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The Monoclonal Antibody E587 Recognizes Growing (New and Regenerating) Retinal Axons in the Goldfish Retinotectal Pathway

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E587 is a new monoclonal antibody against a 200 kDa cell-surface glycoprotein in the fish retinotectal pathway. The E587 antigen probably belongs to the class of cell adhesion molecules, and more specifically, to the family of L1-like molecules. The immunopurified protein is recognized by the antibody against the HNK1/L2 sugar epitope (associated with most cell adhesion molecules) and by a polyclonal antiserum against chick G4, which is related to the cell adhesion molecule L1 in mouse. Moreover the NH_2-terminal sequence of E587 shows similarity with L1 and Ng-CAM.

The E587 immunostaining pattern in the fish retinotectal pathway suggests that the E587 antigen is a growth-associated molecule on fish retinal axons. In fish embryos, all retinal axons are labeled. In adult fish, however, only the young axons from newly added ganglion cells carry E587 staining. After optic nerve transection (ONS) and retinal axonal regeneration, all axons reexpress the E587 antigen into their terminal processes in the tectal retinorecipient layers. The reexpression of the E587 antigen is temporally regulated, and E587 immunoreactivity declines by 7 months and disappears at 12 months after ONS.

We hypothesize that the E587 antigen may mediate axon–axon associations. In its restricted appearance on young axons in normal adult fish, it may contribute to the selective fasciculation of the newest axons with young axons and thus participate in the creation of the age-related fiber organization in the fish optic nerve.

During nervous system development, neurons and their processes interact in a specific and coordinated way leading to the formation of orderly nerve cell connections. Neurons are often located far from their synaptic partners; thus, their growth cones must cover long distances to reach their targets. The growth cones find their paths by interacting with their local environment and by reading and responding to specific guidance cues. One strategy that growth cones employ is to extend along specific subsets of preexisting axon pathways using the surface of other axons as their guidance cue. In some systems, the molecules that are involved in growth cone–environment interactions and selective axon fasciculation have been identified. In the embryonic mouse spinal cord, for instance, commissural axons carry TAG 1, a cell-surface adhesion molecule of the immunoglobulin superfamily (Dodd and Jessell, 1988; Dodd et al., 1988). At the floor plate, the axons express L1 (another cell adhesion molecule) and associate with longitudinally running fiber tracts that also exhibit L1 (Rathjen and Schachner, 1984; Dodd et al., 1988). Such a patterned expression of cell adhesion molecules was also found in developing invertebrates. Two glycoproteins, fasciclin I and II, are expressed on particular portions of embryonic axons in grasshoppers (Bastiani et al., 1987). Fasciclin II is related to NCAM in vertebrates (Harrelson and Goodmann, 1988).

In most invertebrates and vertebrates, neurogenesis and the establishment of axonal connections are events mostly limited to defined periods of embryonic life. Accordingly, antigens with a specific function for the formation of nerve cell connections are seen in embryonic tissues but usually no longer in adults, suggesting that the expression of the molecules is downregulated or that the molecules become biochemically modified (Rougon et al., 1982). Such molecules in the developing nervous system are commonly demonstrated by immunohistochemical techniques using mono- and polyclonal antibodies.

The development of the fish visual system differs from that of most other vertebrates in that it continues beyond the period of embryonic development into and throughout adulthood. The retina continuously produces new ganglion cells at its peripheral margin (Johns, 1977), and these cells send out new axons. Thus, in any adult goldfish, groups of axons are found growing through the retina, the nerve, and optic tract into the tectum. These new axons extend over defined paths following their immediate forerunners in the retina. These axons then form a bundle at the ventral margin of the optic nerve, sweep to the dorsal side of the optic tract, and pass along the dorsal and ventral margin of the tectum (Easter et al., 1981, 1984; Stuermer and Easter, 1984a). As a consequence of the continuous addition of new axons to defined positions, the fish retinotectal pathway acquires its unique age-related fiber organization (Easter et al., 1981). Besides its continuous growth, the other outstanding feature of the fish CNS is the ability of the retinal ganglion cells to regenerate their axons and to reestablish appropriate connections with the tectum (reviewed in Gaze, 1970). This implies that molecules that are crucial for axonal growth and growth cone environment interactions must either be present continuously in the fish CNS or else be reexpressed during axonal regeneration.

To investigate the relation of the special properties of the fish CNS and the expression of molecules involved in retinal axon growth and regeneration, we used in an earlier study a known

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antibody against the neural cell adhesion molecule NCAM (Schlosshauer, 1989; Bastmeyer et al., 1990a). The creation of new monoclonal antibodies (mAbs) against other cell surface molecules of the fish CNS was necessary for studying axonal guidance and regeneration since most antibodies against known molecules do not cross-react with fish neural antigens.

Using the antibody D3, we demonstrated that NCAM immunoreactivity was confined to the new axons in normal adult goldfish. This distribution indicated that the expression of the high molecular form of NCAM is spatially and temporally regulated in normal adults and that its restriction to new axons could be causally related to the selective association of new axons with the forerunning young axons (Bastmeyer et al., 1990a). After optic nerve transection (ONS) and axonal regrowth, all axons exhibited bright D3 immunoreactivity, suggesting that NCAM is reexpressed and probably required during axonal regeneration.

Here, we describe a molecule with a distribution similar (but not identical) to that of NCAM. This molecule was detected by one of our new mAbs, E587, directed against a cell surface glycoprotein of 200 kDa of the fish CNS. Its distribution in the retinotectal system of goldfish was revealed with immunohistochemistry, in normal adults, in fish with regenerating optic nerves, and in embryos of a defined age. The immunohistochemical distribution suggests that E587 selectively recognizes axons in a state of growth. Labeled by E587 are all retinal axons in embryos, only the newest axons in normal adults, and all regenerating retinal axons over 3 months after ONS. Cross-reactivity of the 200 kDa protein with an antibody against HNK1/L2 (Abo and Blach, 1981; Kruse et al., 1984) and an antibody against chick G4 on Western blots suggests that the E587 antigen may be a cell adhesion molecule. It may be related to G4 (Rathjen et al., 1987a) that is related to the mouse cell adhesion molecule L1 (Rathjen and Schachner, 1984) and other members of the family of L1-like molecules (Burgoon et al., 1991).

A short presentation of these results was published in abstract form (Vielmetter and Stuermer, 1989a).

Material and Methods

Preparation of the immunogen. A fraction enriched in cell surface membranes of normal adult goldfish tecta was obtained as described (Vielmetter and Stuermer, 1989b). In brief, the isolated tecta were homogenized in homogenization (HS) buffer (pH 7.4; 10 mM Tris-HCl, 1.5 mM $CaCl_2$, 2,3-dehydro-2-desoxy-*N*-acetylneuraminic acid) and the protease inhibitors spermidine (1 M), aprotinin (25 gm/ml), leupeptin (25 gm), and pepstatin (5 gm/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). The membrane fragments were solubilized in buffer with the detergent octylglucoside [OG lysis buffer: 100 mM octylglucoside, 20 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl, 1 mM CaCl, 1 mg/ml phenylmethylsulfonyl fluoride, pH 7.4) by sonification. The supernatant obtained by centrifugation (1×10^5 *g*, 1 hr, 4°C) was called OG–brain extract. Its protein concentration was determined by a protein assay (Bio-Rad).

To obtain a fraction enriched in membrane glycoproteins, OG–brain extract was passed over 5 ml lectin Sepharose columns (Pharmacia), first over lentin–lectin (LL) Sepharose 4 B and second over wheat germ lectin (WGA) Sepharose 6 MB. LL and WGA columns were rinsed with OG lysis buffer. The proteins bound to the LL column were eluted with elution buffer (PBS, 30 mM octylglycoside, the protease inhibitors listed above) containing 1 M methyl-L,D-mannoside. Proteins bound to the WGA column were eluted with elution buffer containing 1 M *N*-acetyl-D-glucosamine. The LL and WGA eluates were collected, and their protein concentration was determined (protein assay, Bio-Rad). Liposomes were prepared by solubilizing 50 mg of phosphatidylcholine and 100 mg of octylglycoside in chloroform. Chloroform was evaporated, and the mixture of octylglycoside and phosphatidylcholine was vacuum dried overnight. After addition of PBS, the micelles were sterile filtered and stored at room temperature. The LL and WGA eluates were mixed with the micelles at a ratio of 100 μg protein/100 ml micelles. The mixture was dialyzed against PBS in a microdialysis system (Bethesda Research Labs) for 24 hr. Upon concentration of the liposomes by centrifugation (1×10^5 *g*, 12 hr, 4°C), they were stored at −70°C.

Production of monoclonal antibodies. Female 7–8-week old Balb/c mice were injected four times intraperitoneally with a mixture of liposomes containing LL extract and WGA extract in equal amounts (10 mg each) in the following intervals: 2 months, 2 weeks, 2 weeks. Mice were killed 3 d after the last immunization and spleen B-cells were fused with Sp2/0-AG14 myeloma cells using standard fusion protocols (Peters et al., 1985). The supernatants of the hybridoma clones were tested on transverse and sagittal cryostat sections of adult goldfish tecta; on retina whole-mounts, fixed and unfixed; and on dot blots to determine their class specificity. The antibody presented here was mAb E587 IgG.

Immunohistology. Fixed tissue (brain, tract, optic nerve, retina) from normal adult goldfish and from fish whose optic nerves had been cut at various time intervals prior to death was embedded in Tissue-Tek (Miles) and immediately frozen in liquid nitrogen. Goldfish embryos were embedded as a whole. To prepare flat mounts, isolated retinas (Vielmetter and Stuermer, 1989b) were attached to a nitrocellulose filter. The ganglion cell layer was up if whole-mount staining was intended; the ganglion cell layer was down to the filter if horizontal sections were to be cut. Cryostat sections from flat-mounted retinas were made by embedding the retina attached to the filter in Tissue-Tek and cutting sections parallel to the filter. Optic nerves and tecta were serially sectioned in a transverse plane.

Cryostat sections were collected on poly-L-lysine–coated slides, dehydrated for 5 min in ice cold methanol, and rehydrated in PBS at room temperature for 5 min. Sections were incubated with the primary antibodies for 1 hr at 37°C. After two washes (5 min) with PBS, fluorescein isothiocyanate–coupled secondary goat anti-mouse antibodies (Dianova) were added and incubated for 1 hr at 37°C. Retinal whole-mounts were exposed to the primary and secondary antibodies either unfixed or after fixation with paraformaldehyde and methanol.

Retinal explants and culture conditions. Retinal explants were prepared as described previously (Vielmetter and Stuermer, 1989b). In brief, explants, 300 μm wide, 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 of L-15 culture medium (L-15 medium containing 10% fetal calf serum (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 (ice-cold methanol, three rinses in PBS) and exposed to primary and secondary antibodies as described or exposed to the antibody without fixation.

Purification of the E587 antigen. Immunoaffinity absorption of the E587 antigen was performed using columns of CNBr–Sepharose (Pharmacia) to which the purified mAb had been covalently coupled (1 ml with 10 mg protein). OG–brain extract was passed over the affinity column (Pharmacia) (12 hr 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 second with OG lysis buffer with 0.5 M NaCl. The antigen was eluted with buffer (pH 11) of 50 mM triethylamine and 100 mM octylglucoside into a 10% 1 M Na acetate buffer (pH 3) to neutralize the eluate.

The eluted antigen was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 7% gels and 5–15% gradient gels (Laemmli, 1970), respectively. To run the antigen under nonreducing conditions, 10 mM iodoacetamide instead of mercaptoethanol was added to the loading buffer for Western blot analysis (Towbin et al., 1979), and the Immobilon membrane onto which the antigen was transferred was blocked with 10% FCS in PBS (1 hr, 37°C) and then incubated with the appropriate monoclonal antibodies and rinsed three times with buffer (PBS and 0.05% Tween 20). Antibody binding was detected by HRP-coupled goat anti-mouse secondary antibody (Dianova; diluted with FCS in PBS, 1 hr, 37°C). After three washes in buffer (PBS and 0.05% Tween 20), blots were developed in staining solution consisting of 10 ml chloronaphthol (3 mg/ml) in ETOH and 5 μl of 30% H_2O_2.

Cross-reactivity of the E587 antigen was tested with mono- and polyclonal antibodies against G4 (Rathjen et al., 1987a) and neurofascin (Rathjen et al., 1987b; kindly provided by F. Rathjen) and the monoclonal antibody D3 and against chick NCAM 180 (Schlosshauer, 1989;

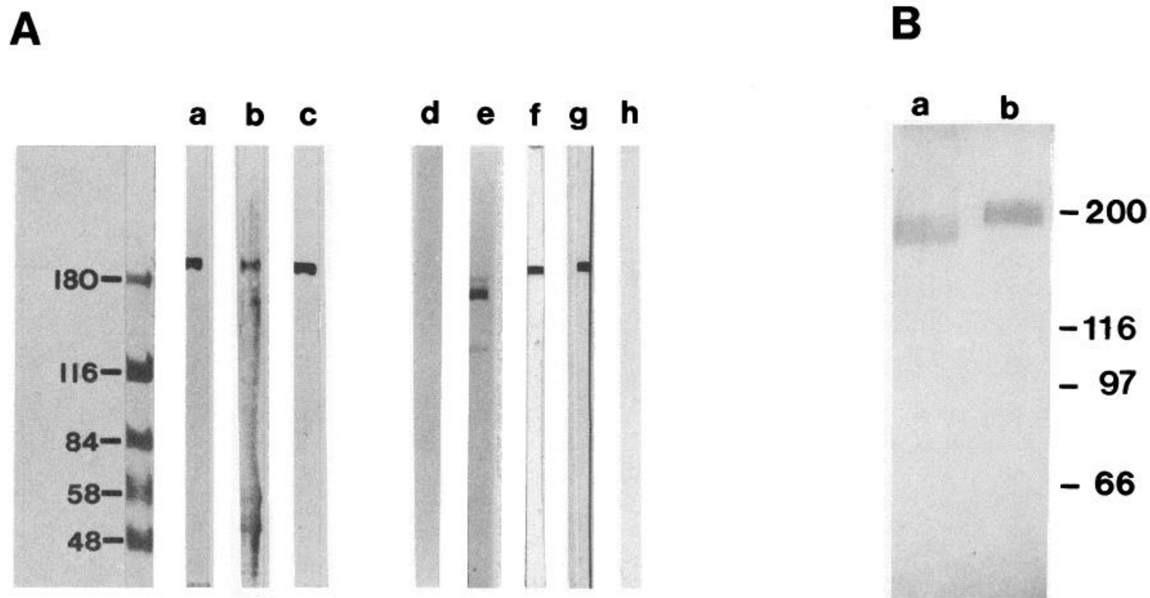

Figure 1. *A,* Western blots of affinity-purified E587 antigen and OG-brain extract separated using 5–15% gradient SDS-PAGE. Molecular mass standards are shown to the *left. Lanes*: *a,* Among the proteins solubilized from brain cell-surface membranes (OG-brain extract) E587 recognizes a protein at 200 kDa. *b,* The affinity-purified antigen appears as major bands at 200 and 50 kDa and as minor bands at about 30, 35, 50, and 55 kDa (stained with Auro-dye). *c,* The 200 kDa band is recognized by mAb E587. *d,* mAb D3 against NCAM does not bind to the affinity-purified E587 antigen. *e,* mAb D3 binds to two bands at 170 and 130 kDa of the OG extract of brain cell-surface membranes (Bastmeyer et al., 1990a). *f,* and *g,* The antibody against the sugar epitope HNK-1 recognizes the affinity-purified E587 antigen; (*f*) and so does a polyclonal antibody against the chick cell adhesion molecule G4 (*g*). *h,* A polyclonal antibody against the chick cell adhesion molecule neurofascin, however, does not cross-react with the E587 antigen. *B,* The affinity-purified E587 antigen was run on 7% SDS-PAGE under nonreducing (*a*) and reducing (*b*) conditions and visualized by silver staining. Molecular mass standards are shown to the *right.* The reduced form of E587 antigen is seen at approximately 200 kDa, and the nonreduced form, at approximately 190 kDa.

Bastmeyer et al., 1990a) and an antibody against HNK1/L2 (kindly provided by M. Schachner).

Protein sequencing (F. Lottspeich). E587 antigen (100 mg, 500 pM) was purified by immunoaffinity chromatography and subjected to SDS-PAGE as described above. The antigen was electroblotted to a glass fiber membrane according to a published procedure (Eckerskorn et al., 1988). The 200 kDa antigen band was cut out from the glass fiber membrane. The amino acid sequence for the NH_2-terminal of the protein was determined with a protein Sequenator (Applied Biosystems).

Results

The mAb E587 recognizes a cell surface glycoprotein on young and regenerating retinal axons in the goldfish retinotectal pathway.

Identification of the E587 antigen

On Western blots of a detergent (octylglycoside)-soluble fraction from cell surface membranes of goldfish brains (OG extract), separated on 5–15% gradient SDS-PAGE, E587 recognizes a single protein band with an apparent molecular mass of 200 kDa (Fig. 1*Aa*). Subjection of the solubilized proteins to immunoadsorption with E587 gave protein bands of which the most prominent ones were at approximately 200 and 50 kDa and with minor ones at about 40, 57, 110, and 170 kDa on SDS-PAGE (Fig. 1*Ab*). The 200 kDa band was recognized by E587 on a Western blot (Fig. 1*Ac*). The 200 kDa band was also recognized by an antibody against the HNK1/L2 carbohydrate epitope (Abo and Blach 1981; kindly provided by M. Schachner) (Fig. 1*Af*), suggesting that the E587 antigen is a glycoprotein and may belong to the class of cell adhesion molecules.

In a separate experiment, the purified E587 antigen was run on 7% SDS-PAGE under reducing and nonreducing conditions and silver stained. The reduced E587 antigen appears at approximately 200 kDa, and the nonreduced form, at 190 kDa (Fig. 1*B*), suggesting that disruption of intrachain disulfide bonds changes the shape of the molecule such that its motility in the gel decreases. Similar effects have been noted with other members of the L1 family of cell adhesion molecules (Bieber et al., 1989; Prince et al., 1989).

A cell surface glycoprotein on fiber tracts in the nervous system of warm-blooded vertebrates with a molecular mass component close to 200 kDa is the cell adhesion molecule L1 of mouse (Rathjen and Schachner, 1984); a related molecule in birds, G4 (Rathjen et al., 1987a); and other members of the L1 family of adhesion molecules (Burgoon et al., 1991). Antibodies

Figure 2. Comparison of the NH2-terminal sequence of E587 antigen, L1 (from Moos et al., 1988), and Ng-CAM (from Burgoon et al., 1991). Identical residues are *boxed.* The first residue of E587 (*) could not be determined unambiguously.

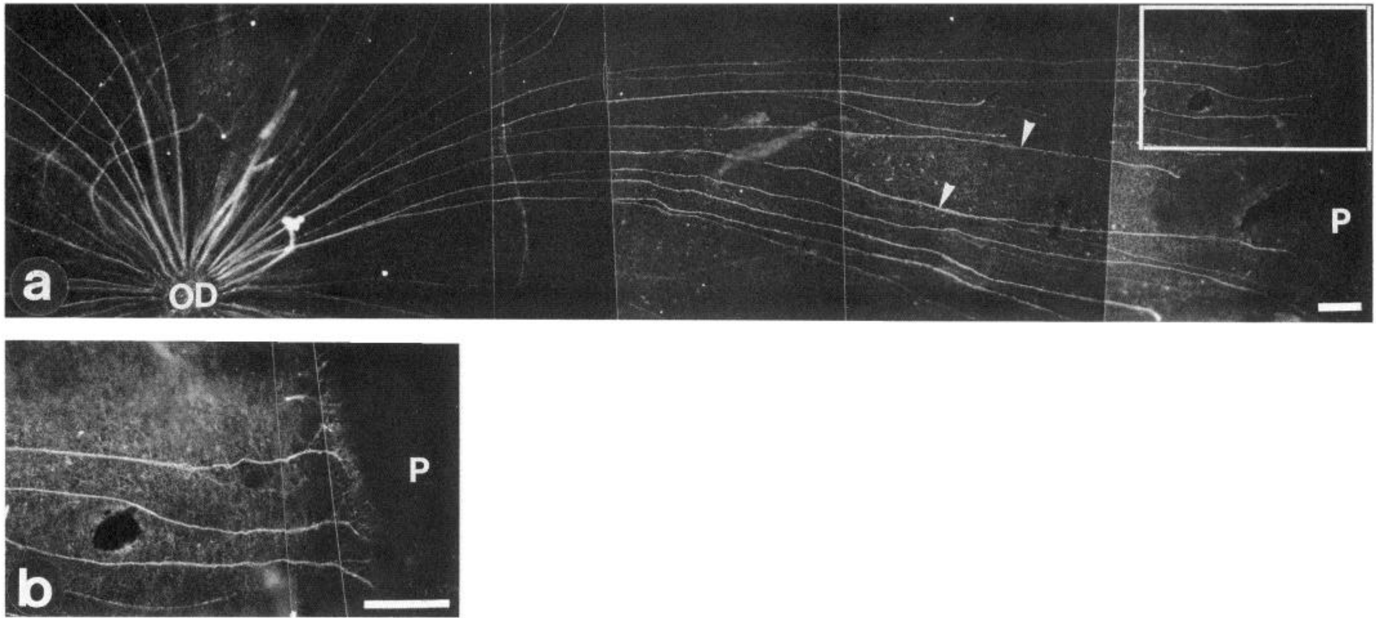

Figure 3. *a,* A flat-mounted unfixed retina was exposed to mAb E587. Only the few new axons (*arrowheads*) originating at the retinal peripheral margin (*P*) are stained. The *boxed region* is shown enlarged in *b. b,* Higher magnification of the retinal margin (*P*) where the E587-positive axons originate. *OD,* optic disk. Scale bars, 100 μm.

against G4 and another cell adhesion molecule, neurofascin (Rathjen et al., 1987b), were applied (kindly provided by F. Rathjen). One polyclonal antiserum against chick G4 did cross-react with the 200 kDa E587 antigen on Western blots (Fig. 1*Ag*) whereas anti-neurofascin did not (Fig. 1*Ah*). This suggests that the fish E587 antigen may be related to the L1 family of adhesion molecules. D3, a mAb against NCAM 180 (Schlosshauer, 1989), cross-reacts with specific sets of axon profiles in the fish nervous system (Bastmeyer et al., 1990a). On Western blots with proteins solubilized from fish brain cell surface membranes (OG-brain extract), D3 binds to two bands at 170 and 130 kDa, (Fig. 1*Ae*; see also Bastmeyer et al., 1990a). D3 did not recognize the immunopurified 200 kDa E587 antigen on Western blots (Fig. 1*Ad*), excluding that E587 recognizes fish NCAM 170/130.

The tentative conclusion that the E587 antigen may belong to the L1 family of cell adhesion molecules was supported from revealing the NH_2-terminal amino acid sequence (F. Lottspeich) of the E587 antigen and comparing it to published sequences of two members of this family, mouse L1 (Moos et al., 1988) and chicken Ng-CAM (Burgoon et al., 1991). As demonstrated in Figure 2, there is a considerable similarity of the NH_2-terminal sequence of E587 antigen and L1 and Ng-CAM.

E587 immunofluorescence in the normal retinotectal pathway

In the retinotectal pathway of normal adult goldfish, the E587 antigen appears restricted to a subpopulation of retinal axons. By their position and paths these E587-positive axons are defined as the young axons from the new ganglion cells born at the retinal peripheral margin (Johns, 1977; Meyer, 1978; Easter et al., 1981). Figure 3*a* shows the E587-positive axons originating from the retinal margin (Fig. 3*b*) in an unfixed flat-mounted normal goldfish retina. Binding of the antibody to axons in the unfixed retina suggests that E587 recognizes an epitope on the outer surface of the axonal membrane. The very same staining pattern was seen in normal retinas that were fixed and permeabilized prior to exposure to E587 and the secondary antibody. In contrast to the normal retina, all or almost all axons were E587 positive in (fixed and unfixed) retinas of fish with regenerating optic nerves (see below). These two results indicate that the appearance of only a few E587-positive axons in the normal retina is specific and not an artifact of poor antibody penetration. This is further supported from other experiments in which one antibody M501 against fish neurofilaments (Wehner, 1990; Wehner and Stuermer, 1990) was used. M501 stained all axons in normal retinas as well as in retinas connected to regenerating optic nerves, but only after fixation and permeabilization of the retinas.

The cross sections through the nerve head (Fig. 4*a*) illustrate that the E587-positive axons meet at the optic disk in a manner typical for the young axons. They cluster in the optic nerve head (Fig. 4*a*), follow the retinal artery (Fig. 4*b,c*), and turn, as known for young axons to the nerve's ventral edge (Fig. 4*d*; Easter et al., 1981). As they continue on their paths, the young retinal

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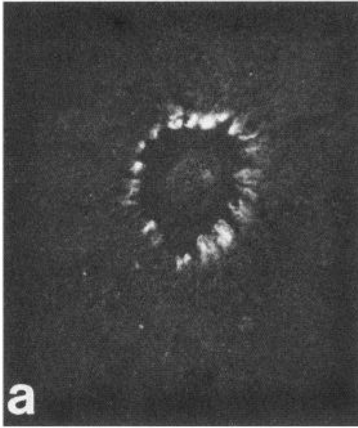
Figure 4. Series of sections through the optic nerve and tract immunostained with mAb E587. *a,* Optic nerve head; the E587-positive (new) axons meet in the center of the retina. *b* and *c,* They join the retinal artery and come to lie at the ventral edge of the optic nerve (*d*). *e,* Close to the bifurcation of the optic tract into two brachia, the new axons change their position. *f,* They acquire a dorsal superficial position in the two brachia (*arrowheads*) before they enter into the tectum. *g,* Cross section through a normal tectum at a midtectal level. mAb E587 does not bind to the older retinal axons, so that the retinorecipient layers *SO* and SFGS (*S*) of the tectum are unstained. Only a few small fascicles in *SO* are labeled (*arrow*) but they are of nonretinal origin. The group of stained axons at the peripheral margin of the tectum, however, are most probably the new retinal axons (*arrowhead*). Diffuse staining is seen in *SM. h,* Higher magnification of the mediolateral edge of the tectum in *g* showing the new retinal axons clustered at the tectal margin. Scale bars, 100 μm.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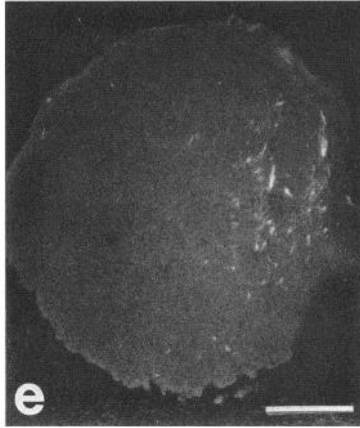
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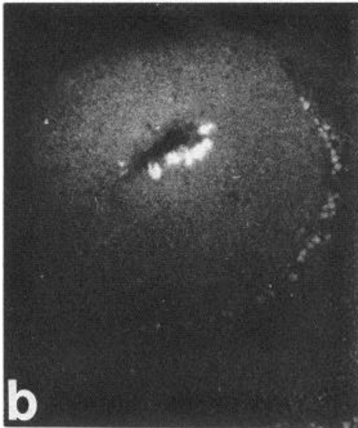
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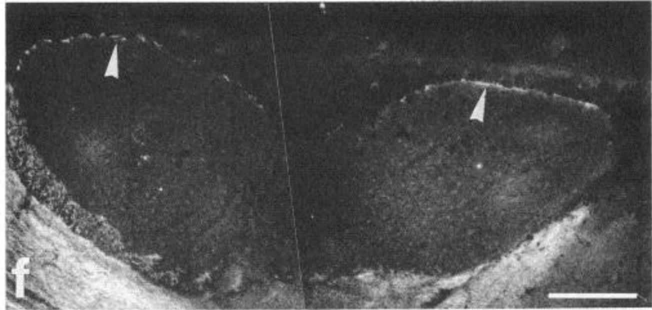
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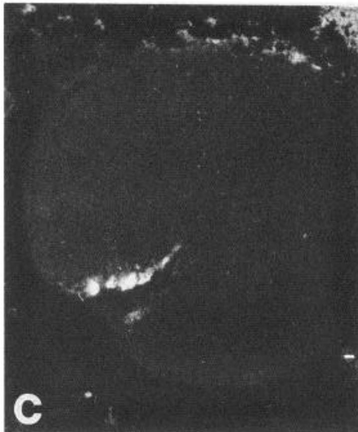
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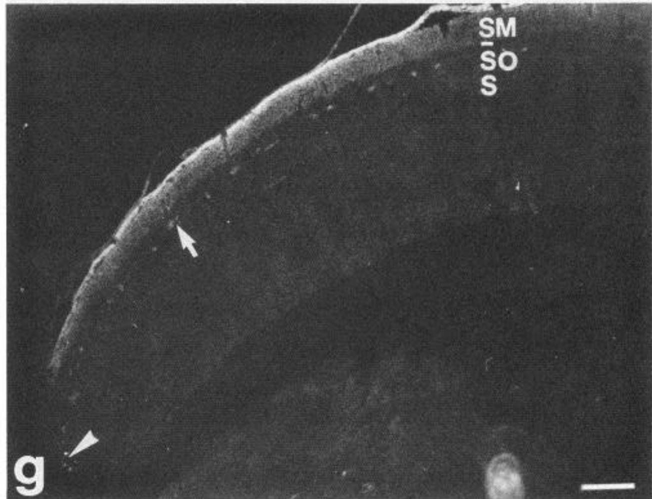
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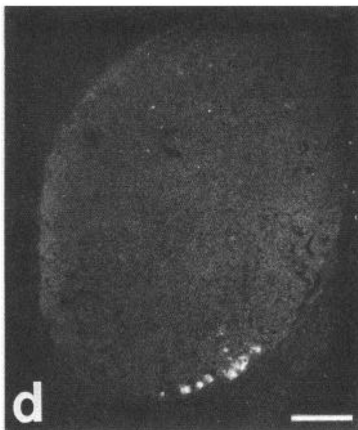
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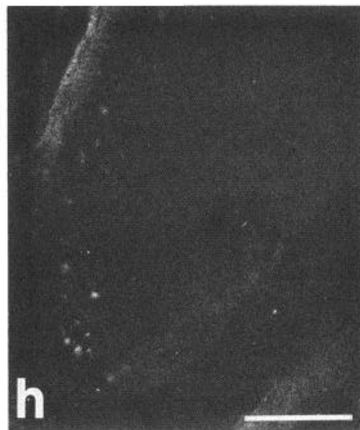
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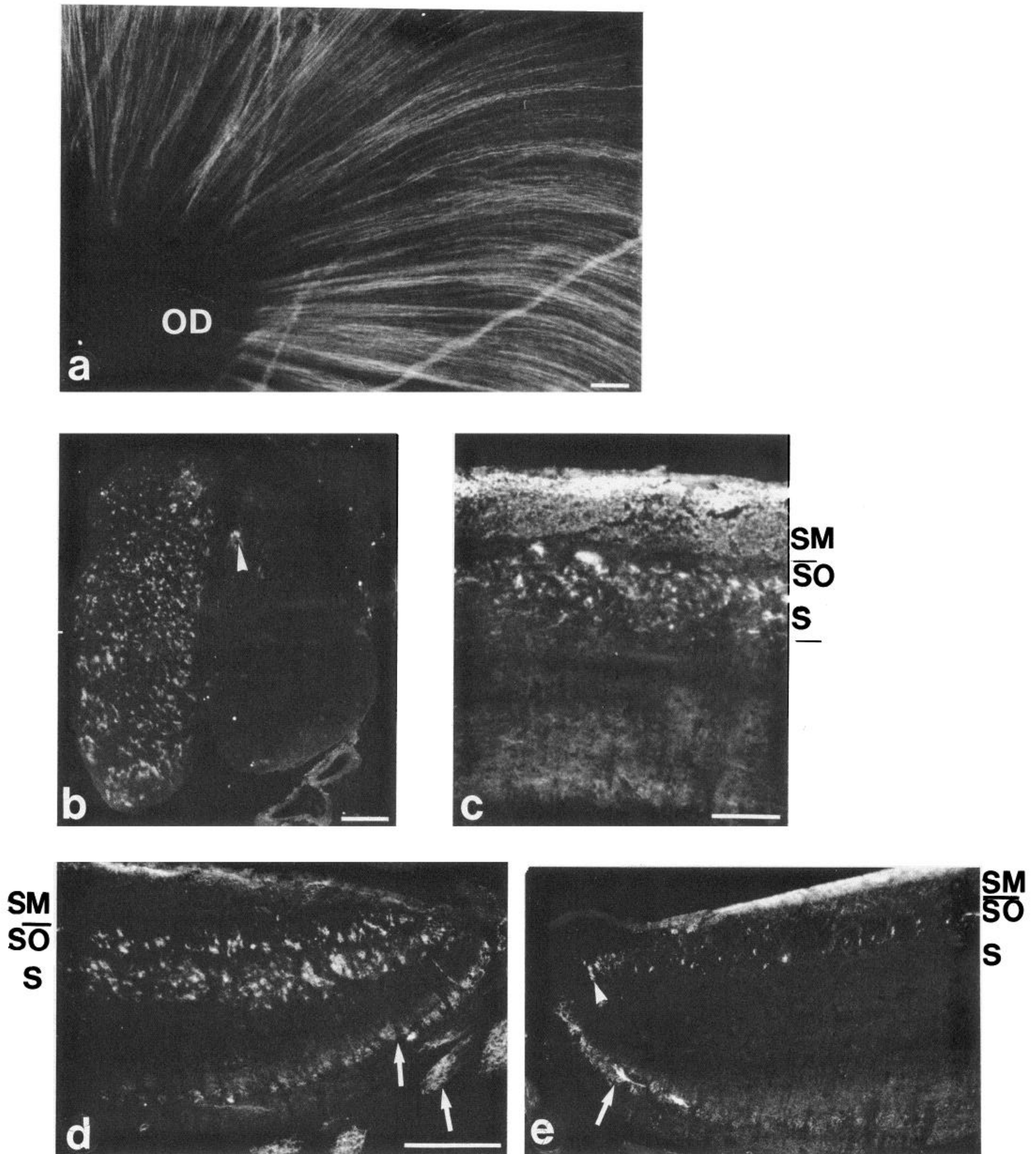


Figure 5. *a,* A retina whole-mount, 3 weeks after optic nerve section. All or almost all axons exhibit E587 staining. *OD,* optic disk. *b,* Cross section through a normal (*right*) and a regenerating (*left*) optic nerve at 3 weeks after left ONS. The previously sectioned nerve shows a large number of E587-positive regenerating axons scattered over the nerve's cross section. The normal nerve exhibits staining only on the small group of new retinal axons (*arrowhead*). *c,* The regenerating retinal axons are stained in the retinorecipient layers *SO* and SFGF (*S*) of the tectum (3 weeks after ONS). As in Figure 4*g,* there is diffuse staining over *SM. d* and *e,* Comparison of the left and right tectum of a fish 3 weeks after left ONS. The right tectum in *d,* innervated by regenerating retinal axons, exhibits E587-positive axons throughout *SO* and SFGS (*S*). The tectum in *e* shows only the nonretinal axons in *SO,* and the new retinal axons at the tectal edge in *SO* (*arrowhead*) and SFGS (*S*) are bare of E587 staining. Groups of tectal efferents (*arrows*) are also labeled. Scale bars *a–c,* 100 μm; *d* and *e,* 200 μm.

axons maintain their marginal position but come to lie dorsally in the optic tract and in its brachia (the two branches of the optic tract) in front of the tectum. The location of the axons labeled with E587 in a cross section through the tract and the brachia (Fig. 4*e,f*) is consistent with the path of the young axons. As they enter into the tectum, the young axons sweep around the dorsomedial and ventrolateral tectal margins (Easter et al., 1981; Stuermer and Easter, 1984a). Accordingly, the tectum at a midtectal level (Fig. 4*g*) exhibits E587 positive retinal axons in the tectal periphery (Fig. 4*g,h*) but not in the remaining areas of the retinorecipient layers stratum opticum (SO) and stratum fibrosum et griseum superficiale (SFGS).

In addition to these peripheral axons, however, small axon bundles in SO are stained by E587 (Fig. 4*g*). These axons are not direct afferents from the retina, because they persist in tecta 1 yr after removal of the contralateral eye (not shown). These axons also remained after ablation of the telencephali, which we considered as one possible origin of these axons. To this point, we have not been able to identify the source of these axons, and therefore we cannot decide whether these axons are new axons from newborn neurons in other regions of the fish brain. In fact, very little is known about neuronal cell proliferation in the growing adult goldfish brain other than in the retina and tectum. In some but not all tecta, light E587 staining was observed in the stratum marginale (SM), the uppermost tectal layer (Fig. 4*g*). Staining in the SM appears diffuse and not, as in the nerve or SO, on distinct axon bundles. Afferents to this layer are unmyelinated axons from the torus longitudinalis. They could perhaps be the source of this diffuse staining.

Except for the small nonretinal fascicles in the SO, the retinorecipient fiber layer SO and the synaptic layer SFGS of the normal tecta were free of E587 staining, and so were the layers below SFGS (Fig. 4*g*). Labeled axons were, however, noted in the layer stratum album centrale (SAC), which is dorsomedially above and ventrolaterally below the tectal cellular layer stratum periventriculare (SPV; Fig. 5*d,e*) and in extratectal axon tracts. These axons in SAC are mainly tectal efferents. We concentrated in this study on retinal axons and did not pursue the labeling pattern in SAC and SPV or in extratectal tracts to any further extent.

The distribution of E587-labeled axons in the normal adult goldfish retinotectal pathway resembles closely the distribution of axons carrying the fish homolog of NCAM 180, detected by mAb D3 (Bastmeyer et al., 1990a). Both bind selectively to young retinal axons from more recently born ganglion cells, and not to older and more mature axons. This implies that the expression of the E587 antigen is, like NCAM, downregulated with time or that the epitope to which E587 binds is no longer accessible to the antibody.

E587 immunofluorescence in the regenerating retinotectal pathway

Retinal axons in fish are able to regenerate upon axotomy. Following intraorbital ONS, the axon segments separated from the ganglion cell body degenerate in the brain-side part of the nerve, in the tract, and in the tectum. The first regenerating retinal growth cones emerge by 4 d at the cut end of the old axons in the eye-side stump of the nerve (Lanners and Grafstein, 1980), traverse the cut, and begin to reinnervate the retinorecipient layers of the tectum. The distribution of E587-positive axons in the regenerating retinotectal pathway suggests that most or all axons carry the E587 antigen during optic nerve regeneration. At 4 d after ONS, the regenerating portions of the optic nerves did not exhibit E587-positive profiles. E587-positive axons are few at 6 d but are present in higher density at 3 weeks after ONS (Fig. 5*b,c*) in the optic nerve. In the retina and the eye-side "old" portion of the optic nerve, the number of E587-positive profile increases as well (Fig. 5*a*). Regenerating retinal axons reach the tectum between 10 and 13 d after ONS (Stuermer and Easter, 1984b) and have by 3–5 weeks reinnervated the tectal retinorecipient layers, SO and SFGS, throughout the tectal rostrocaudal extent. Figure 5*c* exemplifies the E587-positive axons in SO and SFGS in a cross section of a tectum at a midtectal region at 3 weeks after ONS, and Figure 5*d,* at the mediolateral tectal margin in comparison to a normal tectum (Fig. 5*e*). The number of regenerating axons increases in the nerve and tectum at 5 weeks after ONS (Fig. 6*a,b*). Three months after ONS, the E587 immunoreactivity of the regenerating axons is still as high as at 5 weeks. A notable decrease, however, has occurred at 7 months after ONS. The number of brightly stained axons is reduced (Fig. 6*c,d*) as compared to 5 weeks and 3 months after ONS. Finally, in the regenerated retinotectal pathway at 12 months, a small number of axons, nearly as small as in the normal pathway, are labeled by E587 (Fig. 6*e,f*). However, instead of forming a coherent bundle, as in normal fish, they appear as two or more small and separate bundles in the nerve (Fig. 6*e*) and occupy a larger proportion of the tectal margin (Fig. 7*b*). In the retina at 12 months after ONS, the E587-positive axons are the ones that arise from the retinal margin and are new axons. These data lead to the conclusion that retinal axons reexpress the E587 antigen during their regenerative growth. Like newly formed axons from newborn ganglion cells in normal fish, the regenerating axons probably reduce the E587 antigen expression on their surface with time after ONS, or the E587 antigen undergoes changes so that the antibody fails to bind to its epitope.

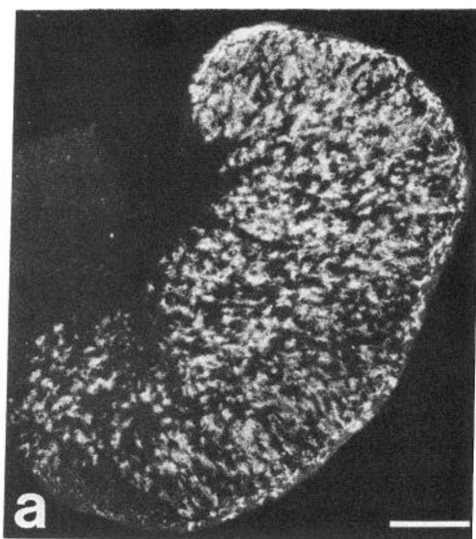
That retinal axons express the E587 antigen while they grow is supported by observations *in vitro.* Axons extending from retinal explants on a laminin-covered surface are stained by the antibody throughout their entire length, including their growth cones and filopodia. E587 labeling was intense whether the axons were prefixed or unfixed. Staining of the axons in Figure 8 is achieved without prior fixation supporting that the antibody is directed against an epitope of a protein on the external surface of the plasma membrane.

E587 immunofluorescence in the embryonic retinotectal pathway

Since E587 recognizes young retinal axons in adult goldfish and growing axons during optic nerve regeneration, we predict that E587 should bind to young retinal axons in embryos or larvae as well.

Goldfish embryos hatch during the third day after fertilization, and retinal axons have already invaded into the tectum at this time (Stuermer and Raymond, 1989). Frontal sections through the entire head of a 60-hr-old goldfish embryo exposed to E587 are shown in Figure 9. In the eyes (Fig. 9*a*), the axons of the retinal ganglion cells are E587 positive. The small optic nerves running horizontally in the plane of section (Fig. 9*b*) are brightly stained as well as the axons in the optic tracts in Figure 9*c*.

These findings suggest that the E587 antigen is associated with retinal axons during their growth, in fish embryogenesis, during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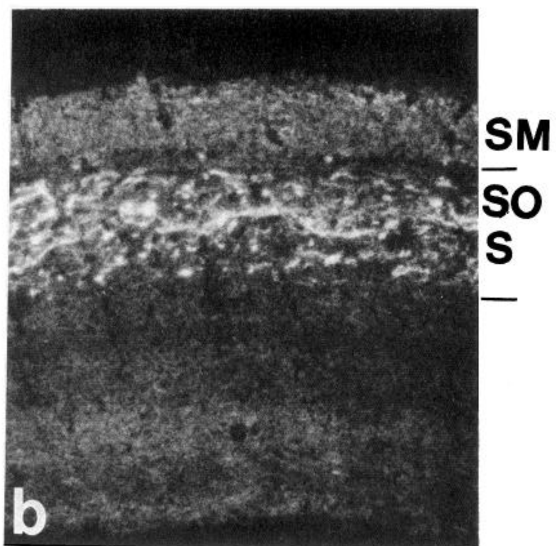
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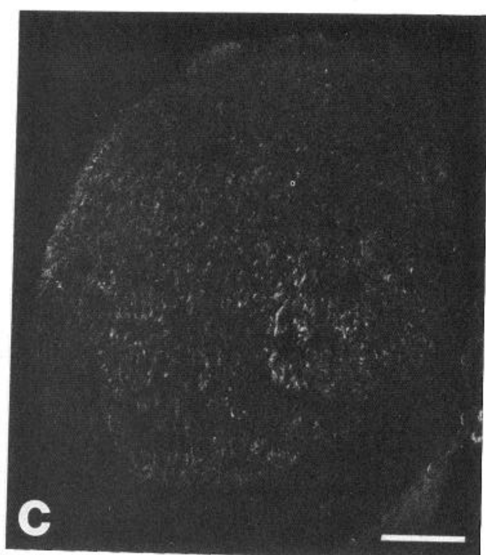
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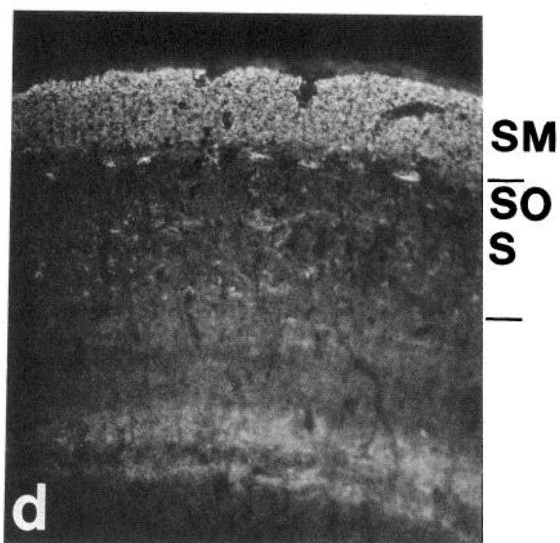
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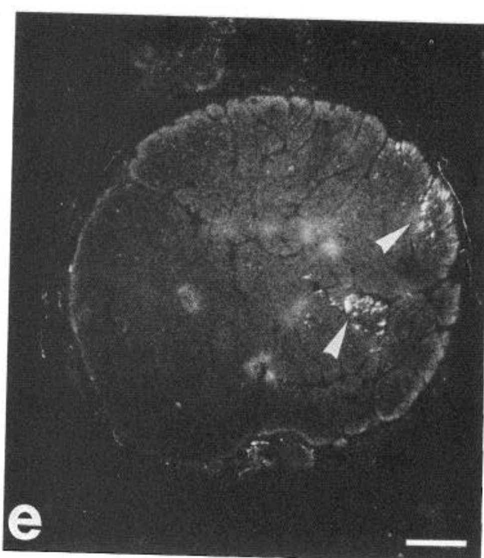
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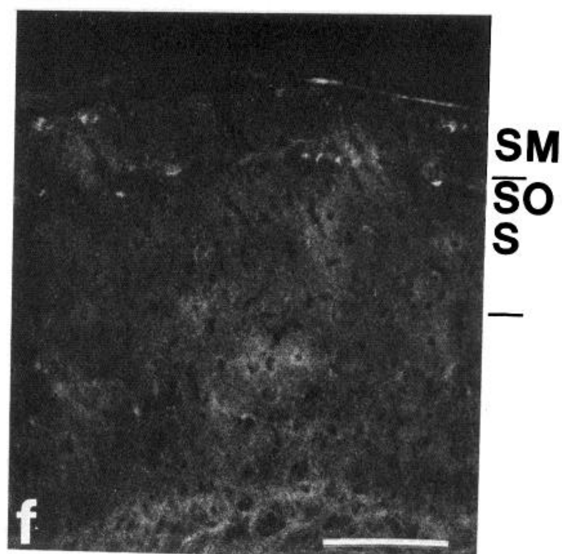
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retinotectal growth in adults, and during optic nerve regeneration.

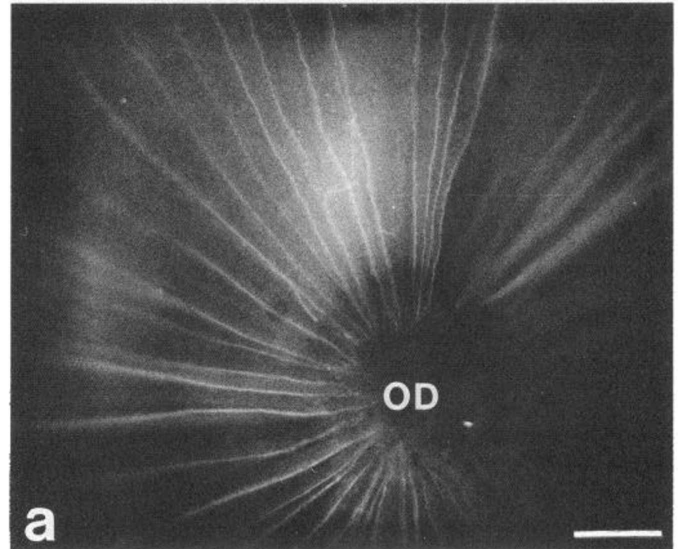
Discussion

Our goal was to identify candidates for axon growth-promoting and axon guidance molecules in the fish visual system. The monoclonal antibody E587 presented here identifies a molecule that may subserve such a function. The E587 antibody bound specifically to growing axons in the fish retinotectal pathway and not to any detectable degree to more mature ones. While all retinal axons were E587 positive in embryos, only a small number of retinal axons exhibited E587 staining in adults. These labeled axons in adults were the young axons from the newly added ganglion cells at the retinal periphery. Upon ONS, however, regenerating retinal axons were stained throughout their length, including their processes in the retinorecipient layers of the tectum. With time and maturation of the regenerated projection, the axons showed progressively less staining.

The characterization of the E587 antigen included immunopurification steps, separation by SDS-PAGE, immunoblotting techniques, and determination of its NH_2-terminal sequence. Together with E587 binding properties in sections, our data showed that on the external surface of the plasma membrane E587 recognizes an epitope of a 200 kDa glycoprotein. The E587 antigen carries the HNK1/L2 carbohydrate epitope (Abo and Blach, 1981) and is recognized by a polyclonal antibody against chick G4 (Rathjen et al., 1987a), a glycoprotein that is related to the murine cell adhesion molecule L1 (Rathjen and Schachner, 1984) and other members of the L1 family of cell adhesion molecules (Burgoon et al., 1991). The NH_2-terminal sequence shows considerable similarities to that of L1 (Moos et al., 1988) and chicken Ng-CAM (Burgoon et al., 1991). Thus, E587 apparently identifies a growth-associated cell-surface glycoprotein on fish retinal axons, probably a cell adhesion molecule, which may belong to the L1 family of cell adhesion molecules.

Distribution of the E587 antigen in the retinotectal pathway of normal goldfish

Molecules with a specific function during axonal growth and pathfinding are found to be expressed during specific phases of nervous system development (for review, see Jessell, 1988; Skene, 1989; Anderson, 1990). Such molecules with a function during axonal growth and pathfinding have been described in mammals, birds, and insects (reviewed in Anderson, 1990) but very few in fish. The E587 immunostaining pattern suggests that E587 identifies a growth-associated molecule in the fish visual pathway. The development of the fish visual system differs from that of most other vertebrates. In most vertebrates, the development of axonal connections is completed during embryogenesis, and the expression of certain molecules that are important for axonal growth ceases or declines with maturation (Jessell, 1988). The embryonic development of the goldfish visual system is rapid and short, but neuro- and axogenesis in this system continue into and throughout adulthood in defined regions (Raymond and Easter, 1983). Accordingly, E587 labels all retinal axons in fish embryos but only a small number in adult fish. The small group of E587-positive axons were readily identified as the young axons from new retinal ganglion cells by their paths, since the paths of these axons have previously been defined by electron microscopic and fiber tracing techniques (Scholes, 1979; Easter et al., 1981, 1984; Bernhard et al., 1988). Thus, the spatial distribution of E587 staining in normal fish suggests that the molecule to which E587 binds is selectively expressed by growing axons in embryos and adults. This is supported by finding that regenerating axons reexpressed the molecule.



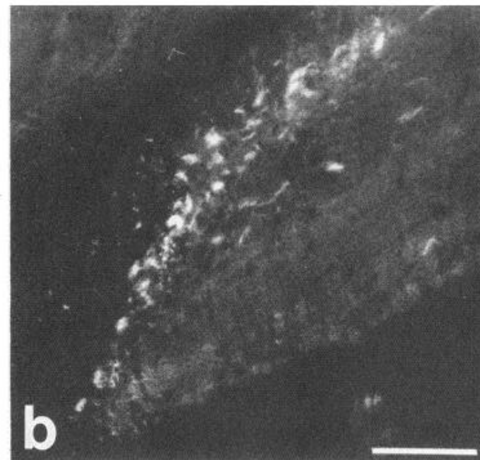


Figure 7. *a,* Retinal whole-mount 12 months after ONS. Only the new axons are E587 labeled, and all other axons are no longer stained (compare Fig. 3*a*). *OD,* optic disk. *b,* In the tectum at 12 months, the E587-positive retinal axons are restricted to the peripheral tectal margin. The group of labeled axons is larger than in a normal tectum (compare Fig. 4*g,h*). Scale bars, 100 μm.

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Figure 6. Cross sections through regenerating optic nerves (*a, c, e*) and tecta (*b, d, f*) at 5 weeks (*a, b*), 7 months (*c, d*), and 12 months (*e, f*) after ONS. *a* and *b,* Regenerating axons at 5 weeks are brightly stained by E587 in the nerve and tectum. *c* and *d,* The E587 positivity on the regenerating axons has markedly decreased at 7 months. *e* and *f,* At 12 months, the regenerating axons are no longer E587 positive. Only a small group, most probably the new axons (see Fig. 7) are labeled in the nerve. *e,* They run in two instead of one group and travel in an abnormal position (*arrowheads*). (*SM, SO, S* as in Figs. 4, 5.) Scale bars, 100 μm.

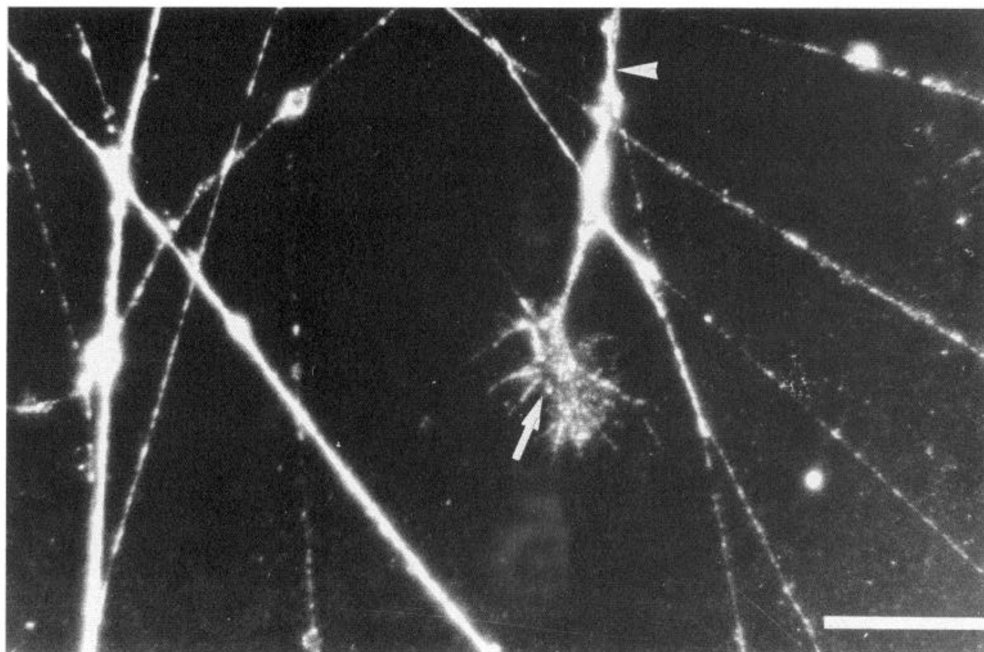


Figure 8. Retinal axons *in vitro,* here stained unfixed, are labeled by E587 into their growth cones (*arrow*) and filopodia. Scale bar, 100 μm.

Our data have provided evidence that the growth-associated cell-surface antigen E587 in fish belongs to the class of cell adhesion molecules and may be a member of the L1 family of cell adhesion molecules (Rathjen and Schachner, 1984).

The avian G4 is like L1 and other members of this family (Burgoon et al., 1991) on several and typically on far-projecting fiber tracts (Weikert et al., 1990), including the retinal axons (Lagenaur and Lemmon, 1987; Kleitmann et al., 1988). Like G4, E587 is not seen on the soma of the ganglion cells (Rathjen et al., 1987a). In this respect, E587 staining differs from that of D3 against NCAM, because NCAM was shown to be present on the surface of fish axons and ganglion cells (Bastmeyer et al., 1990a). Another similarity between L1 and E587 antigen is that both are reexpressed during axonal regeneration (Martini and Schachner, 1988; see also below).

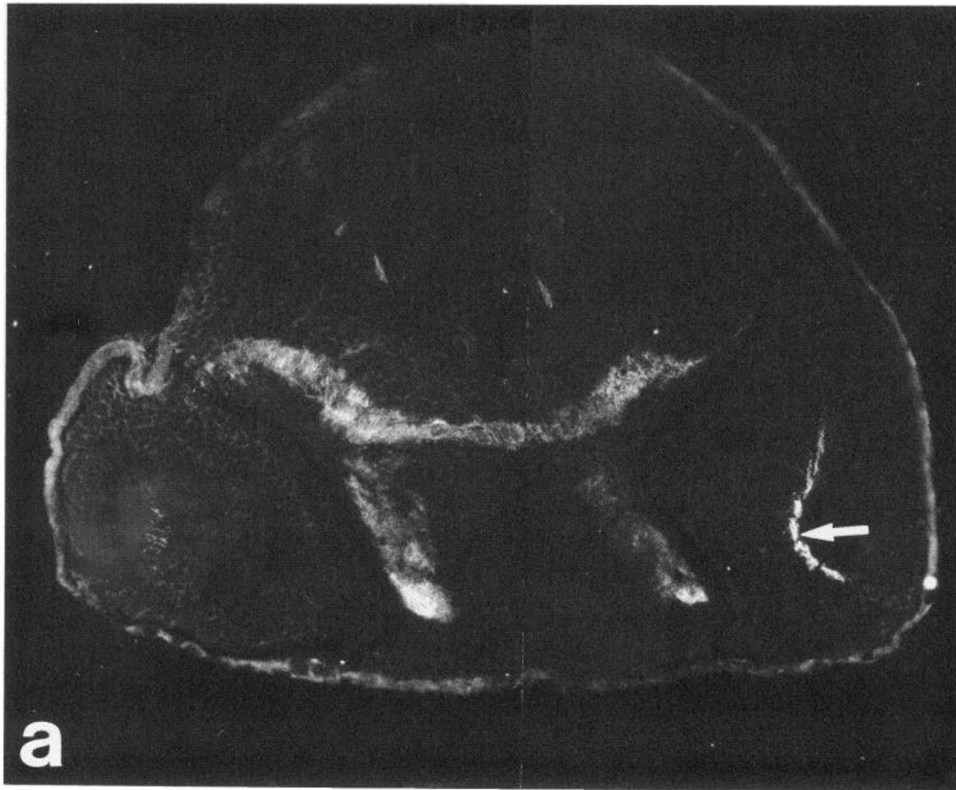
The pattern of E587-positive axons in the retina and nerve closely resembled the distribution of axons labeled with the antibody D3 against NCAM (Bastmeyer et al., 1990a). In its spatially and temporally restricted expression pattern, E587 could, as suggested for NCAM (Bastmeyer et al., 1990a), serve as a guidance cue for the newest axons in the adult fish visual system (Fig. 6 in Stuermer, 1991). The selective association of new with young axons leads over time to the age-related fiber organization that is so typical for the fish optic nerve (Easter et al., 1981).

E587 in the regenerating retinotectal pathway

Axons regenerating after ONS have lost their age-related order (Stuermer and Easter, 1984b; Stuermer, 1986; Bernhard and Easter, 1988), and both E587 antigen and NCAM (Bastmeyer et al., 1990a) are no longer restricted to a few axons but are distributed over all regenerating axons. This finding is consistent with the idea that NCAM- and E587-positive axons are attractive surfaces for the new axons in the normal nerve. During regeneration, any axon is an attractive partner for new growth cones since all axons carry these antigens.

These considerations imply that a molecule or molecules that are at times or places more broadly distributed may gain a function as specific guidance cues when their expression is confined to a few axons over a narrow time window. After optic nerve injury, the regrowing retinal axons *in vitro* and *in vivo* are E587 positive into their growth cones and regenerating axon arbor processes in the tectal retinorecipient layers. Thus, the ganglion cells are apparently induced to reexpress the E587 antigen upon nerve cut and axonal regeneration. The time course over which E587 staining diminishes on the regenerating axons is similar to that seen with D3 against NCAM (Bastmeyer et al., 1990a). Both antibodies stain the regenerating axons brightly up to 3 months after ONS. This rules out for both of them that the formation of synaptic connections would be directly correlated temporally with the decline of the immunoreactivity of the axons. Retinal synapses begin to form upon ingrowth of axons into the tectum by 11–13 d and are seen throughout the synaptic layer by 6 weeks after ONS (Stuermer and Easter, 1984b). Many of these synapses are, however, retinotopically inappropriate and are eliminated later on. Our earlier studies suggest that it takes roughly 6 months for the larger part of axons to form their arbors over their retinotopically appropriate tectal domains and for their ectopic branches and synapses to disappear (Stuermer, 1988a,b). Therefore, the reestablishment of a retinotopically well-organized projection coincides with the time at which the expression of the E587 antigen is reduced. Whether there is any causal relation between these two phenomena remains to be investigated.

In this context, it is noteworthy that axons even in normal fish never remain stable and connected to their original postsynaptic partners. Retinal axons in goldfish shift to maintain the retinotopic order of their terminals in the tectum during the ongoing and geometrically incongruent growth of the retina and the tectum (Easter and Stuermer, 1984). Normal axons except for the new ones were, however, not labeled either by D3 against NCAM or E587. Therefore, shifting, a process of slow growth



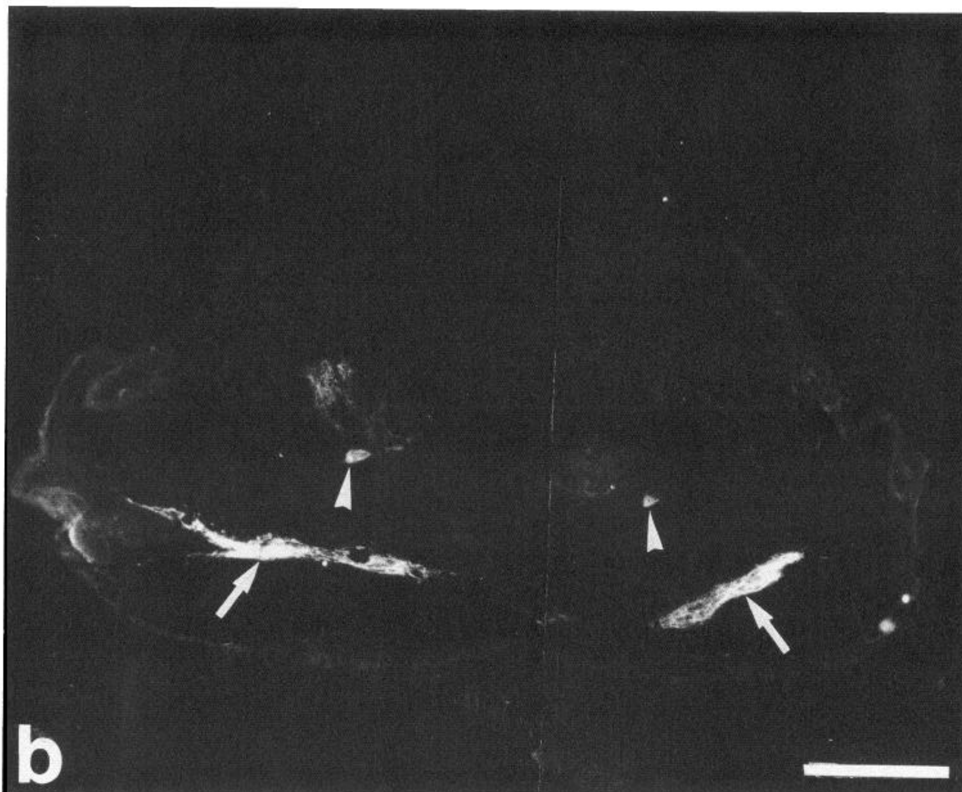


Figure 9. Cross sections through the head of a 60-hr-old goldfish embryo. *a,* In the retina, all axons in the ganglion cell axon layer are stained (*arrow*). *b,* E587 reveals the retinal axons as they exit the eye and form the optic nerve. Stained axons are also seen in the optic tracts (*arrowheads*), which are cut here obliquely across. The other brightly stained structures or parts thereof may represent the tracts of the postoptic commissure and the tract of the posterior commissure (Wilson et al., 1990). Scale bar, 100 μm.

of terminal arbors in the fish tectum, appears not to require an elevated level of these surface molecules, at least not to a degree that would be detectable by immunohistochemical techniques.

E587 always bound to young axons or regenerating ones, and both are known to be unmyelinated. Thus, it would be possible that all axons carried the antigen but that the myelin sheath on more mature axons would hinder the access of the antibody to the axon surface. This, however, is unlikely for the following

reasons. First, remyelination of regenerating axons begins at 6 weeks after ONS and progresses rapidly (Wolburg, 1978). E587 staining, however, was still bright at 3 months and had only declined by 7 months. Furthermore, after ONS E587 staining also increases on axons in the retina and in the old portion of the optic nerve, where axons are and remain myelinated. Thus, myelin is obviously not a barrier for antibody binding to the axons' surface. Therefore, the decline of E587 staining is most likely the result of a downregulation of the antigen by the cell. This has also been suggested for G4. G4 immunoreactivity disappears from the surface of axons after they have reached their targets, and this happens in chick long before myelination sets in (Weikert et al., 1990).

During retinal axonal regeneration in fish, a number of molecules are produced in larger than normal quantities, as was demonstrated in quantitative one- and two-dimensional gels (Giulian et al., 1980; Benowitz and Lewis, 1983; Perry et al., 1985, 1987, 1990). Of these proteins, a few were identified, such as GAP 43 (Benowitz et al., 1981), actin and tubulin (Heacock and Agranoff, 1982), and neurofilaments (Quitschke and Schechter, 1984). The gels used in these experiments also exhibited an increase of proteins of molecular weights similar to that of the E587 antigen.

The finding that the E587 antigen is reexpressed during retinal axonal regeneration indicates that it may subserve a function during axon regrowth. Indeed, L1, which is immunochemically similar to Ng-CAM (Grumet et al., 1984a,b; Grumet and Edelmann, 1988) and NILE (Bock et al., 1985; Friedlander et al., 1986), is also found on glial cells in the mammalian CNS and plays a role in axon–astrocyte adhesion (Bartsch et al., 1989). In the PNS of adult mammals, where injured axons are able to regenerate, L1 and Ng-CAM appear on the regenerating axons of the sciatic nerve and on Schwann cells (Kleitmann et al., 1988; Martini and Schachner, 1988; Seilheimer and Schachner, 1988; Burgoon et al., 1991) and thus probably contribute to the regrowth of the injured axons along the PNS glia. We have isolated oligodendrocyte-like cells from the regenerating fish optic nerve (Bastmeyer et al., 1990a) and seen that these fish glial cells *in vitro* carry the E587 antigen (Bastmeyer et al., 1990b). The fish optic nerve oligodendrocyte-like cells are highly growth supporting surfaces for fish regenerating retinal axons, despite the fact that these glial cells express myelin markers (Bastmeyer et al., 1991; Stuermer, 1991). It is therefore quite likely that the E587 antigen not only mediates axon–axon interactions but also contributes to axon growth on oligodendrocyte-like cells. Experiments addressing these issues are underway.

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