Neurolin, a Cell Surface Glycoprotein on Growing Retinal Axons in the Goldfish Visual System, Is Reexpressed during Retinal Axonal Regeneration

Katja A. Paschke,* Friedrich Lottspeich,‡ and Claudia A. O. Stuermer*

*Faculty of Biology, University of Konstanz, D-7750 Konstanz, Germany; and Max-Planck-Institute for Biochemistry, Genzentrum, D-8033 Martinsried, Germany

Abstract. The mAb E 21 recognizes a cell surface glycoprotein selectively associated with fish retinal ganglion cell axons that are in a state of growth. All retinal axons and ganglion cells in goldfish embryos stained for E 21. In adult fish, however, E 21 immunoreactivity exhibited a patterned distribution in ganglion cells in the marginal growth zone of the continuously enlarging fish retina and the new axons emerging from these cells in the retina, optic nerve, and optic tract. The E 21 antigen was absent from older axons, except the terminal arbor layer in the tectum, the Stratum fibrosum et griseum superficiale where it was uniformly distributed.

Upon optic nerve transection, the previously unlabeled axons reacquired E 21 positivity as they regenerated throughout their path to the tectum. Several months after ONS, however, E 21 staining disappeared from the regenerated axons over most of their lengths but reappeared as in normal fish in the terminal arbor layer.

The immunoaffinity-purified E 21 antigen, called Neurolin, has an apparent molecular mass of 86 kD and contains the HNK1/L2 carbohydrate moiety, like several members of the class of cell adhesion molecules of the Ig superfamily. The NH_2-terminal amino acid sequence has homologies to the cell adhesion molecule DM-Grasp recently described in the chicken.

Thus, retinal ganglion cell axons express Neurolin during their development and are able to reexpress this candidate cell adhesion molecule during axonal regeneration, suggesting that Neurolin is functionally important for fish retinal axon growth.

THE formation of orderly nerve cell connections requires that axons find appropriate substrates for their elongation and cues that guide them along specific routes. To interact with their environment, the developing axons are equipped with sets of cell surface molecules such as the cell adhesion molecules (for review see Jessell, 1988). They participate in the formation of cellular contacts, promote the growth of axons along other axons or other cells (Lagenaur and Lemmon, 1987; Bixby and Zhang, 1990; Chang et al., 1990; Doherty et al., 1990; Furley et al., 1990; Matsunaga et al., 1988), mediate axon fasciculation (Rathjen et al., 1987*a*,*b*), and contribute to axonal guidance. The expression of some of these cell adhesion molecules like fasciclin I and II in grasshopper (Bastiani et al., 1987), TAG-1 in the rat (Dodd et al., 1988), and DM-Grasp (= SCI) in chick (Burns et al., 1991; Tanaka et al., 1991) is restricted to subsets of axons or to specific segments of axonal pathways. Thus they may contribute to highly specific axon–axon associations and pathway choices. Another characteristic of some of these molecules is that they appear on selected groups of neurons and axons in higher concentrations at certain times suggesting that they are needed for specific periods of neuronal differentiation. Thus, neurons possess the mechanism to regulate the expression of cell adhesion molecules both temporally and spatially.

A question of particular interest is whether and to what extent neurons are capable of expressing cell surface molecules and redirecting them to particular sites when neuronal processes are injured in adults and how reexpression may be linked to the success or the failure of axonal regeneration. Little is known about the regulation of cell surface molecules by central nervous system neurons in warmblooded vertebrates, which are unable to spontaneously regenerate their axons (for review see Skene, 1989). Injured axons in the peripheral nervous system, however, regrow and some cell adhesion molecules have indeed been shown to reappear during axonal regeneration (Martini and Schachner, 1988). In contrast to warmblooded vertebrates, central nervous system axons in fish are able to regenerate readily (for review see Gaze, 1970). Although the retinotectal projection of fish has been used for decades to study axonal pathfinding during embryogenesis and during the restoration of this projection in adults, very little is known about the occurrence and distribution of cell surface molecules that are associated with retinal axonal growth and regeneration. The unique mode in which the retinotectal projection develops (Raymond, 1986),

and retinal axons are ordered (Easter et al., 1981), and the capacity of fish retinal ganglion cells to regenerate their axons (for review see Gaze, 1970) make this system especially interesting for studying the appearance and function of growth-associated cell surface molecules.

The development of the fish retinotectal projection is as follows: during the second and third day of embryonic life, retinal axons grow from the eye, reach the tectum, and form a retinotopically organized map (Stuermer, 1988; Stuermer and Raymond, 1989). Subsequently, the retina and tectum continue to grow by adding new neurons into adulthood (Raymond, 1986). The retina grows in annuli (Johns, 1977) and new axons from the new ganglion cells join the axons of the preceding generation and grow towards their target (Easter et al., 1981; Stuermer and Easter, 1984*a*). This unique developmental strategy leads over time to the well-known age-related order of retinal axons in the fish retinotectal pathway (Easter et al., 1981; Stuermer and Easter, 1984*a*). In fish there is, therefore, always a population of growing axons which should express growth-associated molecules. Moreover, such molecules should also be found when axons regrow after injury.

We have discovered two such growth-associated cell surface molecules in the fish retinotectal system with monoclonal antibodies, N-CAM 180 (Bastmeyer et al., 1990), and the E 587-antigen (Vielmetter et al., 1991). This study presents a third antigen that meets the above expectations. This antigen which we named "Neurolin" is recognized by the mAb E 21. Moreover, the pattern of E 21 labeling suggests that ganglion cells in fish can upregulate the expression of growth-associated cell surface molecules such as Neurolin during retinal axonal regeneration. Preliminary accounts of these findings have been represented (Wehner, K. A., and C. A. O. Stuermer. 1990. *Soc. Neurosci. Abstr.* 16).

Materials and Methods

Preparation of the Immunogen

A fraction enriched in cell surface membranes of regenerating adult goldfish optic nerves 4 wk after optic nerve section (ONS)[1] was obtained as described (Vielmetter and Stuermer, 1989). In brief, the isolated nerves were homogenized in homogenization-buffer (pH 7.4, 10 mM Tris-HCl, 1.5 mM $CaCl_2$, 15 μg/ml 2,3-dehydro-2-desoxy-*N*-acetylneuraminic acid), and the protease inhibitors spermidine, (1 mM), aprotinin (25 μg/ml) leupeptin (25 μg), and pepstatin (5 μg/ml). Cell surface membranes were enriched in the interband of a sucrose step gradient (upper phase 20%, lower phase 50% sucrose), by centrifugation (6×10^4 *g*, 10 min, 4°C). These membrane fragments (in PBS) were used for the immunization protocol and fusion that gave the monoclonal antibody E 21. mAb M 501, however, was obtained from a protocol, in which membrane fragments were solubilized in buffer with the detergent octylglucoside (OG) (OG-lysis buffer: 100 mM octylglucoside, 20 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl, 1 mM CaCl, 1 mg/ml PMSF, pH 7.4) by sonication. The supernatant obtained by centrifugation (1×10^5 *g*, 1 h, 4°C), the so-called OG-brain extract, was also used for the determination of the molecular weights of E 21 antigen on SDS-PAGE (see below). Proteins of the OG-brain extract were reconstituted in liposomes, which were prepared according to Vielmetter et al. (1991).

Production of Monoclonal Antibodies

E-21. A female 5-wk-old BALB/c mouse was injected five times intraperitoneally with fractions enriched in cell surface membranes from regenerating optic nerves (4 wk after ONS), in the following intervals and concentrations: d 0, 160 μg membranes in 200 μl Freund's adjuvant; d 19 and d 40, 300 μg in 400 μl; and d 67, 160 μg in 400 μl; d 110, 160 μg in 200 μl incomplete Freund's adjuvant. The mouse was killed 44 d after the last immunization and spleen B cells were fused with SP 2/0-AG 14 myeloma cells using standard fusion protocols (Peters and Baumgarten, 1985).

M 501. Another mouse of the same strain and age was injected with liposomes (0.75 mg) with proteins from the OG brain extract three times at wk intervals, and was killed 3 d later.

The supernatants of the hybridoma clones were tested on transverse cryostat sections of adult goldfish tecta, nerves, and retina, on retina whole mounts (fixed and unfixed), and on dot blots to determine their class specificity. The two antibodies here were E 21 IgG and M 501 IgM.

Immunohistology

Unfixed tissue (brain, tract, optic nerve, retina) from normal adult goldfish and from fish whose optic nerves had been cut at various time intervals before killing was embedded in TISSUE-TEC (Miles Inc., West Haven, CT) and immediately frozen in liquid nitrogen. Goldfish embryos were embedded as a whole. To prepare flatmounts, isolated retinas (Vielmetter and Stuermer, 1989) were attached to a nitrocellulose filter. Optic nerves and tecta were serially sectioned in a transverse plane. Further tissues prepared as above were spinal cord and various inner organs such as liver, kidney, heart, intestinal tract, and gills.

Cryostat sections were collected on HNO_3 precleaned slides, dehydrated, and fixed for 5 min in −20°C cold methanol and rehydrated in PBS at room temperature for 3 × 5 min. Sections were incubated with the primary antibodies for 1 h at 37°C. After three washes (5 min) in PBS, FITC-coupled secondary goat anti-mouse antibodies (Dianova GimbH, Hamburg, Germany) were added and incubated for 1 h at 37°C. Retinal whole mounts were exposed to the primary and secondary antibodies either unfixed or after fixation with methanol.

Retinal Explants and Culture Conditions. Retinal explants were prepared as described (Vielmetter and Stuermer, 1989). In brief, 300-μm-wide explants were placed, ganglion cell layer down, onto laminin-coated coverslips. Two small metal blocks at both ends of the retinal explants kept them in position. The explants were covered with 2 ml L 15 culture medium (L 15 medium containing 10% FCS, 20 mM Hepes, 50 mg/liter Gentamycin, 0.4% methylcellulose, pH 7.4). Axons from retinal explants were allowed to grow for 2 d at 23°C. For immunostaining, explants were fixed (−20°C cold methanol) followed by three rinses in PBS and exposed to primary and secondary antibodies as described or exposed to the antibodies without fixation.

Purification of Neurolin

Immunoaffinity absorption of Neurolin was performed using columns of CNBr-Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) to which the purified E 21 antibody had been covalently coupled (1 ml with 10 mg protein). OG-brain extract was passed over the affinity column (Pharmacia LKB Biotechnology Inc.), (12 h at 4°C under rotation). The affinity column containing the bound antigen was subsequently washed, first with OG-lysis buffer with 0.15 M NaCl and then with OG-lysis buffer with 0.5 M NaCl. The antigen was eluted with buffer (pH 11) of 50 mM triethylamine and 100 mM OG (pH 11.5) into a 10% 1 M Na-acetate buffer (pH 4) to neutralize the eluate.

The eluted antigen was subjected to SDS 7.5 or 10% PAGE in mini-gels (6.5 × 9 cm) (Laemmli, 1970). To run the antigen under nonreducing conditions, mercaptoethanol was omitted from the loading buffer. Gels were silver stained (Heukeshofen, 1985). For Western blot analysis (Towbin et al., 1979), the immobilon (Millipore Corp., Bedford, MA) membrane onto which the antigen was transferred was blocked with 3% BSA in PBS and 0.05% Tween (1 h, 37°C), and then incubated with the appropriate monoclonal or polyclonal antibodies and rinsed three times with buffer (PBS and 0.05% Tween 20). Some of the transferred antigen was stained with Auro Dye (Janssen Virotech, Rüsselsheim, Germany), and then BSA was omitted from the buffer. Antibody binding was detected by alkaline phosphatase-coupled goat anti-mouse secondary antibody (Promega Corp., Madison, WI; diluted 1:10,000 in 1% BSA in PBS, 1 h, 37°C). After three washes in buffer (PBS and 0.05% Tween 20) blots were developed in standard staining solutions.

The affinity-purified antigen was exposed to an antibody against HNK1/L2 (kindly provided by M. Schachner) and, to determine its carbohydrate moieties, to various lectins, each specific for defined carbohydrate groups (Boehringer Mannheim Corp., Indianapolis, IN; glycan differentiation kit).

1. *Abbreviations used in this paper*: OG, octylglucoside; ONS, optic nerve section; SFGS, Stratum fibrosum et griseum superficiale; SO, Stratum opticum.

Molecular Weight Determination of M 501 Antigen

A preparation enriched in cytoskeletal proteins from goldfish optic nerves was obtained according to a protocol in Quitschke and Schechter (1983). The proteins were subjected to two-dimensional gel electrophoresis as described in Giordano et al. (1990); transferred to immobilon membranes (as described above); incubated with M 501 (1 h, 37°C); and after blocking the membrane in 3% BSA and three rinses in PBS, they were incubated with HRP-coupled secondary antibody (goat anti-mouse [Dianova GimbH], 1:100 in PBS and 10% FCS and 0.5% Tween) for 1 h at 37°C. Blots were developed in staining solution consisting of 100 mM Tris, pH 7.4, 10% chloronaphthol (3 mg/ml) in ETOH and 5 μl 30% H_2O_2.

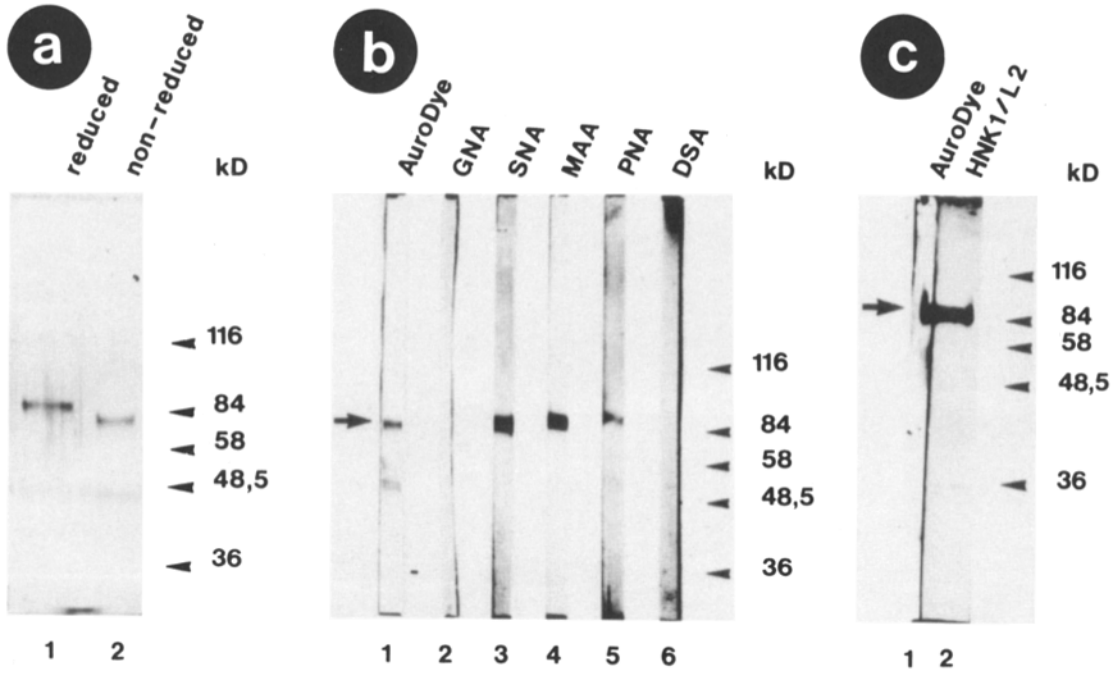
Protein Sequencing

E 21 antigen was purified by immunoaffinity chromatography and subjected to SDS-PAGE as described above. The antigen was electroblotted to glass fiber membrane according to a published procedure (Eckerskorn et al., 1988). The 86-kD antigen band was cut out from the glass fiber membrane. The amino acid sequence for the NH_2-terminal of the protein (30 pm, ~2.5 μg) was determined with a protein Sequenator (Appl. Biosystems, Inc., Foster City, CA).

Results

Biochemical Characterization of Neurolin

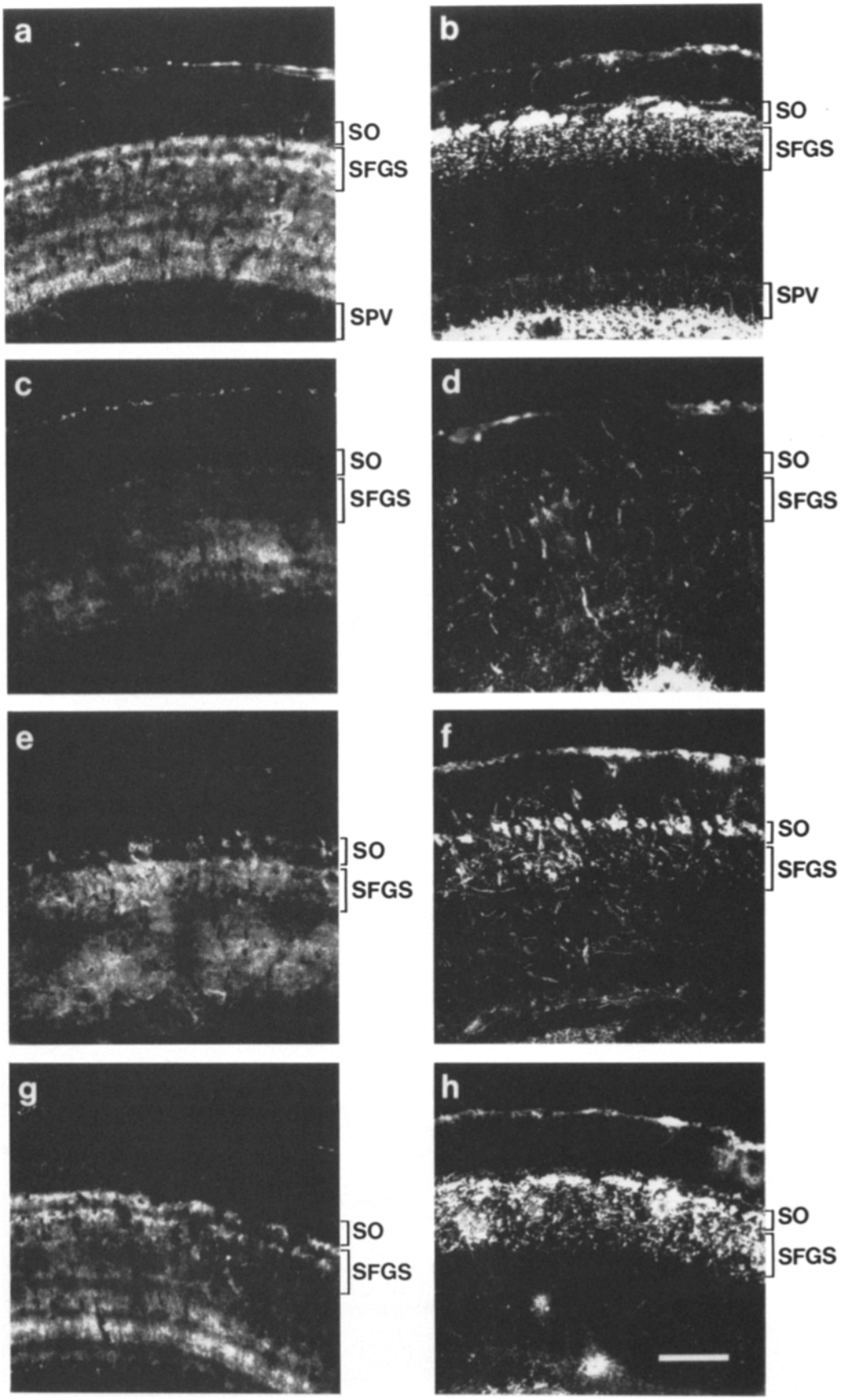
Neurolin was purified with the antibody E 21 over immunoaffinity columns from octylglucoside extracts from either goldfish whole brains or from brain membrane fractions. When the protein eluted from these columns was subjected to SDS-PAGE the apparent molecular mass of Neurolin was



d

Neurolin: H_2N - X G T V I G L Y G E T I V
DM-Grasp: H_2N - L Y T V N A V Y G D T I T

Figure 1. SDS-PAGE and immunoblots of Neurolin. (*a*) Silver-stained SDS gel of the immunopurified protein. Under reducing (lane *1*) and nonreducing (lane *2*) conditions the protein has an apparent molecular mass of 86 and 80 kD, respectively. (*b*) Immunoblots of the immunoaffinity-purified protein after SDS-PAGE. Each membrane strip was incubated with a different Digoxigenin-coupled lectin to identify carbohydrate moieties. (Lane *1*) Control: Neurolin, stained with Auro Dye. The two minor bands below the 86-kD band are degradation products resulting from freezing and thawing of the protein. (Lane *2*) *Galanthus nivalis* agglutinin (*GNA*) recognizes terminal mannose, which is apparently not present on Neurolin. (Lane *3*) *Sambucus nigra* agglutinin (*SNA*) recognizes salic acid-linked α-(2–6) to galactose, present on the protein. (Lane *4*) *Maackia amurensis* agglutinin (*MAA*) recognizes salic acid-linked α-(2–3) to galactose, present on the protein. (Lane *5*) Peanut lectin (*PNA*) recognizes the core disaccharide galactose β-(1–3)-*N*-acetylgalactosamine, present on the protein. (Lane *6*) Digoxigenin coupled to *Datura stramonium* agglutinin (*DSA*). DSA binds to galactose-β-(1–4)-*N*-acetylglucosamine, which is apparently absent from the protein. (*c*) Immunoblot of the purified protein after SDS-PAGE in 10% gel. Neurolin obviously carries the HNK1/L2 carbohydrate moiety. (Lane *1*) Auro Dye; (lane *2*) HNK1/L2 antibody staining. (*d*) Comparison of the NH_2-terminal sequence of Neurolin and DM-Grasp (from Burns et al., 1991). Identical residues are boxed. The first residue of Neurolin could not be determined unambiguously (*X*).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86 kD under reducing and ~80 kD under nonreducing conditions (Fig. 1 *a*). A similar shift of the apparent molecular weight was previously noted for the cell adhesion molecule Neuroglian (Bieber et al., 1989). These molecular weight determinations of the immunopurified protein were made from silver-stained gels since E 21 does not recognize the protein on immunoblots. Using several Digoxigenin-labeled lectins (see Fig. 1 *b*) the carbohydrate epitopes of the purified protein were determined, suggesting that the protein carries α 2–6– and α 2–3–bound sialic acid, and galactose-β (1–3) *N*-acetylgalactosamine. Furthermore, an antibody against the HNK1/L2 carbohydrate moiety associated with a number of known cell adhesion molecules of the Ig superfamily (Naronha et al., 1986) recognized the protein (Fig. 1 *c*). That Neurolin may belong to the class of cell adhesion molecules was supported from revealing the NH_2-terminal amino acid sequence of the protein and comparing it to published sequences. As demonstrated in Fig. 1 *d*, there is a considerable similarity of the NH_2-terminal sequence of the E 21 antigen and DM-Grasp (which is believed to be identical to SC1) (Burns et al., 1991; Tanaka et al., 1991), a recently described and novel cell adhesion molecule in chick. DM-Grasp (SC1) were found in the chick spinal cord but not in the visual system, as in fish.

To evaluate the restricted distribution of Neurolin along the fish retinotectal pathway, we compared the E 21 immunostaining pattern to that of the antibody M 501. M 501 was found to label all retinal ganglion cells and their axons from the eye to their terminal layers in the tectum. M 501 also stained a few processes of radial glial cells in the tectum, which could be clearly distinguished from axonal profiles (Fig. 2, *b*, *d*, *f*, and *h*). M 501 only labeled axons and glial cell processes after treating the tissue with methanol, suggesting an intracellular location of the M 501 antigen. On one-dimensional SDS gels from a preparation enriched in filament proteins, M 501 bound to a protein with an apparent molecular mass of 58 kD, and on two-dimensional gels to four spots between the isoelectric points 5.5 and 5.8. Taken together these data indicate that M 501 probably recognizes the previously described goldfish neuro- and gliafilament proteins ON 1–ON 4 (Tesser et al., 1986).

E 21 Immunostaining Pattern in Normal Adult Goldfish

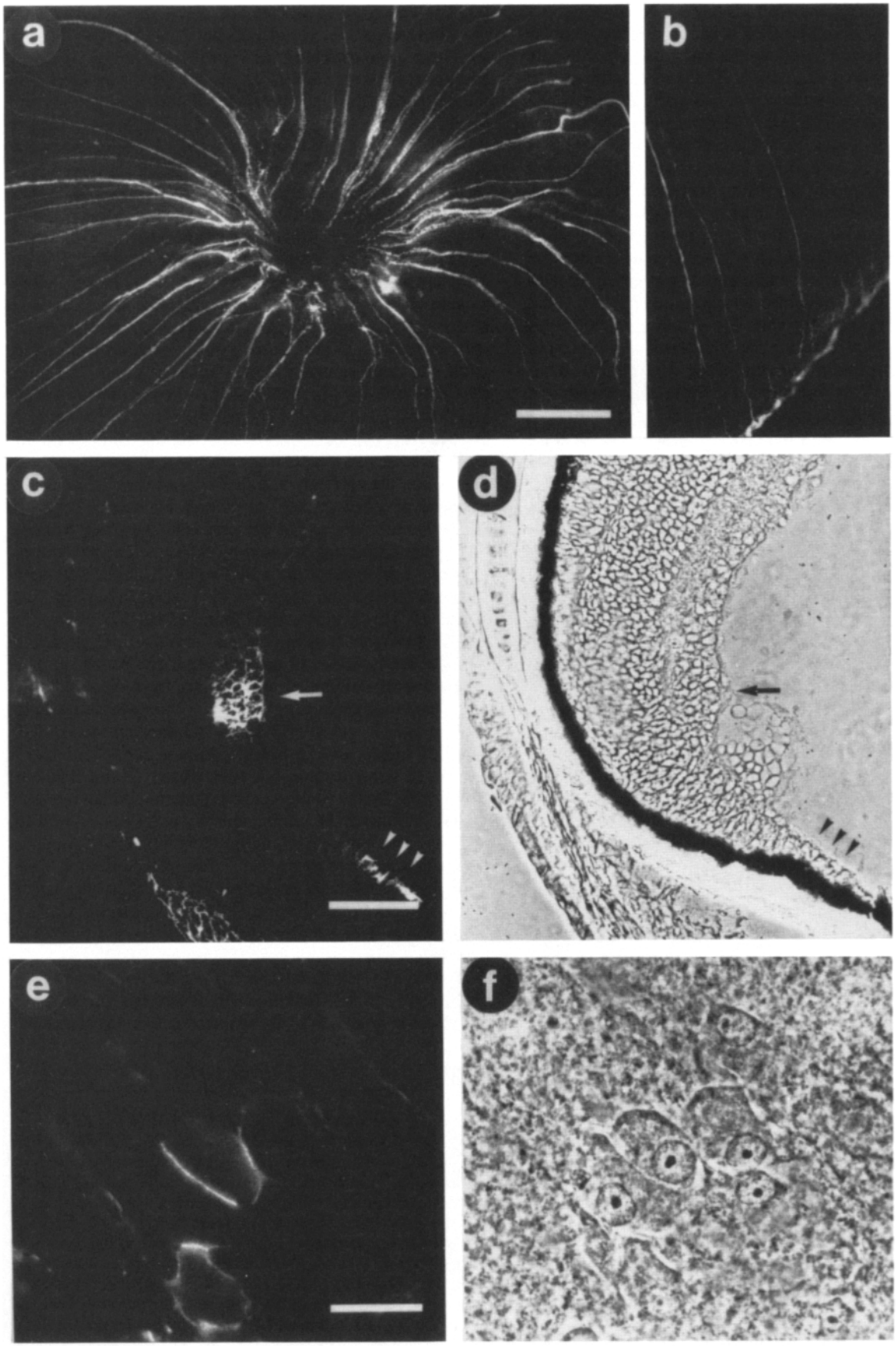
In contrast to M 501, E 21 exhibits a spatially and temporally restricted expression pattern. In flatmounted retinas, either fixed or unfixed, E 21 labeled only a few axons derived from ganglion cells at the retinal peripheral margin (Fig. 3, *a* and *b*). These axons traveled from their marginal origin in a position superficial to older axons, as is typical for axons from the newborn retinal ganglion cells (Easter et al., 1984), and met at the optic disk to leave the eye. Since this staining pattern emerged with and without fixation, we conclude that Neurolin (or at least the epitope to which E 21 binds) is localized to the outer surface of the retinal axonal membranes. For the same reasons, this pattern is not an artifact of poor antibody penetration. M 501 in contrast, did not label the unfixed retinal axons, but recognized all axons after fixation.

On cross sections through the retina, E 21 also labeled the somata of the ganglion cells from which the new axons arise at the retinal margin (Fig. 3, *c* and *d*), and undifferentiated cells in the retinal proliferative zone. In these sections the older and more central ganglion cells appeared E 21 negative. E 21 labeling of retinal explants showed that the antigen was present on these cells but was restricted to sites of cell–cell contact (Fig. 3, *e* and *f*). The staining of cell contacts was observed in explants from normal eyes which had been exposed to the antibody within 3–5 h after excision as well as those which were kept in culture for several days and which had extended axons. Still, we cannot exclude that contact site staining was induced by the excision procedure.

In cross sections of the optic nerve, E 21–labeled axons were clustered at the nerve's ventral margin (see Fig. 5, *a*, *c*, and *e*). This position and the dorsal superficial location of E 21–positive axons in sections from the tract, correlates with known locations of new ganglion cell axons.

The axonal staining pattern of E 21 from the cell bodies to the tectum, therefore, closely resembles that seen with the antibodies D 3 against NCAM 180 (Bastmeyer et al., 1990) and E 587 against an L1-like molecule in fish (Vielmetter et al., 1991). In the tectum, however, where D3 and E 587 are still specific for the youngest axons that travel along the tectal margin, diffuse E 21 immunofluorescence was seen in the Stratum fibrosum et griseum superficiale (SFGS) throughout the mediolateral and rostrocaudal extent of the tectum. These tectal layers contain the majority of the retinal axon terminal arbors (Stuermer and Easter, 1984*b*; Springer and Gaffney, 1981). Since E 21 labeling is spread throughout the SFGS, it includes arbors of all ages (Stuermer, 1984). Moreover, E 21 staining was entirely absent from the layer Stratum opticum (SO), dorsal from the SFGS, through which retinal axons enter and travel through the tectum (Springer and

Figure 2. Cross sections through tecta immunostained with E 21 in *a*, *c*, *e*, and *g* and with M 501 in *b*, *d*, *f*, and *h*. (*a* and *b*) Normal; (*c* and *d*) 10 d after ONS; (*e* and *f*) 30 d after ONS; and (*g* and *h*) 11 mo after ONS. (*a* and *b*) M 501 reveals the distribution of the retinal axons in their major target layers SO and SFGS of the tectum (*b*). Staining is also seen on glial cells in the subependymal layer deep to the cell dense layer Stratum periventriculare (*SPV*) (*b* and *d*) and on a few radially oriented glial profiles (*b*, *d*, and *f*). In the layers receiving the retinal afferents, E 21 (*a*) stains diffusely in SFGS. It does not stain in the SO, which is predominantly occupied by fascicles of retinal axons. E 21 also labels diffusely in variable intensity to the tectal layers between SFGS and SPV, and may represent background staining (*a*, *c*, *e*, and *g*). (*c* and *d*) At 10 d after ONS, only a very few regenerating axons have returned to the tectum, as can be seen in *d*. E 21 staining on these few axons is hardly detectable on these photographs (*c*). As all previous axons have degenerated, the normal E 21 staining pattern has disappeared from the SFGS of the tectum (*c*). (*e* and *f*) At 30 d after ONS, a large number of axons seen with M 501 (*f*) have reoccupied the SO and SFGS of the tectum. The regenerating axons in the SO have reformed small fascicles. E 21 staining (*e*) is associated with the regenerating axons in the fascicles in the SO and also in SFGS. (*g* and *h*) At 11 mo after ONS, the density of regenerating axons in the fascicles of the SO and in the SFGS is as high as in normal tecta (*h*). The staining of E 21 (*g*) in the SFGS closely resembles that in normal tecta (compare with *b*), and is absent from the fiber fascicles in the SO as in normal tecta. Bar, 100 μm.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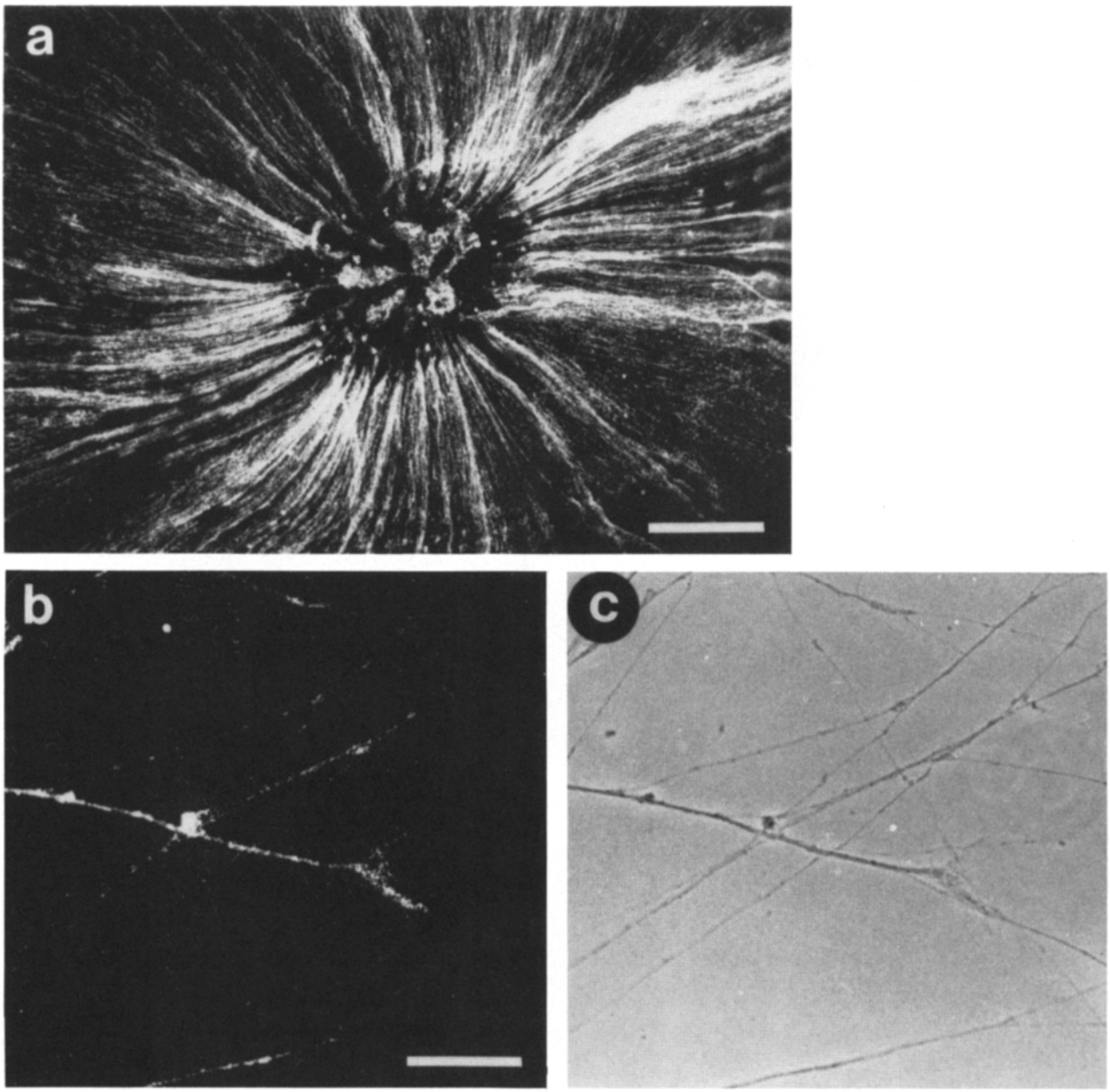
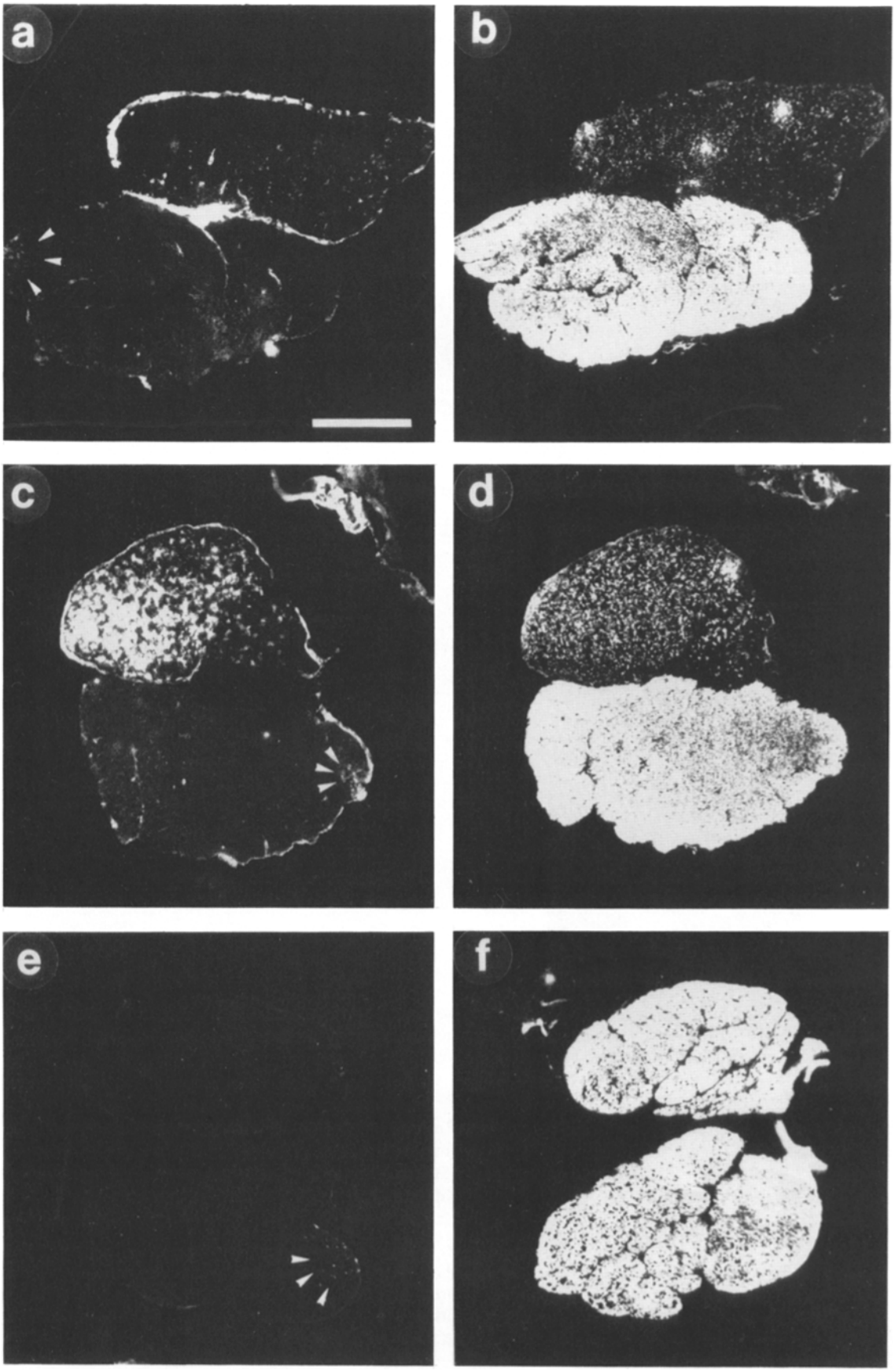


Figure 4. E 21 staining on regenerating axons. (*a*) Whole mount (unfixed) of a retina that has been connected to a regenerating optic nerve, 21 d after ONS. In contrast to normal retinas (compare Fig. 1, *a* and *b*) all retinal axons are stained by E 21 Bar, 200 μm. (*b*) Axons (unfixed), regenerating from a retinal explant in vitro, exhibit E 21 staining over their entire extent up into their growth cones. (*c*) Phase contrast of (*b*). Bar, 20 μm.

Gaffney, 1981) (Fig. 2, *a* and *b*). Thus, the specific association of E 21 with young axons in the pretectal pathways is lost in the tectum, where E 21 labeling is spread over the layers of the retinal axons' terminal arbors. Within the SFGS, E 21 staining was stratified in three sublayers, a feature that is reminiscent of the stratification of retinal axon terminal arbors (Stuermer and Easter, 1984*b*) seen with HRP. Since E 21 labeling in the SFGS is diffuse, it cannot be assigned

Figure 3. E 21 immunostaining in the retina of normal adult goldfish. (*a* and *b*) Retina whole mount which has been exposed to E 21 in an unfixed state. Only the small fascicles composed of new retinal axons are recognized by E 21. (*a*) They meet in the center of the retina to leave the eye and enter into the optic nerve. (*b*) The new axons originate from newborn ganglion cells at the retinal peripheral margin. Bar, 200 μm. (*c* and *d*) The retinal margin in a (fixed) cross section (*c*) immunostained with E 21 and (*d*) in phase contrast. In the retina, only the small population of newly formed ganglion cells (*arrow*) and undifferentiated precursor cells at the extremes of the retinal growth zone (*arrowheads*) are E 21 positive. Note that the new ganglion cells carry E 21 staining over their entire surfaces. Outside the retina E 21 staining is on connective tissues. Bar, 100 μm. (*e* and *f*) Ganglion cells in a retinal explant from more central portions of the retina (*e*) stained with E 21 and (*f*) in phase contrast. On more mature ganglion cells E 21 staining appears highly enriched at contact sites between the cells. Bar, 20 μm.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to specific profiles. E 21 may bind to axon terminal arbors or to elements in their vicinity. To determine whether E 21 immunofluorescence is correlated with the presence of retinal axons, we enucleated one eye or sectioned the optic nerve, which leads to the degeneration and disappearance of the retinal axons. In these tecta, deprived of retinal axons, E 21 labeling vanished from the SFGS (such as in Fig. 2 *c*), suggesting that E 21 immunofluorescence is indeed dependent on the presence of the retinal axons.

E 21 staining was also noted in deeper tectal layers (Fig. 2, *a*, *c*, *e*, and *g*), but this varied from fish to fish, was not systematically linked to the presence or absence of the retinal axons, and may therefore represent background staining.

E 21 Immunostaining in the Regenerating Retinotectal Pathway

In contrast to the normal retina, retinas with regenerating optic nerves exhibited E 21 staining on most or all axons (Fig. 4 *a*). However, on the ganglion cell somata of the regenerating axons, E 21 labeling remained restricted to cell contact sites (Fig. 3, *e* and *f*). At 11 mo after ONS, only the new axons were E 21 positive and these retinas were indistinguishable from the normal. Thus, the E 21 antigen, Neurolin, appears to be reexpressed by the retinal axons in the retina after ONS, even though the intraretinal axonal segments are not new.

After ONS the retinal axons regenerate (starting at day 4 after ONS) from the cut (Lanners ad Grafstein, 1980). Sections of the optic nerves exposed to M 501 demonstrate the presence of a few regenerating axons at 10 d and many more at 30 d after ONS (Fig. 5, *b*, and *d*). The adjacent sections exhibit E 21–labeled profiles in low density at 10 d and in higher density at 30 d after ONS (Fig. 5, *a* and *c*). The number of regenerating axons increases with time after ONS, but at none of the stages did E 21 label homogeneously across the whole nerve. This suggests that Neurolin is reexpressed by the regrowing axons, but possibly during only a limited period of their regenerative growth. The alternative, that Neurolin may be associated with a subpopulation of regenerating axons, is unlikely. When axons regenerated from a retinal explant in vitro on a substrate of laminin, all axons were found to be E 21 positive (Fig. 4, *b* and *c*). Staining was brighter, however, where two or more axons fasciculated with one another, and was weak and punctate on individual axons. Staining extended into the growth cones and into some but not all filopodia. Labeling was observed without fixation further substantiating that Neurolin is on the outer surface of the axonal membrane.

In sections, E 21 staining was markedly reduced at 70 d and further at 90 d after ONS and had entirely disappeared from the regenerated axons in the optic nerve by 11 mo after ONS (Fig. 5 *e*).

In the tecta connected to regenerating optic nerves as opposed to tecta with normal optic nerves, the regenerating axons were E 21 positive all along their intratectal path, including the SO and SFGS. While there were hardly any E 21–positive profiles at 10 d after ONS, there was a typical increase in the density of E 21 labeling in the SO and SFGS at 30 d after ONS (Fig. 2 *e*). The latter coincided with a larger number of regenerating axons as seen with M 501 (Fig. 2 *f*). At 11 mo after ONS, however, axon segments in the SO were again E 21 negative and the diffuse and stratified staining over the SFGS as seen in normal tecta had reappeared (Fig. 2, *g* and *h*).

Thus, Neurolin is reexpressed by the retinal axons during retinal axonal regeneration throughout their entire path. Neurolin expression appears to be downregulated with increasing time after ONS along most of the axon's paths. However, as in normal tecta, it again becomes associated with the layers of the SFGS occupied by retinal axon arbors.

E 21 Staining in the Embryonic Retinotectal System

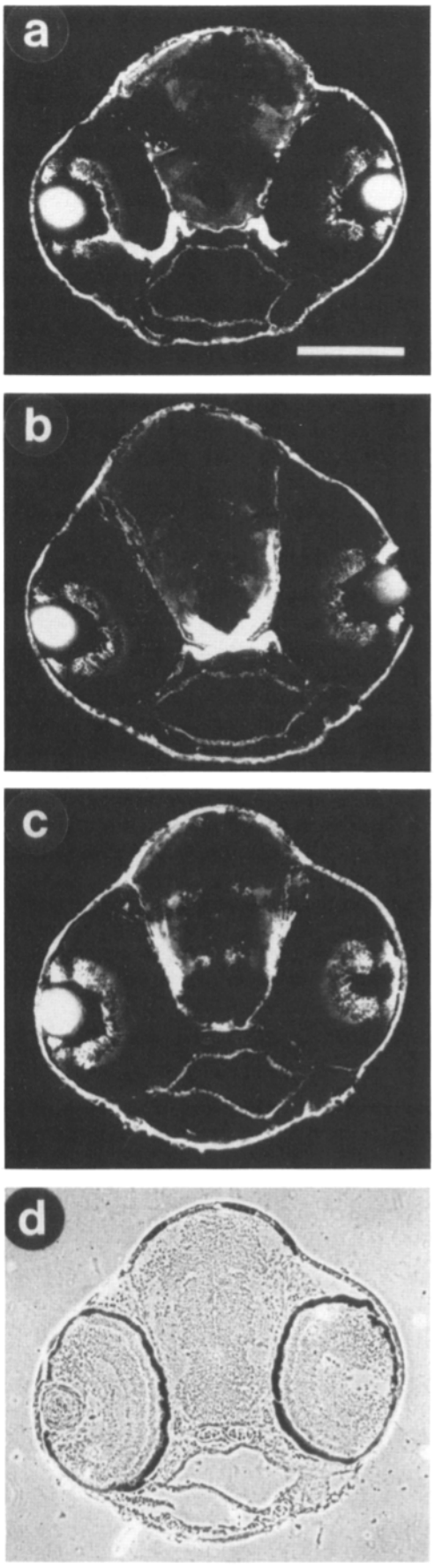
Since E 21 labeling in retinae and nerves of adult goldfish is associated with retinal axons that are in a state of growth, we investigated whether retinal axons in goldfish embryos would also have the antigen. A frontal section through the head of a 5-d-old embryo demonstrates the presence of E 21 immunostaining on retinal ganglion cells and their axons in the optic nerves, optic tracts (Fig. 6, *a* and *b*), and the rostral aspect of the tectum (Fig. 6 *c*). In the tectum, a cloud of immunoreactivity surrounded the region into which the axons had progressed. Regions in which the axons had not yet reached, as judged by M 501 staining, were devoid of E 21 labeling (Fig. 6, *c* and *d*).

These results support the notions (*a*) that growing retinal axons carry Neurolin on their surface while they move towards the tectum, and (*b*) that Neurolin in the tectum is linked to the presence of or is on the surfaces of retinal axons.

The Distribution of Neurolin in the Spinal Cord

In cross sections through the spinal cord of adult fish, E 21 staining was restricted to the lateral edge of the dorsal tract and the incoming sensory fibers (Fig. 7 *d*). In 3-wk-old fish larvae a larger portion of the dorsal tracts were labeled (Fig. 7 *c*). In fish embryos (5-d-old) E 21 staining was even more widely scattered over the developing spinal cord and included cells at the cord's ventral aspect (Fig. 7, *a* and *b*). Judged from their position, the more central cells may represent the

Figure 5. Cross sections of a normal and regenerating optic nerve at the chiasm (*a*, *c*, and *e*) stained with E 21 and (*b*, *d*, and *e*) stained with M 501. (*a* and *b*) 10 d after left ONS; (*c* and *d*) 30 d after left ONS; and (*e* and *f*) 11 mo after left ONS. The regenerating left nerve is in the upper half, the normal nerve in the lower half of the pictures. (*a* and *b*) While M 501 binds to all retinal axons in the normal nerve, E 21 recognizes only the small group of new axons (*arrowheads* in *a*, *c*, and *e*). M 501 also detects the few retinal axons, that have regenerated after 10 d. (*b*) There are also more E 21–labeled axons than in the normal nerve (*a*), scattered over the nerve's cross section. They represent the E 21–positive regenerating axons. (*c* and *d*) At 30 d after ONS, the number of regenerating axons has increased, as seen with M 501 in *d*. Most or all of the regenerating axons are stained by E 21 (*c*). (*e* and *f*) At 11 mo after ONS, the density of axons in the regenerating nerve (*f*) is comparable to that in the normal nerve. E 21 does no longer bind to the regenerating axons, indicating that the expression of Neurolin is downregulated with time after ONS. E 21 sometimes binds also to the meninges surrounding the optic nerves as seen in *a* and *c*. Bar, 200 μm.



floor plate cells and the ventrolaterally located ones, motoneurons. Thus, Neurolin in the spinal cord is more widely distributed during development and becomes restricted to a distinct axon tract with further maturation. Whether axons in this tract persist in adult fish in a state of continuous growth is not known.

The E 21 staining pattern in the developing fish spinal cord resembles, to some extent, the distribution of the recently discovered chick cell adhesion molecules, DM-Grasp (Burns et al., 1991) and SC1 (Tanaka et al., 1991). E 21 also bound to neuronal processes in organs, such as kidney, heart, gill, and the intestinal tract, and this also correlates in part to the distribution of DM-Grasp (Burns et al., 1991).

Discussion

The development of the fish retinotectal system is unique in that it continues throughout life (Raymond, 1986). Similarly unusual is the capacity of the fish retinal ganglion cells to spontaneously regenerate their axons upon injury (for review see Gaze, 1970).

The goal of this study was to identify molecular components that may play a role in the growth and regeneration of retinal axons in fish. The staining pattern with E 21 demonstrated that Neurolin, the E 21 antigen, may subserve such a function. Our experiments have shown that E 21 immunoreactivity is specifically associated with retinal axons (in the eye and the optic nerve and tract) that are in a state of growth. These include all developing retinal axons in the fish embryo, but only the small group of axons from the new ganglion cells of the retinal marginal growth zone in adults (Johns, 1977; Easter et al., 1981). The retinal axons regenerating after optic nerve transection also expressed Neurolin. Thus, Neurolin is defined as a growth associated cell surface molecule.

The absence of E 21 staining from older axons and its disappearance from regenerating axons with increasing time after ONS implies that the expression of this molecule on the axon surface is temporally regulated, declining when the major growth process has been completed.

The expression of Neurolin is apparently not only temporally, but also spatially regulated. For example, E 21 labels axons throughout the tectal synaptic layers SFGS where retinal axon terminal arbors reside (Springer and Gaffney, 1982; Stuermer and Easter, 1984*b*), but not the parent axons giving rise to these arbors. Since labeling extends throughout the SFGS, it predominantly includes the territories of arbors from older axons (Stuermer, 1984). This may seem a contradiction to the above definition of Neurolin as a growth-associated cell surface molecule; however, terminal arbors in the SFGS continue plastic rearrangements throughout the fish's life. In fish, terminal arbors are known to shift (Easter and Stuermer, 1984); i.e., they leave sites which had previously been, but no longer are, retinotopically appropriate as a result of the continuous growth of the retina and tectum in

Figure 6. Cross sections through the head of a 5-d-old goldfish embryo stained with E 21. E 21 recognizes (*a*) the ganglion cells in the retina and the retinal axons in the optic nerves, (*b*) the retinal axons at the chiasm, and (*c*) in the optic tract and the tectal neurophil. (*d*) Phase contrast of *c*. E 21 also binds to the meninges throughout the embryo's head and the lens in the eye. Bar, 200 μm.

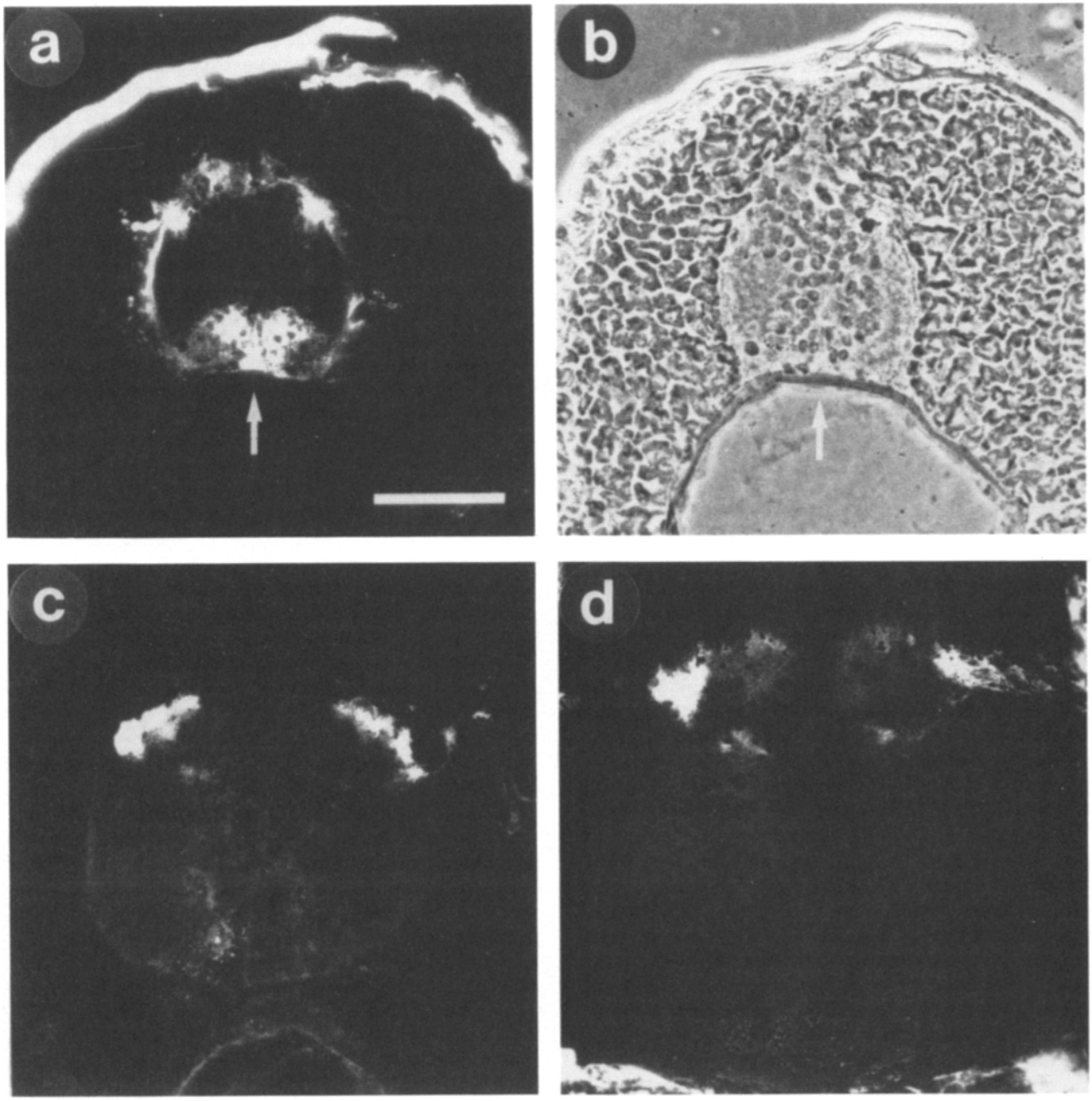


Figure 7. Cross section through the fish spinal cord stained with E 21. (*a*) In a 5-d-old fish larva, E 21 heavily labels cells in the ventral aspect of the developing cord (*arrow*) and a small area dorsally that may represent the tract of ascending sensory fibers. (*b*) Phase contrast of *a*. (*c*) In a 25-d-old larva, E 21 recognizes dorsal fiber tracts the most lateral of which is connected to sensory fibers entering the cord. (*d*) In adult fish the sensory axons, and the most lateral of the dorsal fiber tracts probably carrying the sensory axons are E 21 positive. E 21 staining has, however, disappeared from the other tracts which were still stained at 25 d, and has disappeared from cells that had expressed E 21 in 5-d-old embryos. Bar, 100 μm.

geometrically disparate patterns (Raymond, 1986). In other words, these arbors grow, albeit slowly, probably by adding processes in the direction to which the shift occurs and by deleting processes in the opposite direction (Easter and Stuermer, 1984; Reh and Constantine-Paton, 1984). The pathway of the axons that give rise to these arbors, i.e., the path through the retina, nerve, tract, and the tectal fiber layer SO, is not subject to such alterations but instead remains set (Easter et al., 1981; Stuermer and Easter, 1984*a*). Accordingly, E 21 staining is absent from the older axons in the pretectal segments of the pathway and also from the fiber layer SO.

Whether E 21 staining in the SFGS is directly on the terminal arbors or rather in their vicinity remains an unsolved question. We were unable to localize E 21 at the ultrastructural level, since the antibody did not bind to the antigen after aldehyde fixation. The observation, however, that the typical E 21 staining in the SFGS vanished upon eye enucleation and transiently after transection of the optic nerve (leading to the degeneration of the axons from the tectum), and that

it reappeared during axonal regeneration and was found on retinal axons regenerating in vitro into their growth cones indicates that E 21 staining is at least coupled to the presence of the axons and is in the SFGS most likely localized on the axon arbor processes.

From the distribution of E 21 staining in fish of different developmental stages, a dynamic rearrangement of the antigen can be inferred. While embryonic axons carry Neurolin all along their path, the more mature axons reduce their expression dramatically over their entire lengths except for the territories of their terminal arbors. Likewise, we find that ganglion cells in embryos and the newly differentiated ones at the margins of adult retinae show E 21 labeling on their entire surfaces whereas more mature ganglion cells in more central regions of adult retinas have lost E 21 labeling from most of their surfaces. Stain accumulated at contact sites between the cells at least in freshly excised retinal explants and explants cultured for several days. The latter feature may mean that Neurolin on mature ganglion cells may assist in the stabilization of cell–cell contacts and may, as other cell surface molecules with such a function, include a link to the cytoskeleton (Pollerberg et al., 1987). This speculation, however, remains to be experimentally tested.

A spatiotemporal regulation of the expression at a number of molecules, many of them belonging to the class of neural cell adhesion molecules, in various species such as insects, birds, and mammals, has been demonstrated (for reviews see Jessell, 1988; Anderson, 1990). In fact, it was also described for two other growth-associated cell surface molecules in the retinotectal system of fish, NCAM 180 (Bastmeyer et al., 1990) and E 587 antigen (Vielmetter et al., 1991). The distribution of these cell surface molecules resembles in many respects that of Neurolin. Both were also found on embryonic axons (Vielmetter et al., 1991; Bastmeyer et al., 1990), and on only the new axons in adults, and both were transiently reexpressed on regenerating retinal axons. This suggests that all three molecules are important during retinal axonal growth. There may be more, such as the M 802 antigen (Wehner K. A., and C. A. O. Stuermer. 1989. *Soc. Neurosci. Abstr.* 15.) and yet undiscovered ones. The presence of NCAM 180 and the E 587 antigen on the young retinal axons in adult fish has led us to speculate that these two molecules may participate in the selective fasciculation of new axons (Bastmeyer et al., 1990; Vielmetter et al., 1991). Since E 21 is coextensive with NCAM 180 and E 587 antigen on these axon fascicles, we propose a similar function for E 21 (for review see Stuermer, 1991).

Spatiotemporal regulation in the expression of Neurolin apparently also occurs during axonal regeneration. In fact, the reexpression of Neurolin on the regenerating retinal axons is one of its most interesting characteristics and could mean that this antigen is required for the successful regrowth of the injured axons. A difference from the embryonic situation was that the ganglion cells of the regenerating axons did not reacquire E 21 staining over their entire surface, but as judged from the cells in retinal explants may keep the antigen accumulated at the cell–cell contact sites. From these features we may conclude that the ganglion cells do not entirely recapitulate the developmental situation.

Ganglion cells with regenerating axons are able to downregulate the expression of Neurolin like embryonic cells as shown by the disappearance of E 21 immunoreactivity from the regenerating axons with time after ONS. Concurrently, the expression of Neurolin in the synaptic layers of the SFGS returned to normal. These findings suggest that the ganglion cells in adult fish retain the ability for a coordinated up- and downregulation of molecules such as Neurolin and as E 587 antigen (Vielmetter et al., 1991) and NCAM 180 (Bastmeyer et al., 1990). This coordinated regulation and the success of axonal regeneration in fish are probably causally related.

In proposing that the disappearance of E 21 immunoreactivity from mature axons in development and regeneration is the result of a downregulation of the antigen, alternative explanations for this phenomenon have to be considered. Reasons against the possibility that myelin developing around the axons hinders the antibody from binding to the antigen have been discussed in earlier reports (Bastmeyer et al., 1990; Vielmetter et al., 1991). Another possibility that can neither be substantiated nor rejected at present is that the epitope to which the antibody binds becomes masked (Rabbacchi et al., 1990). In that case, Neurolin masking must be very selective and must spare for instance the Neurolin at the cell–cell contact sites and the terminal arbor layer of the tectum. Thus, it appears more likely that the antigen is indeed downregulated upon axonal maturation.

The upregulation of certain cell surface molecules during axonal regeneration has been noted in the PNS of mammals, such as for L1 and for NCAM on the sciatic nerve of rats (Martini and Schachner, 1988). However, mouse spinal cord neurons older than E13 failed to reexpress TAG-1 the molecule that they carry on the surfaces during their earlier embryonic development (Karagogeos et al., 1991). In light of these findings, it will be interesting to study the appearance of Neurolin-like molecules in the mammalian CNS where spontaneous axonal regeneration fails (Skene, 1989).

Cell adhesion molecules that are expressed on specific fiber tracts in one part of brain are often also found in other areas of the nervous system. TAG-1, for instance, is associated with the commissural axons in the spinal cord, but is also found on dorsal root ganglion neurons and their axons (Karagogeos et al., 1991). Likewise, Neurolin is not solely used in the fish visual system, but also during the development of the fish spinal cord. The limited distribution of E 21 staining in the adult fish spinal cord resembles the distribution of recently published novel cell adhesion molecules, DM-Grasp (Burns et al., 1991) and SC1 (Tanaka et al., 1991) in chick. The NH_2-terminal sequence of Neurolin shows considerable similarities to that of DM-Grasp (SC1) suggesting that Neurolin may represent the fish homologue of DM-Grasp (SC1) or a member of a hypothetical DM-Grasp/SC1 family (Burns et al., 1991). DM-Grasp and SC1, however, were not noted in the chick retinotectal system (Burns et al., 1991; Tanaka et al., 1991). Cloning of the gene (work in progress) will hopefully clarify the relation of Neurolin to DM-Grasp and SC1.

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