Fish E587 Glycoprotein, a Member of the L1 Family of Cell Adhesion Molecules, Participates in Axonal Fasciculation and the Age-related Order of Ganglion Cell Axons in the Goldfish Retina

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Abstract. Axons derived from young ganglion cells in the periphery of the retinae of larval and adult goldfish are known to fasciculate with one another and their immediate forerunners, creating the typical age-related order in the retinotectal pathway. Young axons express the E587 antigen, a member of the L1 family of cell adhesion molecules. Repeated injections of Fab fragments from a polyclonal E587 antiserum (E587 Fabs) into the eye of 3.4 cm goldfish disrupted the orderly fascicle pattern of RGC axons in the retina which was preserved in controls. Instead of bundling tightly, RGC axons crossed one another, grew between fascicles and arrived at the optic disk in a broadened front. When added to RGC axons growing in vitro, E587 Fabs neutralized the preference of growth cones to elongate on lanes of E587 protein, caused defasciculation of axons which normally prefer to grow along each other when explanted on polylysine, and prevented clustering of E587 antigen at axon–axon contact sites. Monoclonal E587 antibody disturbed axonal fasciculation moderately but led to a 30% reduction in growth velocities when axons tracked other axons.

Therefore we conclude that E587 antigen mediates axonal recognition, selective fasciculation and the creation of the age-related order in the fish retina.

THE formation of orderly nerve cell connections in the central nervous system (CNS)[1] depends on the coordinated expression of neuronal recognition molecules. Cell adhesion molecules (CAMs) of the immunoglobulin (Ig) superfamily represent a group of cell surface glycoproteins which have been demonstrated to participate in axonal guidance (reviewed in Rathjen and Jessell, 1991; Brümmendorf and Rathjen, 1993). In some systems, such as in flies, their expression is spatially and temporally regulated (reviewed in Hortsch and Goodman, 1991). They thus allow specific sets of growth cones to recognize and grow along pre-existing and CAM-expressing pathways which take the elongating axons close to their targets. Growth cone guidance by selective fasciculation is found in invertebrates as well as in vertebrates, including man (Jouet and Kenwrick, 1995), and seems to be an economical strategy for axon growth once a scaffold of early tracts has formed (Dodd and Jessell, 1988).

Aside from analyses of mutant flies (reviewed in Grenningloh and Goodman, 1992), insights into the function of individual CAMs were provided by in vivo and in vitro studies which used antibodies against relevant portions of these CAMs (for examples see Rathjen et al., 1987; Chang et al., 1987; Lemmon et al., 1989; Furley et al., 1990; Stoeckli et al., 1991; Burns et al., 1991; Pollerberg and Mack, 1994; Doherty et al., 1995). Moreover, the expression of certain axonal CAMs is downregulated when axons have completed phases of long distance growth, when they establish contacts with their targets and/or when they become myelinated (Bartsch et al., 1989; Weikert et al., 1990; Stuermer et al., 1992).

An interesting pattern of CNS growth was found in teleosts (Johns, 1977; Scholes, 1979). Goldfish and other carps grow throughout most of their lives. The fish visual system (the best studied CNS portion in this context) is well adapted to the increase in the fish's body size, and continues to add new neurons in the retina and optic tectum over years (Raymond, 1986). The optic tectum is the target of retinal ganglion cell (RGC) axons. In the retina, new neurons are born in a circumferential zone at the retinal peripheral margin (Johns, 1981). This annular growth pattern begins quite early in the fish's life, shortly after the RGCs of the central retina have made connections with the optic tectum (Laessing, U., and C. A. O. Stuermer, unpublished results). It proceeds through larval development and into adulthood (Johns, 1977). Each new generation of RGCs sends their axons to the brain, and these axons fasciculate with one another and with their immediate forerunners along significant portions of their path (Scholes,

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1. *Abbreviations used in this paper.* CAMs, cell adhesion molecules; CNS, central nervous system; Fab, Fragment of antigen binding; mAb, monoclonal antibody; RGC, retinal ganglion cell.

1979; Easter et al., 1981, 1984; Stuermer and Easter, 1984). This behavior leads to the age-related order of retinal axons in the retinotectal pathway whose discovery caused a search for similar principles of fiber order in higher vertebrates (Walsh, 1986).

Using monoclonal antibodies, we identified several cell adhesion/surface proteins in goldfish whose expression on RGC axons is regulated (Bastmeyer et al., 1990; Vielmetter et al., 1991; Paschke et al., 1992; Schulte, T., F. Lottspeich, and C. A. O. Stuermer, 1994. *Soc. Neurosci. Abstr.* 20:1080). Highest expression levels are found on axons growing in embryos and in adults after optic nerve transection. In larval and adult goldfish, antibodies against these proteins selectively label the young axons from peripheral RGCs (reviewed in Stuermer et al., 1992). This suggested that the proteins might contribute to aspects of RGC axonal growth and to their association into age-related bundles.

The E587 antigen is one of these proteins. It was named after the monoclonal antibody E587 (Vielmetter et al., 1991). E587 antigen is a 200-kD glycoprotein whose NH_2 terminus has homologies to members of the L1 family of CAMs (Vielmetter et al., 1991). Additional, unpublished sequence information (Giordano, S., and C. A. O. Stuermer, unpublished results) suggests moderate and comparable degrees of homologies to all known L1 family members. It is therefore undecided whether E587 antigen is the homologue of L1, Ng CAM/G4, Bravo/Nr CAM, or is a new family member.

We have used mAb E587 and polyclonal antibodies against the protein to study the function of E587 antigen. Here, we report in vitro and in vivo results showing that E587 antibodies block axonal fasciculation, negatively affect growth cone velocities and, most importantly, perturb the order of age-related bundles in the goldfish eye.

Parts of this work were presented in abstracts (Giordano, S., M. Bastmeyer, A. Y. Loos, and C. A. O. Stuermer, 1993. *Soc. Neurosci. Abstr.* 19:1090; Bastmeyer, M., C. A. Leppert, H. Ott, and C. A. O. Stuermer, 1995. Proc. 23. Göttingen Neurobiol. Conference).

Material and Methods

Animals

Common goldfish (*Carrassius auratus*, 5–7 cm body length) obtained from a local supplier were used for protein isolation and retinal explants. In vivo studies were performed with juvenile goldfish from our laboratory breeding colony. 10 individuals were kept in 100 liter tanks at 22°C and fed twice a day to accelerate their growth. All experiments performed in this study are in compliance with the national animal welfare legislation.

Antibodies

mAb E587 was produced as previously described in detail (Vielmetter et al., 1991). A purified IgG fraction of mAb E587 in citrate buffer was prepared. Purified IgGs of mAb C183 were used for control experiments. This antibody recognizes an unidentified antigen and binds to living goldfish retinal axons in culture much like mAb E587 as judged by immunofluorescence.

Polyclonal antibodies were produced by injecting rabbits subcutaneously with immunopurified E587 antigen. Complete Freund's adjuvant was used for the first injection and incomplete for three subsequent injections every third week. Fab fragments were obtained from the IgG fraction by using a Papain digestion kit (Pierce, Sankt Augustin, Germany). The specificity of Fab fragments for E587 antigen (in brief E587 Fabs) was verified on Western blots and on cryosections of goldfish brains. Fab fragments from non-immune rabbits served as controls.

Goldfish Retinal Explants

In vitro experiments were performed with regenerating retinal axons which readily extend from retinal explants when the fish optic nerve has been transected 14–17 d before preparation. Retinae of embryonic fish are too small (200 μm in diameter) for assays of the present study, and embryonic axon outgrowth is poor as compared to that from adults. Goldfish retinal explants were prepared as previously described (Vielmetter and Stuermer, 1989). In brief, the optic nerves of adult goldfish were cut under MS-222 (3-aminobenzoic acid ethyl ester; Sigma, Deisenhofen, Germany) anesthesia. The retina was isolated and attached to a nylon filter (Hybond; Amersham, Braunschweig, Germany). Retina and filter were cut into segments 300-μm wide and explanted, ganglion cell layer down, onto coated coverslips. Small metal blocks were placed on the ends of the segments to keep the retina in contact with the substrate. The cultures were kept in Leibovitz medium (L-15; GIBCO, Eggenstein, Germany), supplemented with 10% fetal calf serum and 0.4% methyl cellulose at 22°C.

Substrate Preparation

Sterilized coverslips were coated with a Poly-L-lysine solution (0.1 mg/ml in distilled water) for 1 h at room temperature, washed with distilled water and air dried. Immunopurified E587 antigen (protein concentration 1 μg/ml) was applied to polylysine-coated coverslips in 50 μm wide lanes using a special silicone matrix as described by Vielmetter et al. (1990). 10 μl of E587 antigen solution in phosphate buffer was injected into the channels of the silicone matrix. After 2 h at room temperature, the matrix was removed and the coverslips were washed twice with L-15. This resulted in a patterned substrate of alternating lanes of polylysine alone and antigen bound to polylysine. Retina segments were explanted onto the patterned substrate either directly or after blocking the antigen lanes with E587 Fabs (500 μg/ml in L-15) for 1 h. Fab fragments (200 μg/ml) were also added to the medium to block E587 antigen on growing axons. Control cultures were treated with Fab fragments of non-immune serum in the same way.

Fasciculation of Retinal Axons In Vitro

For defasciculation assays, either mAb E587 (100 or 200 μg/ml), mAb C-183 (100 or 200 μg/ml), E587 Fabs (0.2 or 0.8 mg/ml), or Fab fragments of non-immune serum (0.2 or 0.8 mg/ml) diluted in medium were added to the cultures at the time when the retina segments were explanted onto polylysine-coated coverslips. Control cultures received the equivalent volume of citrate buffer. Living cultures were photographed after 1 and 2 d. After 2 d, cultures were fixed and processed for immunocytochemistry. Growing axons of retinal cultures treated with mAb E587, mAb C183, or controls, were monitored with time-lapse videomicroscopy. Living axons were viewed with a 40× lens under phase contrast in an inverted microscope (Zeiss Axiovert) to which a camera was attached. The camera was connected to an image processor (Hamamatsu, Garsching, Germany) and an S-VHS time-lapse recorder (Panasonic, Osaka, Japan). To avoid continuous illumination, a shutter which opened every 5 s for 200 ms was inserted into the light path. Four images were taken and averaged and recorded. Axon growth was recorded in randomly selected fields for 1.5–2 h. These video sequences served to measure the growth velocity of growth cones in fascicles and on polylysine, and to evaluate their behavior upon encountering another axon. Only growth cones that were monitored for at least 1 h were included in velocity measurements.

Fasciculation of Retinal Axons In Vivo

For in vivo functional assays, goldfish of equal size (3.4 cm mean body length, head to tail; 1.4 mm mean lens diameter) were selected from our laboratory breeding colony with 10 fish for each experimental group. Antibodies or control substances were injected into the eyes of these fish using a glass micropipette. The fish were anaesthetized in MS-222 prior to each injection. The sclera and iris were penetrated with a syringe and antibody solution was pressure injected through the preformed hole using a picospritzer (Transjector; Eppendorf, Hamburg, Germany). Injected eyes that were processed for immunocytochemistry with the secondary antibodies alone revealed that the antibodies have access to the retinal fiber layer, where they are still seen 4 d after injection. During the first 5 wk, 0.1 μl were injected every third or fourth day. The injected volume was increased to 0.2 μl for the next 5 wk. This treatment resulted in antibody concentrations of 0.3–0.5 mg/ml in the vitreous. Four groups of fish, all of equal size, were injected as follows: (*a*) 6-mo-old goldfish, right eye mAb E587 (11.5 mg/ml), left eye untreated; (*b*) 6-mo-old goldfish, right eye E587 Fabs (23 mg/ml), left eye untreated; (*c*) 9-mo-old goldfish, right eye E587 Fabs (23 mg/ml), left eye untreated; (*d*) 9-mo-old goldfish,

right eye Fab fragments of non-immune sera (17 mg/ml), left eye buffer only. During these 10 wk, the goldfish grew by 50% in length to a mean body length of 5.1 cm (head to tail). The injections had no negative effect on growth of the eye. Both the injected and the control eye had the same size (mean lens diameter 2.1 mm) and the retinal wholemounts of these eyes had equal areal dimensions (mean of 28 mm^2).

Immunohistochemistry on Retinal Cultures

For immunostaining, the cultured axons were washed in L-15, fixed in methanol for 5 min at −20°C and postfixed in 2% paraformaldehyde in PBS at room temperature for 5 min. Cultures were washed three times in PBS (5 min each), and incubated with primary antibodies. For double labeling, cultures received a mixture of E587 Fabs and mAb E587 (10 and 50 μg/ml in PBS, respectively). After three washes in PBS they were incubated simultaneously in fluoresceine (FITC)-coupled goat anti–rabbit antibodies and rhodamine isothiocyanate (RITC)-coupled goat anti–mouse antibodies (Dianova, Hamburg, Germany) for 1 h at room temperature, washed again in PBS, and mounted in Mowiol, containing n-propylgallate to reduce fading. For labeling with mAb C183, living axons were treated with the antibody (10 μg/ml in L-15) for 30 min, fixed, and immunostained as described above. Cultures that were grown in the presence of either polyclonal or monoclonal antibodies were washed in L-15, fixed, and incubated in secondary antibodies. Retinal cultures were viewed in a Zeiss Axiophot using the appropriate filter sets.

Immunohistochemistry on Retinal Whole Mounts

Cornea and pigment epithelium of isolated eyes were removed and the retina (photoreceptor layer down) attached to a nylon filter (Hybond; Amersham). To make axons accessible to antibodies, the vitreous and the inner limiting membrane together with retinal blood vessels were carefully removed. In some cases the inner limiting membrane including blood vessels was left intact to exclude the possibility that abnormal axonal growth patterns were artifacts caused by the preparation. The retinae were fixed in methanol (−20°C) for 5 min and treated with primary antibodies overnight (at 4°C). Control eyes received a mixture of E587 Fabs and mAb E587 (10 and 100 μg/ml, respectively, in PBS) to stain young axons. Fab-injected eyes received only mAb E587 and mAb-injected eyes only E587 Fabs. After three washes in PBS (10 min each) retinae were incubated in a mixture of FITC-coupled goat anti–rabbit antibodies and RITC-coupled goat anti–mouse antibodies. After 2 h, retinae were washed three times in PBS, coverslipped in Mowiol, and viewed in a Zeiss Axiophot using the appropriate filter sets.

Results

The E587 Antigen Is Enriched at Axon/Axon Contact Sites

Goldfish retinal axons growing in culture are labeled by mAb E587 over their entire length including growth cones and filopodia (Fig. 1 *A*) (Vielmetter et al., 1991). However, when axons crossed each other (Fig. 1, *B* and *C*) or grew in fascicles (Fig. 1, *D* and *E*), E587 immunoreactivity was greatly enhanced at axon/axon contact sites. This staining pattern was specific for antibodies against E587 antigen and visible with both divalent IgGs (Fig. 1, *C* and *E*) and monovalent Fab fragments of polyclonal sera (Fig. 1 *F*). Other antibodies such as mAb C183 (Fig. 1 *G*) or mAb D3 against NCAM 140/180 (Bastmeyer et al., 1990) do not show enhanced labeling at these contact sites. When retinal axons grew in the presence of E587 Fabs (200 μg/ml), clustering at contact sites was no longer visible (Fig. 1 *H*). However, clustering of the antigen could not be blocked by the addition of mAb E587 (Fig. 1 *I*). The clustering of E587 antigen at axon/axon contact sites suggests that this glycoprotein mediates axonal interactions.

The E587 Antigen Serves as a Substrate for Retinal Axons

Whether immobilized E587 antigen promotes axonal growth was investigated in vitro using regenerating goldfish retinal axons. When E587 antigen was presented in a patterned substrate consisting of alternating lanes of antigen on polylysine and polylysine alone, the axons grew almost exclusively on the lanes containing antigen (Fig. 2 *A*). Although less than on polylysine, axonal outgrowth on E587 antigen was still fasciculated. This is attributable to the low concentration of the antigen used here which is confirmed by the weak immunofluorescence of the antigen lanes (Fig. 2 *A*). For most axons the preferred substrate were still other axons under these conditions. To prove that this outgrowth pattern was specific for E587 antigen and not simply caused by the higher protein concentration in the preferred lane, explants were grown in the presence of monovalent E587 antibodies. As shown in Fig. 2 *B*, the addition of E587 Fabs at a concentration of 200 μg/ml resulted in a completely random and defasciculated growth pattern. The preference for the antigen lanes was no longer seen. Non-immune Fab fragments had no such effect. Interestingly, the addition of mAb E587 (200 μg/ml) caused a less fasciculated growth pattern but had no effect on the preference for the antigen lanes.

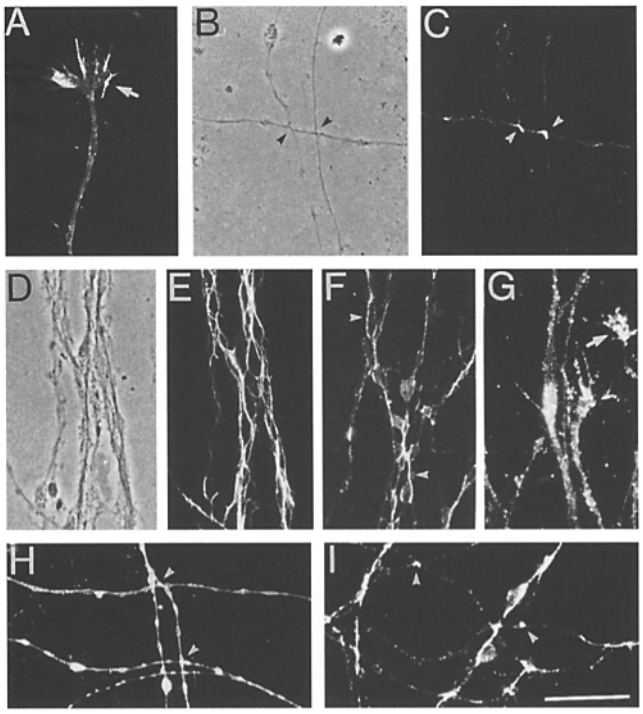

Figure 1. Retinal axons growing on a polylysine substrate and labeled with mAb E587 (*A*, *C*, *E*, and *I*), E587 Fabs (*F* and *H*), and mAb C183 (*G*). E587 antigen is expressed over the entire axon including the growth cone (*A*, *arrow*). Phase contrast (*B*) and corresponding immunofluorescence (*C*) micrograph of three axons crossing each other. E587 immunoreactivity is highly enhanced at contact sites (*B* and *C*, *arrowheads*). Phase contrast (*D*) and corresponding immunofluorescence (*E*) micrograph of fasciculated axons. Enhanced immunoreactivity at contact sites is also visible with E587 Fabs (*F*, *arrowheads*). Axons grown in the presence of E587 Fabs do not accumulate the antigen at contact sites (*H*, *arrowheads*). The addition of mAb E587 to the culture medium had no such effect, enhanced labeling at contact sites is still detectable (*I*, *arrowheads*). mAb C183, which served as a control in the functional assays, binds to living axons including their growth cones (*G*, *arrow*). Bar, 20 μm.

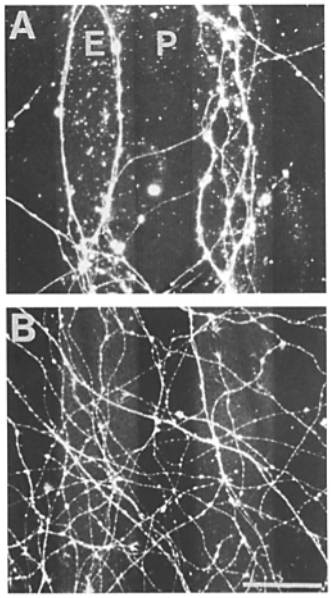

Figure 2. Retinal axons growing on a patterned substrate of polylysine (*P*) and E587 antigen (*E*) bound to polylysine. The retinal explant is toward the bottom. In control medium (*A*) the axons elongate almost exclusively on the antigen lanes, whereas the addition of E587 Fabs (200 μg/ml) results in a random growth pattern (*B*). Axons were labeled with a polyclonal serum against E587 antigen. The micrographs were overexposed to show the antigen lanes and therefore do not reveal the typical concentration of E587 antigen at axon/axon contact sites (compare to Fig. 1). Bar, 50 μm.

In Vitro Perturbation of Axon Fasciculation with Antibodies against E587 Antigen

To test whether the E587 antigen is involved in axonal fasciculation, we used antibodies to perturb the growth pattern of retinal axons growing in culture. When retinal segments were explanted onto polylysine-coated coverslips, the retinal axons grew in thick fascicles (Fig. 3 *A*). The addition of mAb E587 resulted in a marked decrease of fasciculation particularly near the explant but had no effect on axon outgrowth per se or on axon morphology (Fig. 3 *C*). Fascicles reformed, however, at a distance of 100–150 μm from the explant. To verify that the defasciculation is not caused by the binding of IgGs to axonal surfaces, the IgG C183, which also binds to the surface of living retinal axons (Fig. 1 *G*), was applied in controls. mAb C183 had no effect on axonal fasciculation (Fig. 3 *B*).

To quantify the effects of mAb E587 on axonal defasciculation we counted the number of growth cones growing on polylysine or on the surface of other axons. Since growth cones elongating in a fascicle are difficult to recognize in static images we used time-lapse videomicroscopy. Randomly selected fields were monitored for 1–2 h and the video sequences later analyzed. In control cultures without antibodies (231 growth cones) or with mAb C183 (82 growth cones), 55% of the growth cones were associated with other axons. With mAb E587 added to the culture medium (217 growth cones), only 35% of the growth cones were associated with other axons (Fig. 4). A similar effect was observed when single growth cones elongating on polylysine encountered another axon. In control cultures, 51% (19 of 37) of the growth cones changed their direction and fasciculated with the other axon. In cultures treated with mAb E587 only 29% (14 of 49) fasciculated (Fig. 5). These findings indicate that fasciculation of goldfish retinal axons can be partially inhibited (by 30%) through the addition of mAb E587.

In a second set of experiments E587 Fabs were used instead of mAb E587. The addition of these Fab fragments (0.2 or 0.8 mg/ml) resulted in marked defasciculation (Fig. 3 *D*) and over the entire extent of the axons. Growth cones

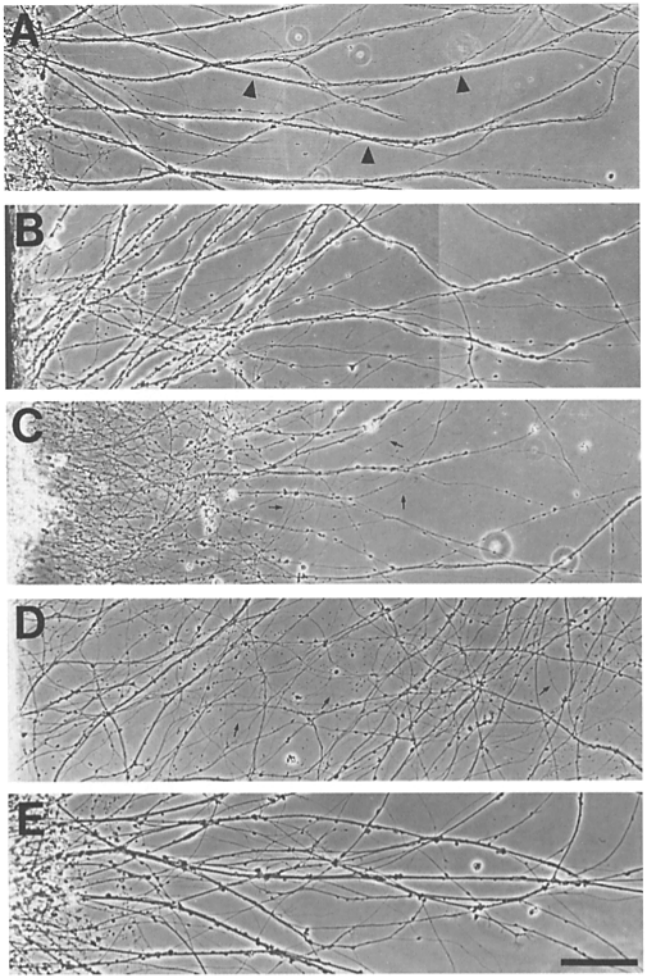

Figure 3. Phase contrast micrographs of retinal axons growing on a polylysine substrate with different antibodies added to the culture medium. The retinal explant is to the left. In control medium (*A*) and with mAb C183 (*B*) axonal outgrowth is strongly fasciculated (*A*, *arrowheads*). The addition of mAb E587 (*C*) or E587 Fabs (*D*) results in a defasciculated growth pattern with numerous single axons (*C* and *D*, arrows). Fab fragments of non-immune sera (*E*) have no effect on axonal defasciculation. Bar, 30 μm.

elongating along other axons were rarely seen in such cultures. Again, Fab fragments of non-immune sera used at the same concentrations had no effect on neurite outgrowth or axonal fasciculation (Fig. 3 *E*).

Influence of mAb E587 on Growth Cone Velocity

The growth velocities of axons elongating on polylysine or on the surface of other axons were measured using time-lapse videomicroscopy. In control cultures, growth cones elongated with a mean velocity of 53.4 ± 17.2 μm/h (n = 33) on polylysine and 76.6 ± 20.7 μm/h (n = 38) on other axons. This 30% increase in velocity is statistically significant (P = 0.01, Kolmogorov-Smirnov two group test). The addition of mAb 587 to the culture medium had no effect on growth velocities on polylysine (mean: 53.8 ± 15.3 μm/h; n = 56), but the mean velocity of growth cones in a fascicle fell to 55.6 ± 13.0 μm/h (n = 33). As seen in growth velocity diagrams (Fig. 6), the presence of mAb E587 reduced axon growth to the velocity of axons grow-

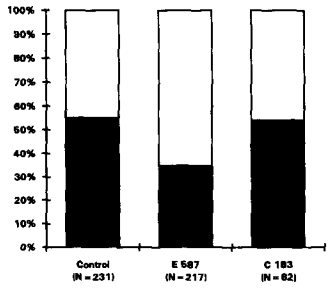

Figure 4. Retinal axons were grown on a polylysine substrate in control medium, in the presence of mAb E587, or mAb C183. The number of growth cones elongating on polylysine or fasciculated with other axons was determined. In control medium or cultures treated with antibody mAb C183, 55% of the growth cones are found in a fascicle. In contrast, only 35% grow fasciculated with other axons in the presence of mAb E587. □, On polylysine; ■, in fascicle.

ing on polylysine alone (Fig. 6 *B*). The addition of control antibody mAb C183 had no such effect. Growth cones elongating along other axons grew about 35% faster than those on polylysine. Thus, mAb E587 not only interferes with axonal fasciculation but also reduces the growth velocity of axons extending along other axons to control levels found on polylysine.

In Vivo Perturbation of Axon Fasciculation with Antibodies against E587 Antigen

The E587 antigen is selectively expressed by young axons derived from new retinal ganglion cells at the retinal peripheral margin (Fig. 7) indicating that the antigen may contribute to the intraretinal path of these axons. Therefore, juvenile and rapidly growing goldfish received repeated injections of E587 Fabs or control solutions into one eye. The diameter of the lens increased on average from 1.4 (± 0.1) mm to 2.1 (± 0.2) mm and the retinal area from 14 mm^2 to 28 (± 4) mm^2 over the 10-wk period. Injected eyes showed the same growth as uninjected controls. From earlier counts in goldfish of 5 cm size (Raymond-Johns and Easter, 1977) we estimate that roughly 50,000 new RGCs were added during the growth of our fish. Axons of these new RGCs are targets of E587 antibody while they are elongating from their cell of origin to the optic disk. Whole mounts of control goldfish retinae immunostained with mAb E587 exemplify the orderly array of fascicles of newly formed axons. At the retinal peripheral margin close to their origin, axons meet to form small bundles (Fig. 7 *B*). On their further path to the center of the eye, the smaller bundles converge to form fewer but thicker fascicles (Fig. 7 *B*) which leave the eye at the optic disk. These fascicles are straight and ordered. The pattern of fascicles in eyes injected with E587 Fabs was markedly disordered (Fig. 7 *A*). The fascicles of new axons often exhibited an irregular, wavy appearance and were

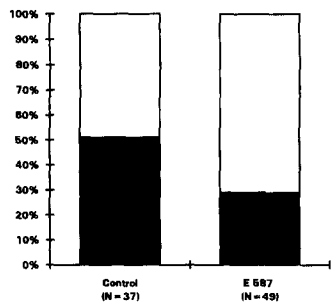

Figure 5. Behavior of growth cones that elongate on a polylysine substrate and meet other axons. In control cultures, 51% of the growth cones fasciculate with the other neurite. In the presence of mAb E587, this level dropped to 29%. □, No fasciculation; ■, fasciculation.

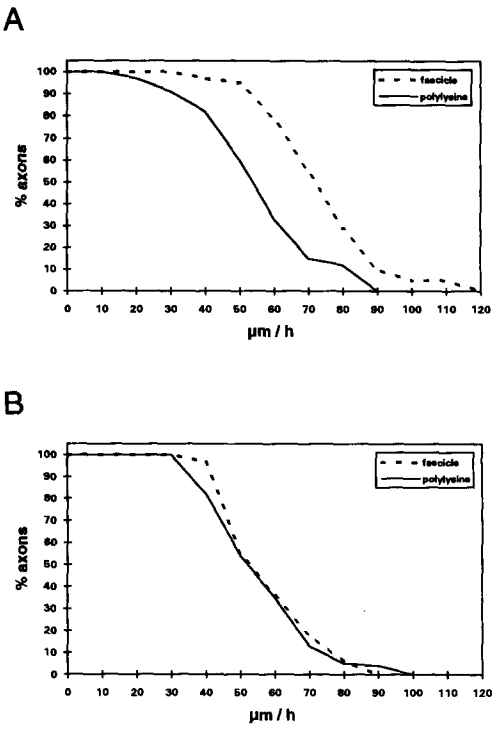

Figure 6. Distribution of growth velocities in the absence (*A*) or presence (*B*) of mAb E587. The percentage of axons (vertical axis) growing faster than a given velocity (horizontal axis) was determined for growth cones elongating on polylysine (*solid line*) or on other axons (*dashed line*). mAb E587 had no effect on growth velocities on polylysine, but for axons growing on other axons the velocity distribution curve is shifted towards smaller values (*B*) compared to control cultures (*A*).

broader than in control eyes. Moreover, bundles of E587 positive axons frequently leave their original fascicle to join another one (Fig. 7 *A*). This is rarely observed in control eyes. While newly formed axons normally fasciculate within the first 50 µm of their path and form small bundles that run more or less straight towards the optic disk (Fig. 8 *B*), fasciculation of the new axons in treated eyes appears delayed. Groups of few or perhaps single axons were still visible at distances of 250 µm away from the margin (Fig. 8 *A*). Further to the center, the fascicles in the treated eyes had a less ordered appearance, most obvious in regions midway between the margin and the optic disk (Fig. 8 *C*). While fascicles in the control eye were of more or less similar diameters and were orderly and straight (Fig. 8 *D*), fascicles in the treated eye were of variable diameters and curved (Fig. 8 *C*). Moreover, small bundles or single axons appear to switch fascicles or run alone (Fig. 8 *C*). Fascicles of young axons converge to fewer but distinct bundles at the optic disk of control eyes (Fig. 8 *F*). In injected eyes, fascicles of new axons associate into broader bundles at the optic disk and with numerous E587-positive axons remaining outside the bundles (Fig. 8 *E*).

These effects were seen in all animals injected with E587 Fabs (9-mo-old fish: $n = 9$; 6-mo-old fish: $n = 8$). Animals injected with mAb E587 ($n = 8$) had less severe but notable degrees of disorder in the axonal outgrowth pattern. Bundles of axons switching fascicles were rarely seen, but single axons and broadened fascicles were frequent compared to control eyes. The injection of Fab fragments of

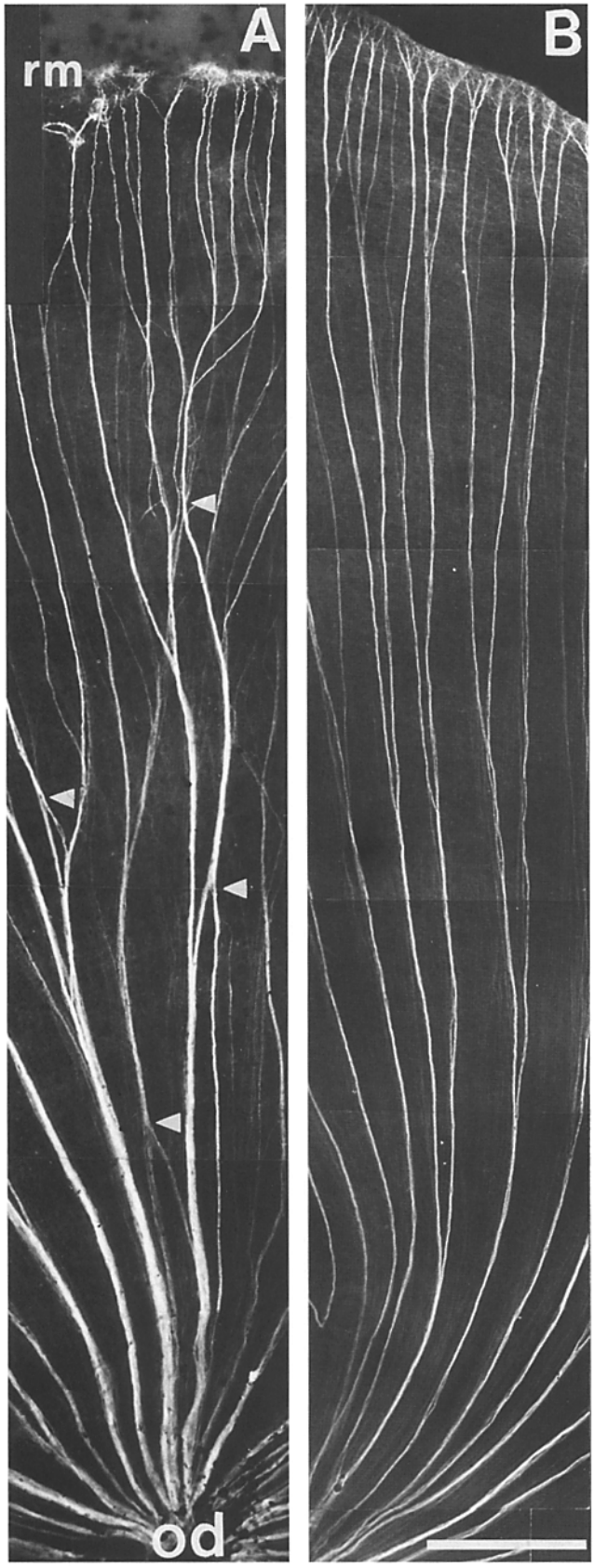

Figure 7. Retinal wholemounts labeled with E587 antibodies. Segments spanning the distance from the retinal peripheral margin (*rm*) to the optic disk (*od*) from an eye injected with E587 Fabs (*A*) and a control eye (*B*). The two eyes were obtained from different goldfish and therefore differ slightly in size. E587 selectively labels young axons from the new retinal ganglion cells at the peripheral margin. They form small fascicles and their path is more or less straight and orderly in the control eye (*B*). Note the wavy appearance of axon fascicles and the groups of labeled axons that leave their original fascicle (*A*, *arrowheads*) in the injected eye. Bar, 200 μm.

non-immune sera or buffer alone had no effect on the outgrowth pattern. Thus, the E587 antigen is involved in the fasciculation of retinal axons and contributes to the growth-related order in the goldfish eye.

Discussion

The fish visual system is well known for its unique age-related order of retinal ganglion cell axons which develops through the tight fasciculation of young axons which express the E587 antigen. In this context, the present results have revealed the functional significance of E587 antigen in that this cell surface protein contributes to the establishment of the RGC axonal order. Axons growing in the presence of E587 antibodies formed disorderly fascicles, crossed one another, or migrated towards the optic disk through areas between E587-positive fascicles. The loss of preference for like axons was mimicked by RGC axons in vitro. In the presence of Fab fragments of polyclonal sera against E587 antigen, growth cones no longer favored other axons as substrates for their growth, lost their ability to cluster the antigen at axon/axon contact sites, and crossed E587 antigen lanes freely. With mAb E587, growth cones reduced their velocity when tracking other axons.

The present results therefore confirm a function of E587 antigen which had been anticipated earlier after discovering its discrete expression in larval and adult fish. E587 antigen is selectively associated with young axons from newly formed RGCs and is downregulated by older axons (Vielmetter et al., 1991). Before they merge into one coherent bundle in the optic nerve, young axons are grouped in smaller age-matched fascicles in the retina. This grouping results from the preference of new axons to associate with one another and to track their forerunner on their path to the optic disk. The position of young axons superficial to older axons was first detected by Easter et al. (1984) who found their growth cones close to the basal lamina which separates the retina from the vitreous. Axons of earlier generations, i.e., from more centrally located RGCs, lie deep to the younger ones and are, as is typical of goldfish, myelinated (Wolburg, 1980).

Our present findings indicate that E587 antigen is responsible for the preferential growth of new axons along forerunners. E587 antibodies interfere with growth cone fasciculation and cause a loss of order in newly forming fascicles. The path of E587-positive axons in routes outside the fascicles suggests that, in the presence of antibodies, growth cones lose their preference and elongate on alternate substrates. These may be the surface of RGCs, glial cell processes, older axons, or the vitread basal lamina (Easter et al., 1984). The evaluation of loss of RGC axonal order is confined here to the retina because injected antibodies do not diffuse into the optic nerve.

The fact that axons from the newly formed periphery

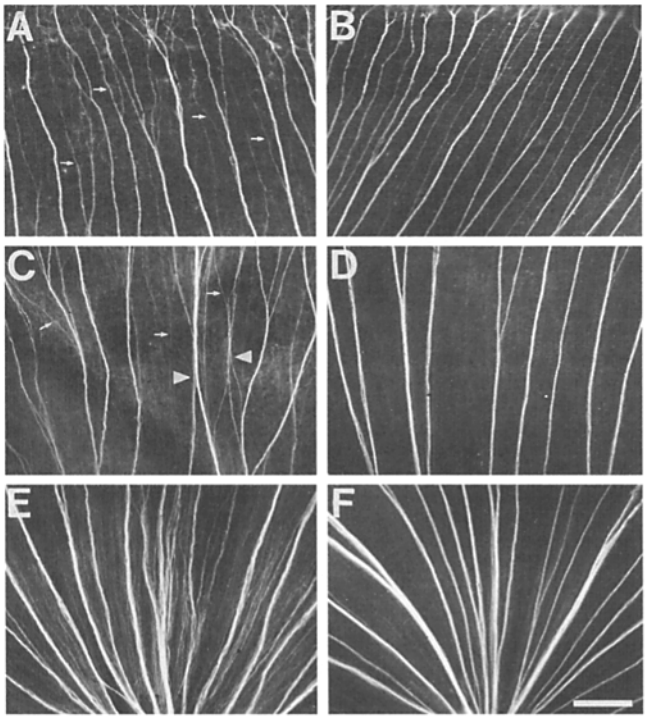

Figure 8. Retinal wholemounts labeled with E587 antibodies. Matching pairs of eyes injected with E587 Fabs (*A*, *C*, and *E*) and the uninjected control eyes (*B*, *D*, and *F*) are shown. In the retinal periphery (at the top of the micrographs) of the control eye (*B*) newly formed axons fasciculate within the first 50 μm and form small bundles. In the treated eye, fine E587-positive axons (*A*, *arrows*) are still visible up to 250 μm from the margin. In regions midway between the margin and the optic disk, fascicles have equal diameters and run more or less straight in the control eye (*D*). Fascicles in the treated eye have variable diameters and a wavy appearance (*C*). In addition, several small bundles or single axons that either switch between fascicles (*C*, *arrowheads*) or run alone (*C*, *arrows*) are visible. Fascicles of young axons converge at the optic disk but are still clearly discernible bundles in control eyes (*F*). In injected eyes, bundles of new axons are less fasciculated and numerous E587-positive axons lie between the fascicles (*E*). Bar, 100 μm.

succeed in elongating from the margin of the retina to the optic disk despite repeated antibody injections shows that axogenesis and axonal elongation per se are not blocked by E587 antibodies. That aberrant routes and defasciculated growth are not caused by the injection procedure itself or by increase of intraocular pressure is shown in control experiments. Retinae receiving buffer injections or Fab fragments of non-immune sera showed normal fascicle patterns. This is consistent with observations of tissue culture where axons did grow in the presence of E587 Fab fragments. With E587 Fabs, nearly all growth cones elongated on the polylysine substrate instead of tracking other axons indicating that they no longer preferred other axons as substrates for their growth.

However, in cultures treated with mAb E587 instead of E587 Fabs, only 30% of the growth cones left the fascicles. The 70% growth cones which migrated along their forerunners showed a marked reduction in growth velocity. mAb injections in vivo also produced a more moderate effect on axonal fasciculation than E587 Fabs. Whether the antibodies affect the in vivo growth velocity of new RGC axons is not known. However, inferred from their distribution, new RGC axons take novel routes in the presence of Fabs, implying that growth cones explore their environment to find alternative pathways to the optic disk. In other systems, they advance more slowly in such situations (Kaethner and Stuermer, 1992; Sretavan and Reichardt, 1993).

The present finding that E587 Fabs disturb RGC axonal growth more severely than mAb E587 suggests that E587 antigen provides more than one domain for the present aspects of axon-axon interactions. mAbs bind to one epitope, but polyclonal sera bind to several. Therefore, we conclude that E587 antigen has at least two sites/domains which are involved in mediating axon growth. This is common for members of the L1 family as well as for other CAMs of the Ig superfamily (Brümmendorf et al., 1993; Doherty et al., 1992; Frei et al., 1992). That E587 antigen has more than one functional domain is further substantiated by the observation that axons fail to cluster E587 antigen at contact sites in the presence of Fabs but not when mAb E587 is added to axons in culture.

Cellular mechanisms which assist in moving and holding the protein at contact sites are likely to involve interactions with the cytoskeleton as were reported for NCAM 180 (Pollerberg et al., 1987) but data in support of such interactions in the case of E587 antigen are not yet available. Fasciculation and stimulation of axon growth (seen here by the gain in velocity of growth cone advancement on E587-expressing surfaces) are thought to be mediated through activation of intracellular signaling cascades (reviewed in Doherty and Walsh, 1994). The increase in axon growth velocities requires increased rates of polymerization of cytoskeletal proteins at the growth cone's advancing edge. Signaling cascades involved are often activated by cytoplasmic domains of CAMs (reviewed in Doherty and Walsh, 1994). In the case of E587 antigen this intracellular domain is markedly homologous to that of CAMs of the L1-family (Vielmetter et al., 1991). Still, as indicated in the Introduction, it is not clear whose homologue fish E587 antigen represents. The limited (40%) degree of homology with several known members of the L1 family suggests that E587 antigen may be a new member of this family of cell adhesion molecules (Giordano, S., and C. A. O. Stuermer, unpublished results).

Our experiments have revealed a function of E587 antigen which is consistent with its putative identity as a multidomain cell adhesion molecule. The interesting regulation of expression of this and other cell surface proteins in fish is most likely responsible for the creation of the age-related order of retinal axons. E587 antigen, as well as Neurolin (Paschke et al., 1992) and NCAM 180/140 (Bastmeyer et al., 1990) are found on axons in embryos. They are downregulated on older axons but expressed by new ganglion cells and their axons which are continuously added to the growing fish retina. Expression of E587 antigen on new axons leads them to selectively fasciculate with one another while growing in between older, E587-negative axons. The age-related order is thus a consequence and epiphenomenon of this behavior.

Whether the function(s) of E587 antigen revealed here result from homophilic or heterophilic interactions cannot be determined at present. Growing fish RGC axons ex-

press several additional surface proteins (reviewed in Stuermer et al., 1992), two of which are CAMs of the Ig superfamily (Bastmeyer et al., 1990; Paschke et al., 1992).

In light of these findings it is perhaps surprising that blockage of E587 function by antibodies causes such a striking effect in vivo and in vitro. Antibody perturbation experiments with members of the L1 family of cell adhesion molecules resulted in effects comparable to those observed here. L1 is a potent substrate for neurite outgrowth (Lemmon et al., 1989) and blockage of epitopes of chick G4 lead to a reduction in sympathetic neurite length when they were offered sympathetic axons as a substrate for their growth (Chang et al., 1987). Injection of antibodies also impaired the concerted action of G4 and NCAM in chick motoneurons (Landmesser et al., 1990). Also, commissural axons in the chick spinal cord committed pathway mistakes when antibodies against relevant cell adhesion molecules were administered to embryos (Stoeckli, E. T., and L. Landmesser. 1994. *Soc. Neurosci. Abstr.* 20:1296). Finally, antibodies against rat L1 caused errant growth of rat RGC axons during retinal development (Brittis, P. A., V. Lemmon, U. Rutishauser, and J. Silver, 1993. *Soc. Neurosci. Abstr.* 19:236).

We suggest that the growth behavior of E587-positive RGC axons in vitro is directly comparable to that in vivo. It should be emphasized that axons observed here in vivo are from newborn RGCs (i.e., embryonic axons), whereas those observed in vitro are regenerating axons from RGCs which had received a conditioning lesion of their axons 14 d prior to their explantation in tissue culture. Lesioned RGCs are known to re-express E587 antigen in vivo as well as in vitro (Vielmetter et al., 1991). This implies that E587 antigen may also be required for some aspects of axonal regeneration not only in fish, but perhaps in mammals as well (Schaden, H., B. Stecher, M. Bähr, and C. A. O. Stuermer, 1993. *Soc. Neurosci. Abstr.* 19:677).

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