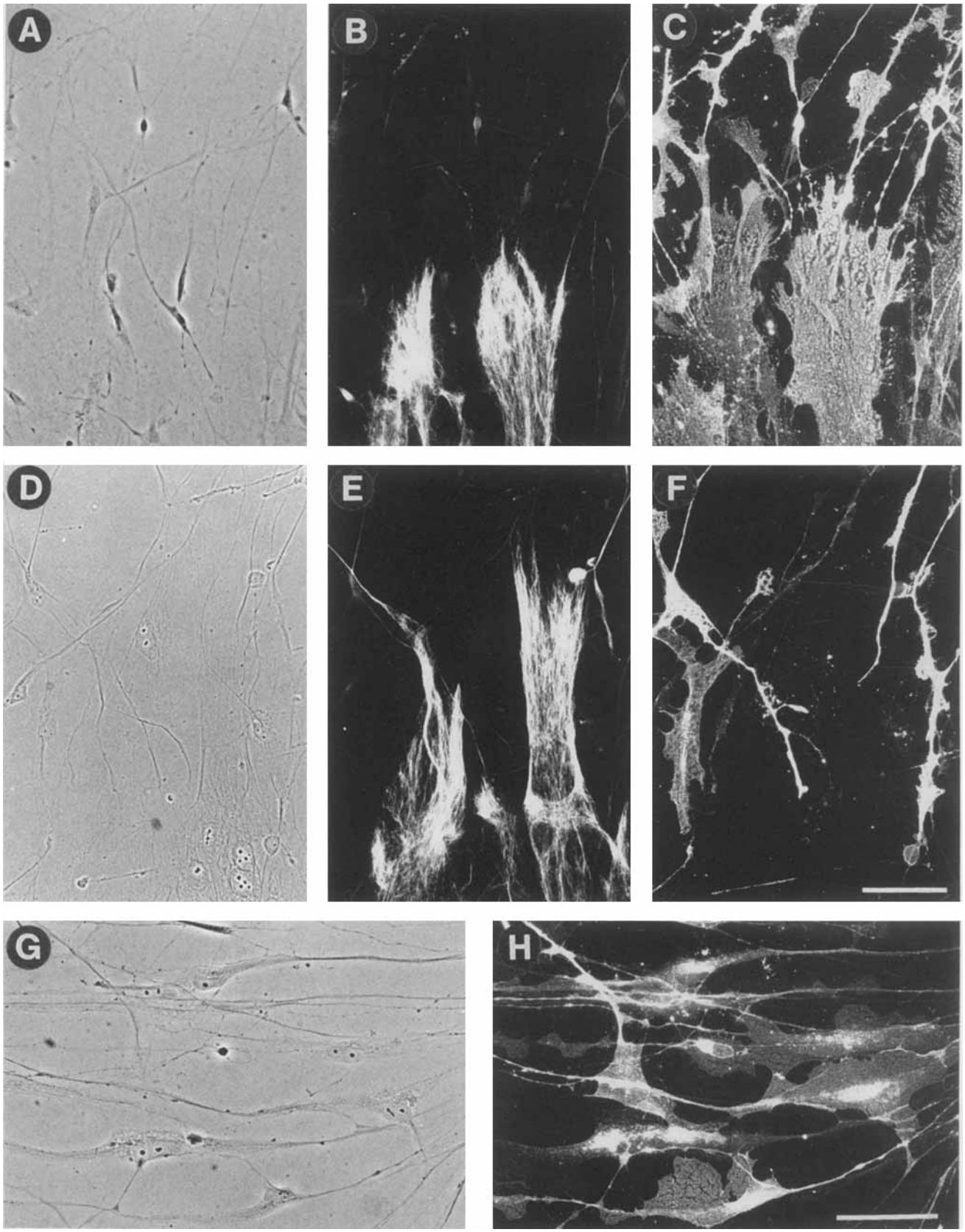


Fig. 1. Immunocytochemical characterization of CNS glial cells. All panels show goldfish optic nerve glial cells after 3 weeks in vitro. **A–C** show the same field in phase contrast (A) and after a double label with anti-GFAP (B) and A2B5 (C) antibodies. Both cell types, GFAP-positive astrocytes (B) with a compact morphology and GFAP-negative oligodendrocytes with a bi- or multi-polar morphology are labeled by Mab A2B5 (C). The same field is shown in phase contrast (**D**) and after a double label with anti-GFAP (E) and O4 (F) antibodies. Only the oligodendrocytes and not the GFAP-positive astrocytes (E) are labeled by Mab O4 (F). **G,H** show corresponding phase contrast (G) and immunofluorescence micrographs of goldfish oligodendrocytes stained with Mab 6D2 (H). All oligodendrocytes express the myelin proteins IP1/2 (H). Scale bar in F: 50 μm, applies to A–F. Scale bar in H: 50 μm, applies to G,H.

TABLE 1. Immunocytochemical properties of goldfish CNS and PNS glial cells

Antibody	Astrocytes	Oligodendrocytes	Schwann cells	Fibroblasts
α-GFAP (goldfish	+	±[b]	−	−
α-GFAP (bovine)	+	±[b]	+	−
A2B5	+	+	+	+
O4	−	+	+	−
O1/ α-GalC	−	−	−	−
6D2		+	+	-
α-36k myelin protein	−	+[a]	−	−
α-fibronectin	−	−	−	+
192-IgG (NGF-rec)	−	−	+	−
L2/HNK1	+	+	+	+[b]
D3 (NCAM)	+[c]	+	+	−
ε587 (L1-like)	+[c]	+	+	−
E21 (Neurolin)	−	−	+	+[b]

[a]When co-cultured with retinal axons.
[b]Subpopulations.
[c]Weaker expression than oligodendrocytes.

differentiation. We tested whether the serum (FCS) concentration or animal source of serum, the type of substrate or the elevation of intracellular cAMP by forscolin (Morgan et al., 1991) would influence the antigenic phenotype of the cells.

Changing the FCS concentration from 10% to 20% or to 1% and 5% and replacing FCS with 10% carp serum failed to induce 36K and Gal-C expression in oligodendrocytes. Furthermore, when polylysine, fibronectin, or Concanavalin A instead of polylysine/laminin were offered as a substrate, oligodendrocytes did not express 36K or Gal-C. The addition of 100 μM forscolin to the medium also had no effect. Concentrations of 200–500 μM forscolin led to cell death within 3–4 h. However, when co-cultured with retinal axons, oligodendrocytes did acquire the 36K protein (Fig. 4). When retinal segments are explanted onto 3-week-old oligodendrocytes, the axons readily extend along these glial cells (Bastmeyer et al., 1993), apparently using oligodendrocytes as substrate for growth. In subsequent long-term cocultures, 3–4 weeks after explantation of the retinal segments, the relation of some oligodendrocytes and axons changed. Some oligodendrocytes apparently had left the substrate and joined the axon fascicles (Fig. 5). These oligodendrocytes were arranged alongside the axons in a focal plane above the substratum-attached oligodendrocytes. The visualization of the nuclei with DAPI best shows the elevated position of the axon-associated oligodendrocytes as compared to their substrate-associated counterparts (Fig. 5C). These axon-associated oligodendrocytes expressed the 36K protein (Fig. 5B) but the others did not. Axon-associated oligodendrocytes, however, did not exhibit Gal-C. In control glial cell cultures of similar and older ages but raised without retinal explants, 36K-immunoreactivity was never observed. These observations indicate that in vitro oligodendrocyte differentiation leading to the expression of 36K protein requires extensive interaction with axons. This interaction, however, does not induce the production of Gal-C by oligodendrocytes.

The CNS/PNS Boundary in Adult Goldfish Brains

The sources of fish Schwann cells in culture (considered below) were the PNS portions of the fifth to tenth cranial nerves. To confirm that myelin (and hence myelin-forming cells) of these cranial PNS areas is distinct from that in the CNS, cryostat sections through these fish brain areas were exposed to an antiserum against 36K which is CNS specific (Jeserich and Waehneldt, 1986) and to Mab 6D2 against IP1 and IP2 proteins of CNS and PNS myelin (Jeserich and Rauen, 1990). Figure 6 illustrates a clear boundary between the CNS and PNS, in that the anti-36K anti-serum selectively recognized CNS but not PNS myelin (Fig. 6C). As expected, Mab 6D2 stained both CNS and PNS myelin (Fig. 6B).

Fish Schwann Cells

PNS glia cells obtained from the V. to X. cranial nerves grew well and to highest density when the nerve pieces were sandwiched between two coverslips (see Materials and Methods). These cultures contained two morphologically and immunocytochemically distinct cell types: compact cells and spindle shaped cells with extremely long processes. Compact cells represent fibroblasts since they were immunoreactive for the polyclonal serum against fibronectin (Fig. 7A,B) but did not crossreact with the glial-marker antibodies O4 (Fig. 7C,D), 6D2 (not shown), and anti-GFAP (not shown; see Table 1). Spindle-shaped and typically bi- or tri-polar cells with processes of up to 600 μm in length were O4-, (Fig. 7C,D) and 6D2-, (Fig. 7E,F) positive and thus were identified as Schwann cells. Similar to fish oligodendrocytes, their number increased with time in culture.

In mammals, Schwann cells but not oligodendrocytes express nerve growth factor (NGF) receptor (Lemke and Chao, 1988). This is also true in the fish. Fish Schwann cells exhibited a typical punctate staining (Fig. 8C,D) when exposed to Mab 192-IgG against the low affinity NGF-receptor (Chadler et al., 1984), whereas oligodendrocytes did not (Fig. 8A,B).

Interestingly, fish Schwann cells were recognized by polyclonal antibodies against bovine GFAP (not shown) but not by the serum against goldfish GFAP (Nona et al., 1989), suggesting that they express a different subtype of GFAP than CNS glial cells. Thus, fish Schwann cells in vitro are antigenically distinct from fish optic nerve/tract-derived oligodendrocytes. This was further

Fig. 2. Cell proliferation in CNS cultures. Corresponding phase-contrast (A, E) and fluorescence images (B–D, F–H) of goldfish CNS glial cells after a BrdU pulse for 12 h and a double label with either O4 or GFAP and anti-BrdU antibodies. After 1 week in vitro (A–D) all cell types proliferate. In the field shown, three O4-positive oligodendrocytes (B) have incorporated BrdU (C). All nuclei in this field are shown by DAPI staining (D). After 4 weeks in vitro (E–H) oligodendrocytes no longer proliferate as much as before, but BrdU-positive nuclei (G) are still found in the GFAP-positive astrocyte clusters (F). All nuclei in this field are shown by DAPI staining (H). Scale bar in D: 30 μm, applies to A–D; scale bar in H: 50 μm, applies to E–H. (Figure appears on overleaf).

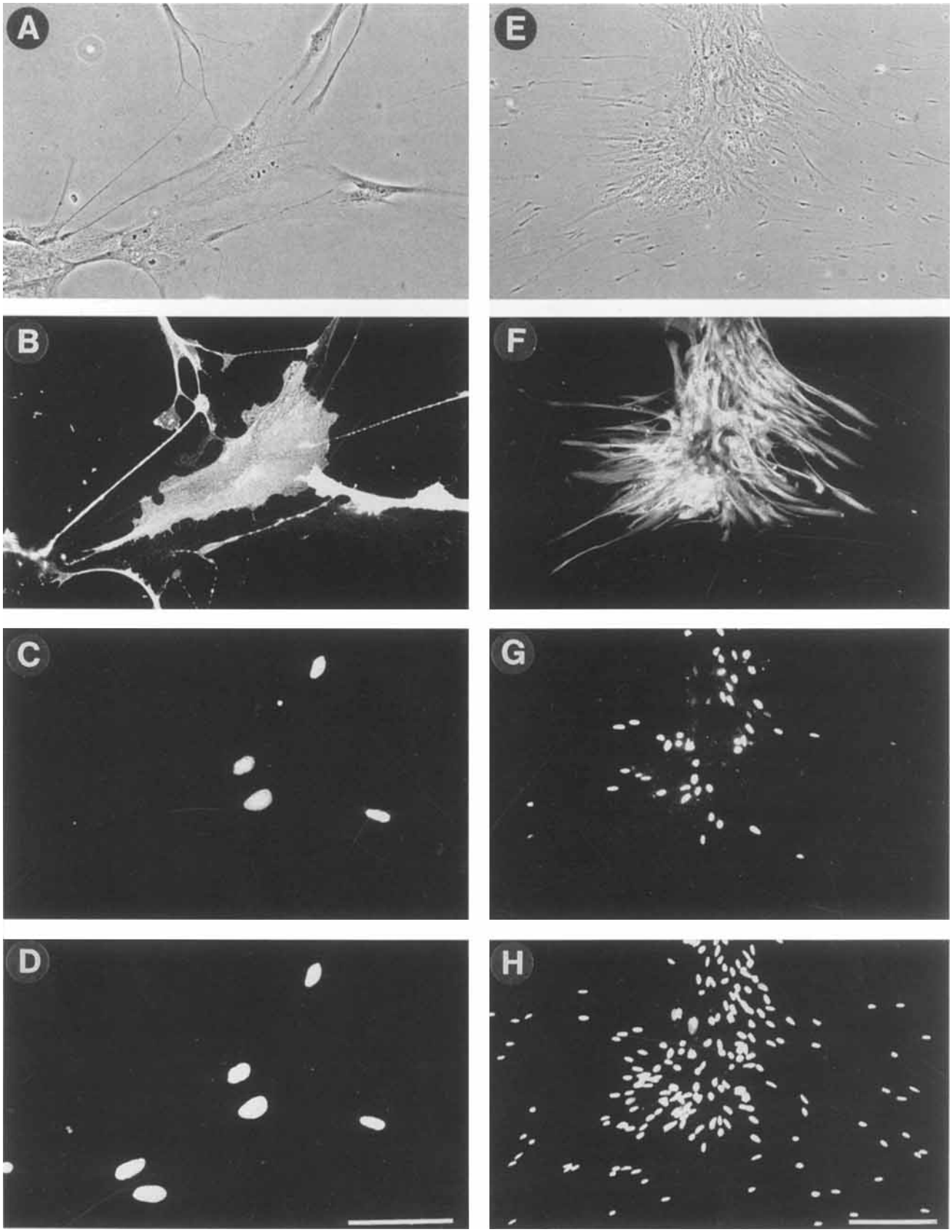


Fig. 2

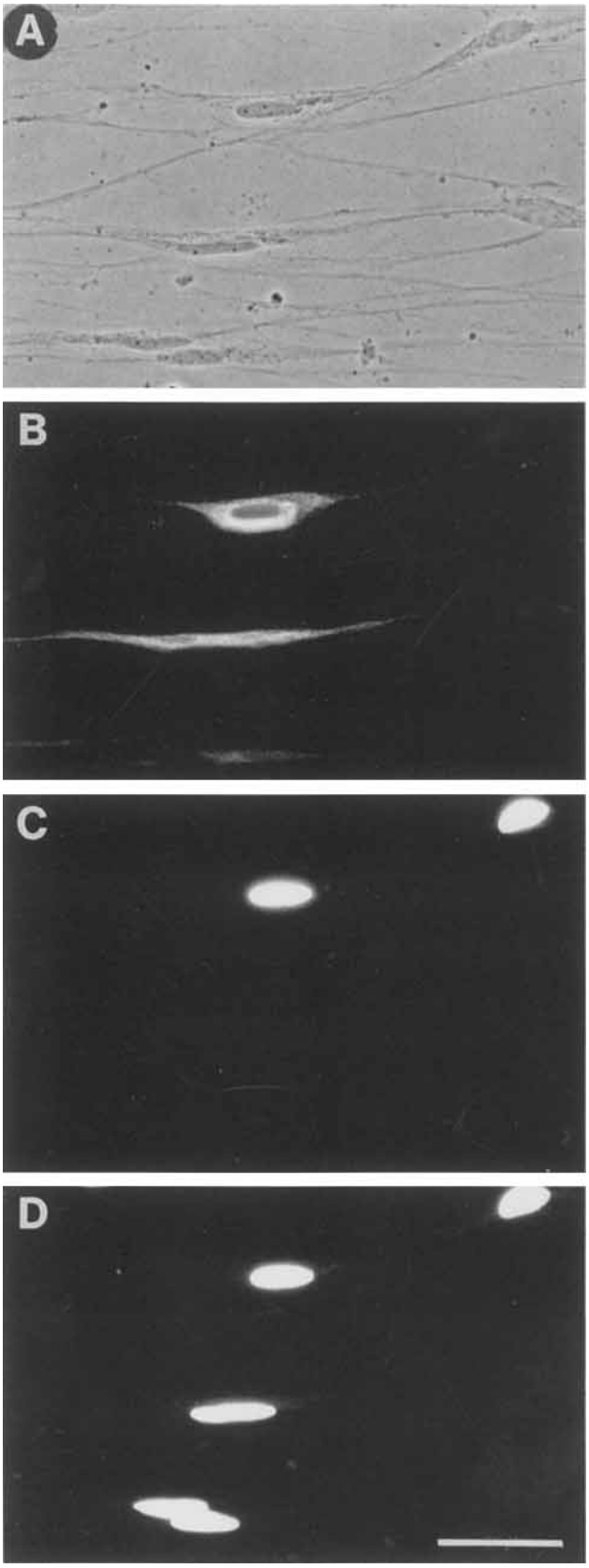


Fig. 3. BrdU incorporation by GFAP-positive and -negative oligodendrocytes. Phase-contrast micrograph (A) of oligodendrocytes in a 3-week-old culture. In this portion of the culture two oligodendrocytes (B) exhibit GFAP. BrdU was incorporated by one of the two GFAP-positive oligodendrocytes and by one GFAP-negative oligodendrocyte (C). The nuclei of the cells are revealed by DAPI stain (D). Scale bar in D applies to A–D: 25 μm.

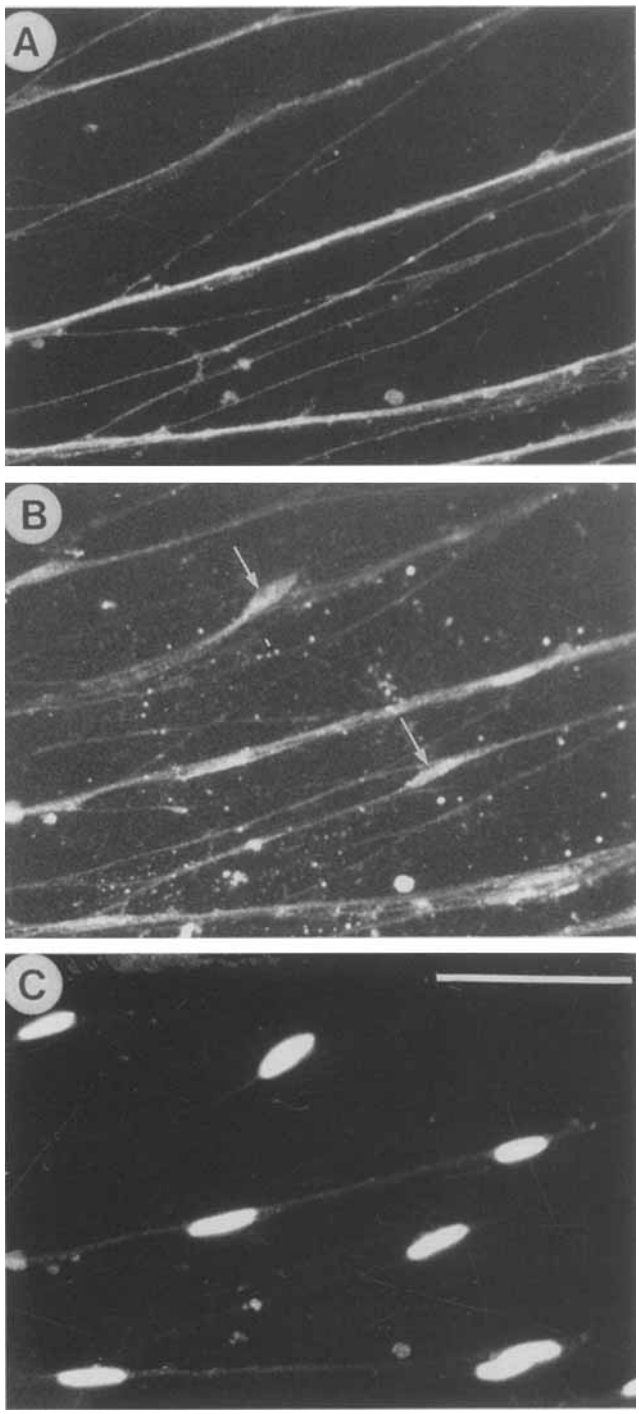
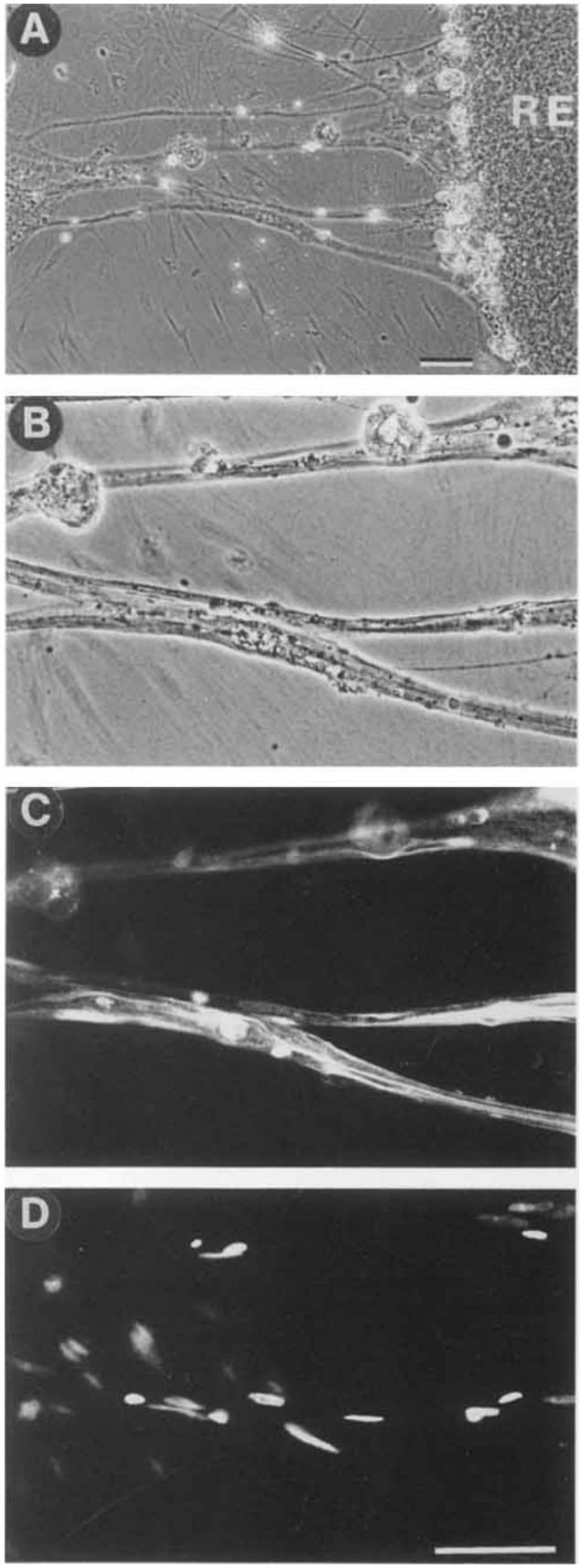


Fig. 4. Expression of 36K-myelin protein in oligodendrocyte/axonal cocultures. Corresponding images of a 3-week-old axon-glia co-culture. A: The axons are labeled by the neurofilament antibody SMI 31. SMI 31 staining of axons was bright so that it is unavoidably also seen in B. B: Oligodendrocytes which are associated with the axons exhibit bright anti-36K immunoreactivity (arrows) which is of markedly higher intensity than the SMI 35 staining of the axons in the background. C: The nuclei of the glial cells exhibit DAPI-stain. Bar in C: 50 μm, applies to A–C.



supported by examining the cell specific expression of several cell adhesion molecules.

Cell Adhesion Molecules on Oligodendrocytes and Schwann Cells

As previously shown, goldfish oligodendrocytes provide effective growth-supportive surfaces for regenerating axons of fish and rat retinae in vitro (Bastmeyer et al., 1991, 1993). As expected, fish Schwann cells also allowed the growth of fish axons along their surface (not shown). Since axonal growth along mammalian Schwann cells is at least in part mediated by cell adhesion molecules (reviewed in Bunge and Hopkins, 1990), we determined which of the cell adhesion molecules previously defined in the goldfish nervous system (Stuermer et al., 1992) are expressed by oligodendrocytes and/or Schwann cells (Table 1). Both oligodendrocytes (Fig. 9A,B) and Schwann cells (Fig. 9C,D) were positive for Mab L2 against the carbohydrate epitope HNK-1 (Abo and Blach, 1981; Künemund et al., 1988). Both goldfish oligodendrocytes and Schwann cells expressed the neural cell adhesion molecule (NCAM) defined by Mab D3 (Schlosshauer, 1989; Bastmeyer et al., 1990) (Fig. 9E–H). Both also carried the E 587 antigen (Fig. 9I–M), a cell adhesion molecule belonging to the L1 family (Vielmetter et al., 1991). Mab E 21 recognizes the cell adhesion molecule belonging to the L1 family (Vielmetter et al., 1991). Mab E 21 recognizes the cell adhesion molecule Neurolin (Paschke et al., 1992) that has homologies to the cell adhesion molecule DM-GRASP (Burns et al., 1991; Lässing et al., 1993). Mab E 21 stained Schwann cells (Fig. 9P,Q) but not the oligodendrocytes (Fig. 9N,O). Mab E 21 immunoreactivity was also absent from oligodendrocytes in coculture with retinal axons, indicating that axons do not induce Neurolin expression in these glial cells. Thus, both glial cell types commonly express a variety of cell adhesion molecules, but only fish Schwann cells carry Mab E 21 immunoreactivity.

DISCUSSION

In the present study an extended characterization of oligodendrocytes derived from the regenerating adult goldfish optic nerve/tract was performed. Interestingly these cells share several features with Schwann cells which are known to support nerve repair in the vertebrate PNS. 1) Like Schwann cells, fish optic nerve/tract-derived oligodendrocytes proliferate extensively in vitro.

Fig. 5. Elevated position of oligodendrocytes expressing 36K-myelin protein in oligodendrocyte/axonal co-cultures. A: Phase-contrast micrograph showing retinal axons extending from an explant (RE) on a 3-week-old glial cell carpet. B–D show a portion of A at higher magnification, (B) phase-contrast, and (C) and (D) fluorescence micrographs. Some oligodendrocytes associated with axons in the fascicles express the myelin protein 36K (C). The nuclei of these cells are revealed by DAPI-stain (D). Scale bars: 50 μm. Scale bar in D applies to B–D.

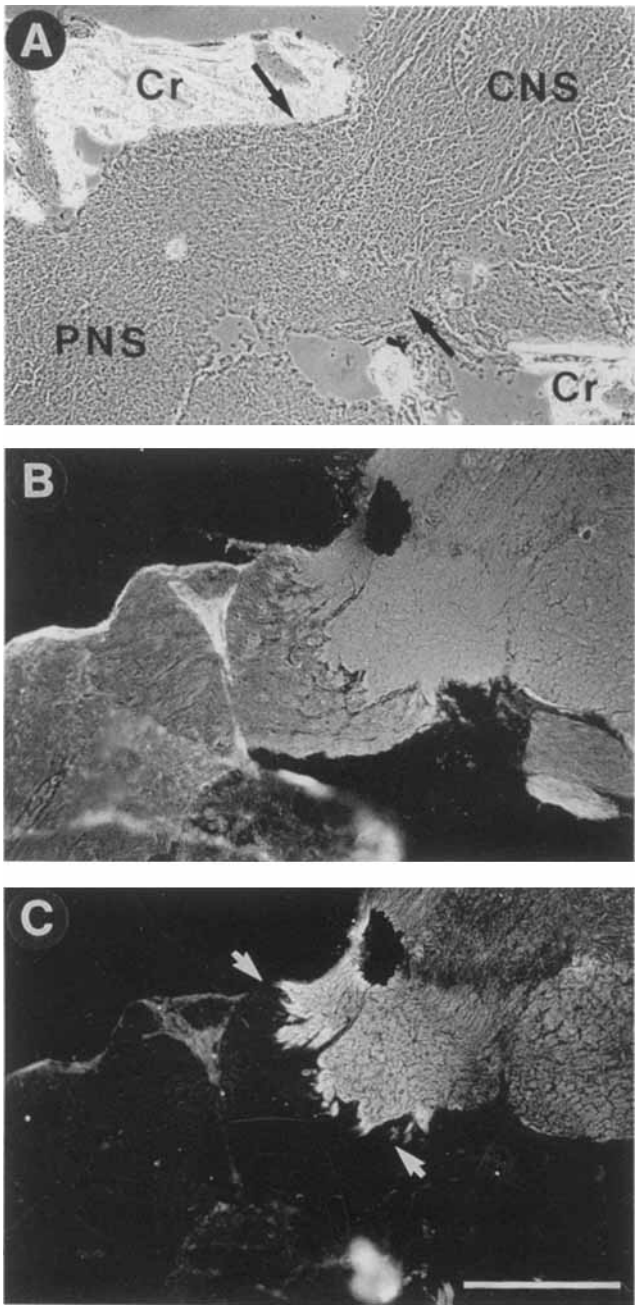


Fig. 6. Immunohistochemical localization of myelin proteins at the PNS/CNS boundary. Phase contrast micrograph (A) of a cryosection at a level where the IX. cranial nerve leaves the cranium (Cr, arrows in A and C). The same section was double-labeled with Mab 6D2 (B) and a serum against 36K (C). Note that Mab 6D2 stains PNS and CNS myelin, whereas the 36K myelin protein is specific for the CNS. Scale bar in C: 300 μm.

2) They express over extended periods of time in vitro, cell adhesion molecules that most likely contribute to the growth of axons along their surface (Bastmeyer et al., 1993; Stuermer et al., 1992). 3) They can associate with axons and this association is required for an ongoing production of the myelin protein 36K. Still, these oligodendrocytes are distinct from mammalian and fish Schwann cells in several respects. Goldfish Schwann cells identified by the 6D2 antibody like their mammalian counterparts exhibit the low affinity NGF receptor which is absent from fish oligodendrocytes. Schwann cells, but not oligodendrocytes, carry the cell adhesion molecule defined by Mab E 21 (Paschke et al., 1992), which distinguishes them further from oligodendrocytes. As known from earlier studies (Jeserich and Waehneldt, 1986b), oligodendrocytes but not Schwann cells express the CNS-specific myelin protein 36K. Despite their striking similarities, optic nerve/tract-derived oligodendrocytes and fish Schwann cells can now be distinguished in vitro on the basis of their antigenic phenotype.

That oligodendrocytes of the fish CNS resemble Schwann cells has been suggested in earlier studies. They not only share the glycosylated myelin proteins IP1 and IP2 (Jeserich and Waehneldt, 1986a), both being related to the mammalian PNS myelin protein P0 (Schliess and Stoffel, 1991) but also fail to differentiate in vitro beyond the level of IP-expression (Jeserich and Stratmann, 1992) when kept in dissociated cell culture from the larval trout brain. This suggests that, like Schwann cells, they might require axonal contact and/or other exogenous stimuli for the expression of a more advanced myelinogenic phenotype (Mirsky and Jessen, 1970; Politis et al., 1982). The present results confirmed for optic nerve/tract-derived goldfish oligodendrocytes that the induction of at least one major CNS myelin constituent, the 36K protein, is mediated through axonal contact (Jeserich and Rauen, 1990; Jeserich and Stratmann, 1992). No other exogenous stimuli, such as the elevation of cAMP or serum components or certain substrate molecules, were capable of mimicking this effect. Factors from astrocytes obviously are not sufficient to evoke 36K induction since cells of this type always coexisted with oligodendrocytes in these glial cell cultures (Bastmeyer et al., 1991, 1993). Control cultures which did not receive retinal axons never showed 36K expression by oligodendrocytes.

The induction of 36K is probably the result of a close interaction between the axons and oligodendrocytes and is not elicited through the mere presence of axons. This is concluded from the absence of 36K in substrate-associated oligodendrocytes, which axons use as a surface for their growth (Bastmeyer et al., 1993). Interestingly enough, only those oligodendrocytes which had left their substrate-adherent partner oligodendrocytes and resided in an elevated position alongside axons fascicles produced 36K. However, they still did not express Gal-C.

In their dependence on axonal contact for induction of advanced myelogenic proteins, fish oligodendrocytes differ from their mammalian counterparts which spontaneously differentiate in vitro from precursor cells into mature oligodendrocytes (reviewed in Pfeiffer et al., 1993). A further difference lies in an ongoing expression of cell adhesion molecules by fish glial cells. This is another property that puts fish optic nerve/tract-derived oligodendrocytes closer to Schwann cells (Bunge and Hopkins, 1990). We may expect that fish Schwann cells and oligodendrocytes express more growth-promoting mole-

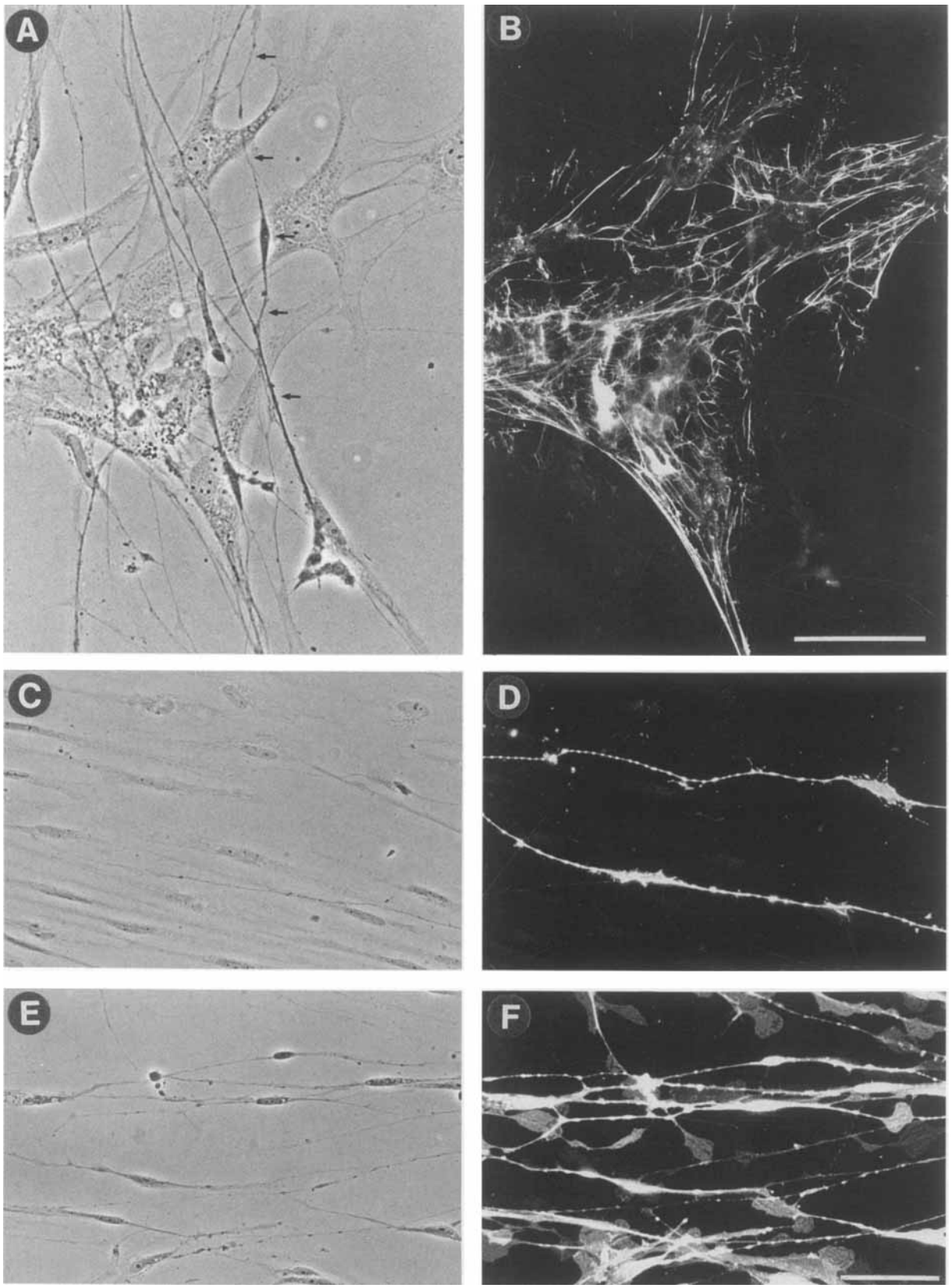


Fig. 7. Immunocytochemical characterization of PNS glial cells. All panels show goldfish cranial nerve glial cells after 3 weeks in vitro. A and B show the same field in phase contrast (A) and after immunostaining with anti-fibronectin (B). Fibroblasts with a compact morphology express fibronectin (B). The spindle-shaped Schwann cells (one is depicted by arrows in A) are fibronectin-negative. Schwann cells, but not the fibroblasts, are stained with Mab O4 (C, D). These Schwann cells also express myelin proteins as shown by 6D2 staining (E, F). Scale bar in B: 50 μm, applies to A, B. Scale bar in F: 50 μm, applies to C–F.

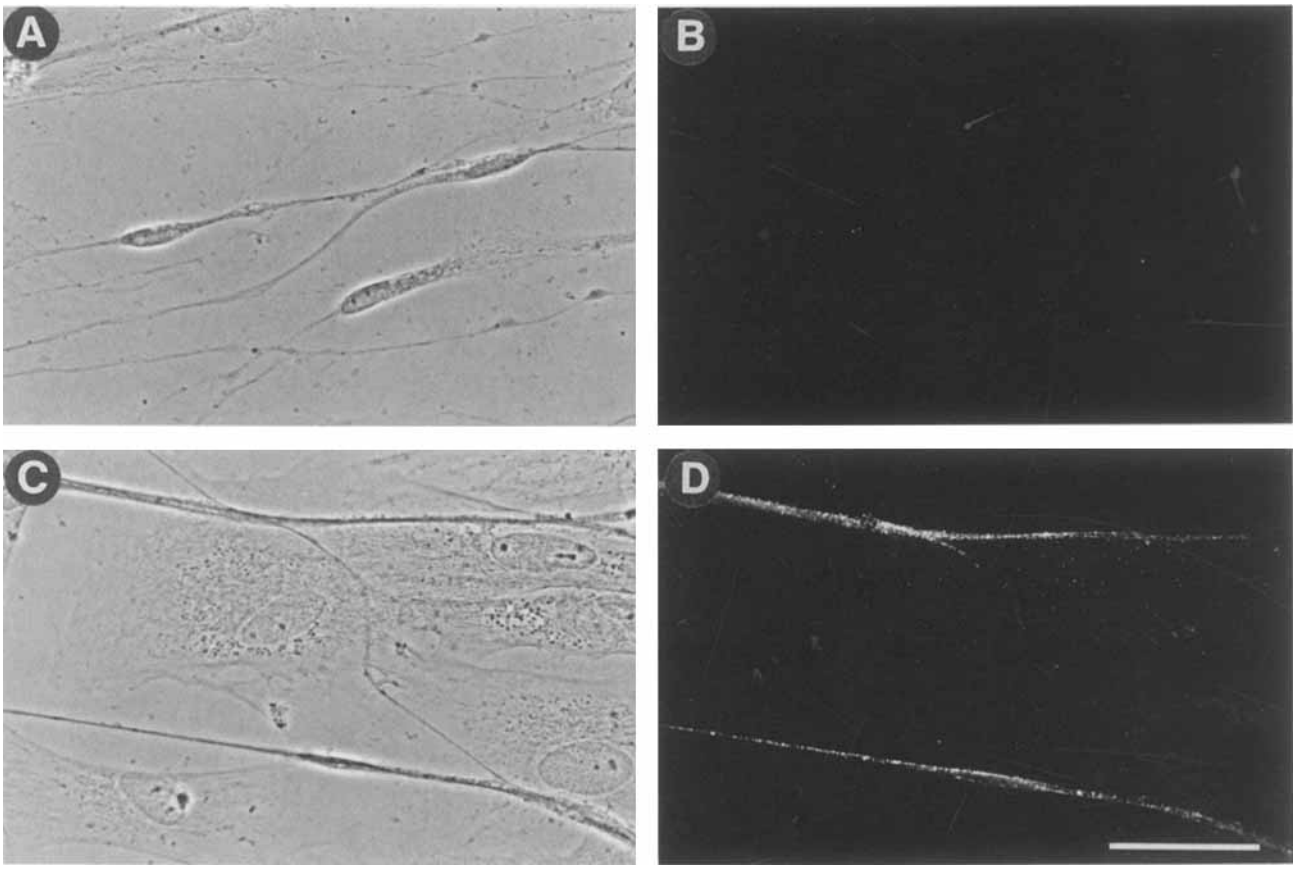


Fig. 8. Expression of NGF-receptor on goldfish Schwann cells. Corresponding phase contrast (**A, C**) and immunofluorescence (**B, D**) micrographs of CNS (A, B) and PNS (C, D) glial cell cultures treated with Mab 192-IgG against low affinity NGF receptor. Oligodendrocytes and astrocytes do not express NGF receptor (A, B). In PNS cultures, only the Schwann cells show a punctate staining pattern (C, D). Scale bar in D: 30 μm, applies to all panels.

cules than we have been able to determine here. Oligodendrocytes, however, lack Neurolin, which is found on fish Schwann cells. But the fact that fish optic nerve/tract-derived oligodendrocytes produce molecules such as NCAM and the L1-like molecule E 587 is consistent with their ability to promote growth of axons along their surface (Bastmeyer et al., 1993). When compared with mammalian and avian oligodendrocytes this property appears unique (Keirstead et al., 1992; Schwab and Caroni, 1988). It is at present unclear, however, whether fish optic nerve/tract oligodendrocytes also produce these molecules in vivo and assist injured axons in their regenerative growth within the fish visual pathway.

The place of origin of the oligodendrocytes that divide in vitro and those that remyelinate axons in vivo (Wolburg and Bouzehouane, 1986) remains unknown. Our present results suggest that the proliferation of oligodendrocytes and astrocytes, at least in vitro, does not depend on the continued division of a pool of undifferentiated precursors but occurs instead through divisions of 6D2/04-positive oligodendrocytes and astrocytes. For the identification of dividing oligodendrocytes in double-labeling experiments with BrdU, we used instead of 6D2 the O4 antibody which, in mammals, is a marker for early, undifferentiated oligodendrocytes (Sommer and Schachner, 1982). From earlier experiments on fish, however, we know that O4-positive oligodendrocytes are also 6D2 positive (Bastmeyer et al., 1991, 1993). BrdU was incorporated by both oligodendrocytes that exhibited GFAP-positive fibrils and by those (the majority) that were GFAP negative, suggesting that both are capable of division. Furthermore, the progeny of individual oligodendrocytes consisted of 04/GFAP-positive cells and cells that were 04 positive and GFAP negative. This and the observation that the number of 04-positive oligodendrocytes, which co-express GFAP in 1 week and older cultures (in F12 at 28°C) was always small, is compatible with two possible modes of their in vitro development. Oligodendrocytes derived from a recently divided oligodendrocyte either do not necessarily go through a phase in which they transiently express GFAP, or the duration of their transient GFAP expression is very brief. While A2B5 did not define a specific differentiation state of glial cells considered here, a precursor for oligodendrocytes reminiscent of the A2B5-positive progenitor of mammals (Raff et al., 1983) appears to exist in fish larvae (Jeserich and Stratmann, 1992) and mature brain tissue, too (Maggs and Scholes, 1986; Jeserich, unpublished). They are obviously needed,

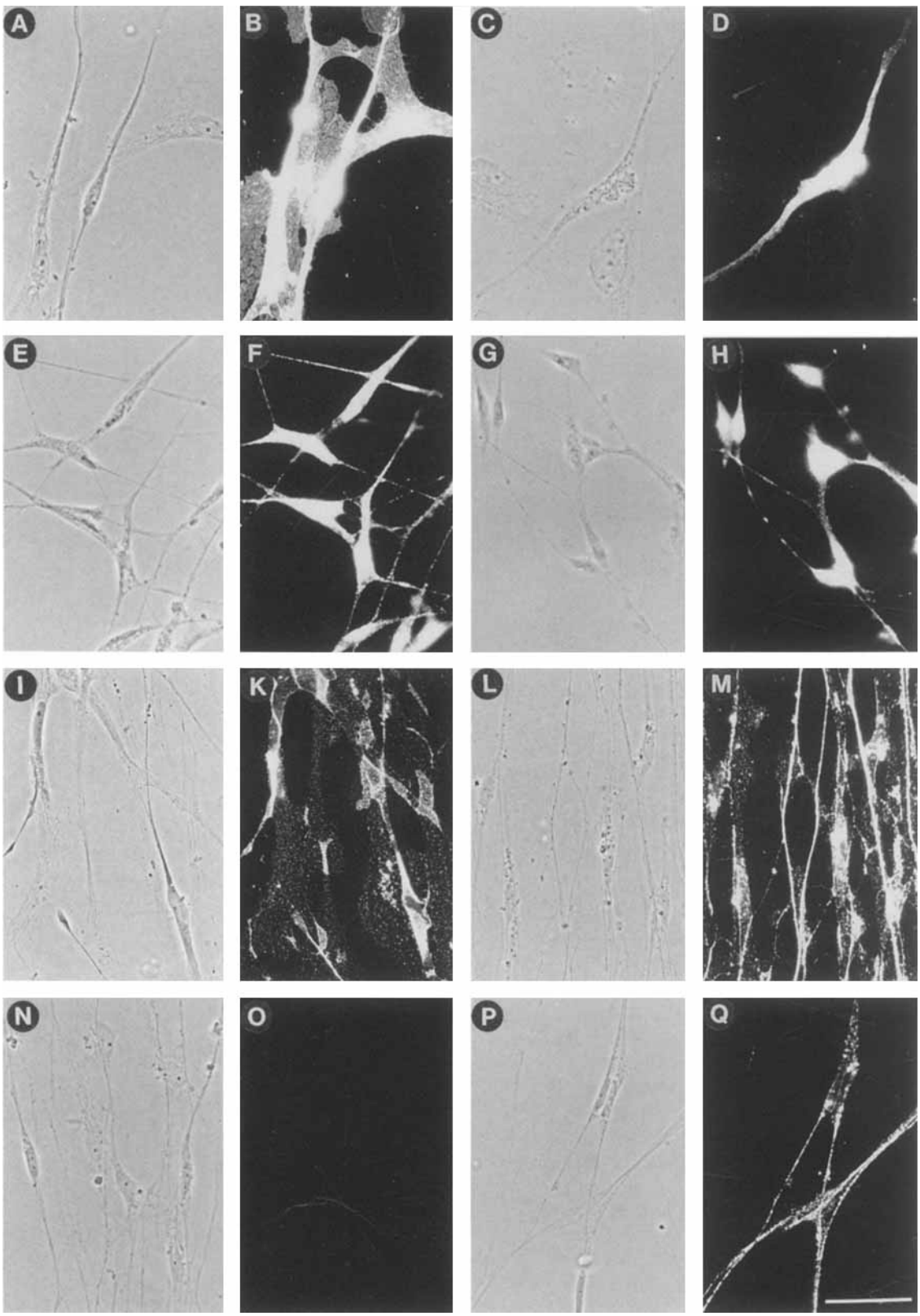


Fig. 9. Cell adhesion molecules on goldfish oligodendrocytes and Schwann cells. Corresponding phase contrast (A, C, E, G, I, L, N, P) and immunofluorescence (B, D, F, H, K, M, O, Q) micrographs of oligodendrocytes (left two columns) and Schwann cells (right two columns) stained with antibodies against cell adhesion molecules. Oligodendrocytes express the L2/HNK1 epitope (A, B), NCAM, recognized by Mab D3 (E, F), and an L1-like molecule, recognized by Mab E587 (I, K). They do not express the candidate cell adhesion molecule Neurolin recognized by Mab E21 (N, O). Schwann cells are positive for the L2/HNK1 epitope (C, D), Mab D3 (G, H), Mab E587 (L, M), and Mab E21 (P, Q). Scale bar in Q: 50 μm, applies to all panels.

since new retinal axons are continuously added in adult fish optic nerves (Easter et al., 1981) which have to become myelinated as they mature.

It appears that transection of the optic nerve and the cellular and molecular events following injury stimulate oligodendrocyte division. Whether through cell dissociation or through explantation of nerve pieces, we always obtained greater numbers of and more rapidly dividing oligodendrocytes in cultures from injured than from normal optic nerves. This suggests that optic nerve injury either triggers precursors resident in vivo which divide and subsequently produce oligodendrocytes that can further divide (as seen in our culture) or it stimulates resident oligodendrocytes to divide and to continue to do so in vitro. This trigger may lie in the deprivation of the axons and/or factors produced by microglial cells (Guilian et al., 1985).

Together with results on larval and juvenile trout oligodendrocytes (Jeserich and Rauen, 1990; Jeserich and Stratmann, 1992) the present observations indicate that certain myelogenic features are instable in fish oligodendrocytes (Jeserich and Rauen, 1990). That adult goldfish optic nerve/tract oligodendrocytes lacked 36K throughout their growth in vitro unless they interacted with axons argues for the idea that they dedifferentiate rapidly and completely and remain in a de-differentiated state until a relevant stimulus reaches them. The axon-mediated stimulus that provokes 36K expression, however, does not suffice to induce synthesis of the glycolipid Gal-C. While Gal-C in mammalian Schwann cells can be re-induced by agents elevating intracellular cAMP levels (Morgan et al., 1991; Sobue and Pleasure, 1984), forscolin, which elevates cAMP in vertebrate cells, failed to evoke GalC-production in optic nerve/tract-derived goldfish oligodendrocytes. Fish optic nerve/tract-derived oligodendrocytes therefore require more signals to maintain their differentiated phenotype in vivo than those tested here. During further in vivo maturation additional stimuli may come from the axons once appropriate synaptic contacts have been established. In this context, it will be interesting to learn whether the concomitant maturational changes of axons and of glial cells lead to a downregulation of the cell surface-associated cell adhesion molecules in goldfish oligodendrocytes.

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