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Axonal guidance in the chicken retina

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SUMMARY

During retina development, ganglion cells extend their axons exclusively into the innermost tissue layer, but not into outer retina layers.

In order to elucidate guiding mechanisms for axons, tissue strips of embryonic chicken retinae were explanted onto retinal cryosections (cryoculture). Ganglion cell axons originating from the explant grew preferentially on the innermost retina layer of cryosections, whereas outer tissue layers were avoided, very much as in vivo.

Stereotropism, interaction with laminin of the basal lamina and axonal fasciculation did not significantly affect oriented axonal outgrowth, so that stereotropism as a guidance mechanism could be excluded. Ganglion cell axons were not directed by physical barriers, e.g. microstructured silicon oxide chips. Similarly, UV induced protein inactivation revealed that laminin present in the inner retina did not provide a guidance cue. Even in the absence of ganglion cell axons in retinal cryosections due to prior optic nerve transection in ovo, the growth preference for the innermost retina layer was maintained in cryocultures.

However, oriented elongation of axons along the innermost retina layer was lost when radial glial endfeet were selectively eliminated in retinal cryosections. In addition, glial endfeet provided an excellent growth substratum when pure preparations of endfeet were employed in explant cultures. The preference for glial endfeet positioned at the inner retina surface was accompanied by the avoidance of outer retina layers, most likely because of inhibitory components in this region. This assumption is corroborated by the finding that addition of exogenous growth-promoting laminin to cryosections did not abolish the inhibition. Laminin on glass surfaces provided an excellent substratum. Axonal outgrowth was also seriously hampered on specifically purified cells of the outer retina. Most notable, however, in cryocultures aberrant innervation of outer retina layers could be induced by prior heat or protease treatment of cryosections, which pointed to proteins as potential inhibitory components.

In summary the data substantiate the hypothesis that within the retina, ganglion cell axons are guided by a dual mechanism based on a permissive and an inhibitory zone. Radial glia is likely to be instructive in this process.

Key words: cell polarity, chicken retina, cryoculture, neurite outgrowth, radial glia

INTRODUCTION

The orientation of afferent dendrites versus efferent axons of a given neuron is crucial for directed information processing in the adult nervous system. The developmental mechanisms that guarantee the differently oriented extension of different neurites of one and the same cell remain elusive. The cell pole from where neurites emerge might be predetermined by the direction of the cell axis, as has been suggested for outgrowing dendrites of pyramidal neurons in the cerebral cortex (Globus and Scheibel, 1967) and, specifically, for somatosensory neurons (Valverde et al., 1989). Cell intrinsic regulation of initial axon formation is also likely to take place in retinal ganglion cells, which extend their axons perpendicular to the axis of mitosis in the neuroepithelium (Hinds and Hinds, 1974). Subsequently, however, extrinsic factors appear to affect further neurite extension.

A regulatory influence of astroglia on neuronal differentiation has been documented in several in vitro systems. Diffusible factors, derived from astroglia originating from the neural target area, enhance axonal outgrowth of hippocampal neurons (Qian et al., 1992). Similarly, striatal glia induces axon-like neurites in mesencephalic neuronal cultures (Rousselet et al., 1988). In contrast, dendrite-like neurites are initiated in mesencephalic neurons by mesencephalic astroglia (Autillo-Touati et al., 1988). Although the molecular components active in neuron-glia interactions have not yet been identified, some glycosaminoglycans obviously mimic distinct differentiation effects. In mesencephalic cultures, dermatan sulfate enhances dendritogenesis preferentially, whereas chondroitin sulfate and heparan sulfate support axonal outgrowth (Lafont et al., 1992). Various extracellular matrix molecules including laminin and cytotactin/tenascin/J1 facilitate or inhibit extension of different neurite types (Snow et al., 1991; Lafont et al., 1992; Snow and Letourneau, 1992; Oohira et al., 1991; Chiquet, 1989; Edelman, 1992; Lochter and Schachner, 1993). The spatiotemporal regulation of the expression of these extracellular components may define permissive and inhibitory microenvironments. Although controversial data have been presented, matrix molecules might function as guiding cues in a concerted action.

The well characterized and relatively simple architecture of the chicken retina makes it an ideal system for developmental studies concerning cell polarity and directed neurite extension. There are several neuronal cell types but only one nonneuronal cell, radial Müller glia (Ramon Y Cajal, 1933). Blood vessels with endothelial cells and pericytes as well as other glia cell types are absent from the chicken retina. Ganglion cells are the only axon bearing projection neurons of the retina. Their axons are positioned in the innermost tissue layer (optic fiber layer, OFL), the corresponding dendrites are extended exclusively in the opposite direction into the inner plexiform layer (IPL) (Ramon Y Cajal, 1933). We hypothesize that axons and dendrites of a neuron are likely to have different guidance cues. Consequently, a given microenvironment could be nonpermissive for axons but permissive for dendrites.

During early stages of development the neural retina represents a neuroepithelium with a proliferative zone at the ventricular outer tissue surface (Prada et al., 1981; Hinds and Hinds, 1974; Spence and Robson, 1989; Prada et al., 1991). Initially, cells are oriented radially with the cell body migrating along the width of the epithelium. At later stages of development, when the retina tissue thickens, cell cycle dependent migration is confined to outer retina layers. Leaving the cell cycle, postmitotic ganglion cells extend a process towards the inner retina surface where a growth cone forms at the leading edge of the presumptive axon. Subsequently the juvenile axon grows along the endfeet of neighboring neuroepithelial cells towards the optic fissure. Axonal extension remains restricted to the innermost retina layer.

Retinal cells are generated in a columnar fashion, as deduced from lineage studies using fluorescent tracer and retrovirus mediated gene transfer techniques (Wetts and Fraser, 1988; Turner and Cepko, 1987). Centrally positioned cells within individual columns of the neuroepithelium appear to function as a guiding scaffold for radially migrating siblings and as master progenitor cells for neurons and glia (Prada et al., 1989; Meller and Tetzlaff, 1976). Very similiar features are evident in the optic tectum of the chicken (Gray and Sanes, 1992). In the retina the principal morphology of radially oriented cells is preserved even in adulthood in the form of radial Müller glia cells (Ramon Y Cajal, 1933). This has led several authors to call radial cells, even in early retinal histogenesis, Müller glia cells (Meller and Tetzlaff, 1976; Bhattacharjee and Sanyal, 1975). For simplicity we use the term 'radial glia' as an operational term describing all cells being radially oriented with endfeet at the innermost retina surface independent of the developmental stage.

Using seven different in vitro and in vivo approaches, we have analysed guiding cues for ganglion cell axons within the retina. By dissecting radial glia with the aid of a specific purification method, it became feasible to analyse selectively the surface properties of glial endfeet. Endfeet located in the innermost retina layer provided an superb growth substratum, whereas outer tissue layers were inhibitory. Our observations suggest that ganglion cell axons are guided by a dual mechanism partially based on radial glia.

MATERIALS AND METHODS

Materials

Retinae were dissected from White Leghorn chickens. All chemicals used were from Sigma, including trypsin (T-8253) and laminin (L-2020), unless stated otherwise as follows. Poly-D-lysine, fetal calf serum and chicken serum (Boehringer); DMEM and rabbit serum (Gibco); monoclonal antibodies: mAb C4 (Schlosshauer and Dütting, 1991), mAb D1 and D3 (Schlosshauer, 1989), mAb 1W5 (Schlosshauer and Herzog, 1990), mAb 2A1 (Schlosshauer et al., 1990), mAb 2A10 (Schlosshauer et al., 1993), mAb 2M6 (Schlosshauer et al., 1991); antibodies specific for UV-labile laminin epitopes were from Sigma (L-9393). Secondary antibodies: DTAF and TRITC-conjugated goat anti-mouse IgG and M and goat anti-rabbit IgG (Jackson Immuno Research).

Optic nerve transection

For quantitative elimination of retinal ganglion cells and their axons, the presumptive optic nerve formed by ganglion cell axons was transected in ovo at embryonic day (E) 4 as outlined by Schlosshauer et al. (1990). Transected specimens were kindly provided by D. Dütting, Tübingen.

Cryocultures

For cryocultures, isolated chicken retinae of different developmental stages (E4,5 to postnatal day (P) 10) and with or without prior optic nerve transection were frozen without prior fixation and cryosectioned without using OCT compound. In some experiments neural retinae were dissected out of the eye ball, flat mounted onto an adhesive nitrocellulose filter with the innermost tissue layer facing upwards, and cryosectioned as above. Cryocultures from chicken embryos younger than E4.5 were not possible due to rapid deterioration of the tissue in vitro. Sections were attached to dry, poly-lysine coated glass coverslips (500 μg/ml, 1 hour, 37˚C), air dried (15 minutes) and washed several times (14 hours, 4˚C) in HBSS. Pretreatment of cryosections was performed before washing as follows: 70˚C for 10-60 minutes; incubation with laminin (50-500 μg/ml, 14 hours, 4˚C) or methanol (15 minutes, −20˚C) or 0.1 N HCl or 0.01 N NaOH or 1% Triton X-100 or 0.005% trypsin (15-30 minutes, 22˚C). Endogenous laminin was inactivated by exposing dry cryosections or purified glial endfeet (see below) to UV light (254 nm, 30 minutes; Hammarback et al., 1985). After additional washing, retina explants were placed onto these cryosections. Binding of laminin to cryosections was verified by indirect immunofluorescence using a laminin-specific antibody. The antibody recognizes a UV-labile epitope and therefore could be used to monitor UV inactivation of laminin. Cryosections devoid of glial endfeet were prepared by physical detachment of endfeet (see below) before cryosectioning. Cryocultures were established by placing 300 μm wide viable retina tissue strips (E6), attached to nitrocellulose filters (Bonhoeffer and Huf, 1980), onto retinal cryosections for 48 hours, at 37˚C, in 5% CO_2. Culture medium (Walter et al., 1987b) contained 1.2% serum but no methylcellulose.

Alternative substrata

Retinal tissue strips were also explanted onto patterned laminin substrata, microstructured silicon, isolated glial endfeet and viable tissue layers of the outer retina. Patterned laminin substrata consisting of alternating stripes of native and denaturated laminin were prepared in the following way. Glass coverslips were homogenously coated with laminin (50 μg/ml, 14 hours, 4˚C), covered with a non-translucent mask (PVC foil, stripe width approximatly 200 μm) and illuminated with UV light (see above). Microstructured silicon chips (kindly provided by Mr Bach, IMIT, Villingen-Schwenningen, FRG) had been prepared by photolitography in conjunction with a reactive ion- or an anisotropic wet etching procedure to gain three-dimensional structures of 10 μm height. In order to reduce the hydrophobicity, the material surface was covered with silicon oxide. Anisotropic wet etching resulted in the inclination of 54.7˚ at the long sides and 50-90˚ at the narrow sides (Seidel et al., 1995). For explant cultures, microstructured silicon chips were plasma etched (3 minutes oxygen/argon) and homogenously coated with poly-lysine (100 μg/ml, 1 hour, 37˚C) and laminin (50 μg/ml, 14 hours, 4˚C).

Isolation of glial endfeet

Isolation of glial endfeet from different developmental stages (E4-P10) was based on the method of Halfter et al. (1987). Embryonic retinae were flat mounted onto adhesive nitrocellulose filters with the endfeet facing upwards. A poly-lysine coated glass coverslip was carefully pressed onto the retinal tissue surface, left for 10 minutes at 37˚C and 5% CO_2 and removed again to tear off glial endfeet. The absence of axonal fragments in the resulting preparation was monitored by the axon specific mAb C4. UV irradiation of glial endfeet was performed as described above. The detachment procedure was also employed to generate tissue sections devoid of glial endfeet for cryocultures. Viable tissue layers of the outer retina were prepared by three successive detachment steps (once with poly-lysine-coated glass coverslips and subsequently twice with nitrocellulose filters; Sartorius 13006-50-ACN, 0.45 μm) resulting in retinae without the OFL and the ganglion cell layer (GCL). The detachment process was controlled by toluidine blue staining of cross sections of 'delayered' tissue.

Visualisation of axonal growth

Visualisation of outgrowing ganglion cell axons was performed either by prelabeling retina tissue with the fluorescent dye rhodamine isothiocyanate (Bonhoeffer and Huf, 1980) or by postlabeling paraformaldehyde-fixed cultures on Müller glial endfeet after 24 hours or on cryosections after 48 hours in vitro, employing the axon-specific monoclonal antibody mAb 2A1 or the neuronal marker mAb 2A10. Axonal outgrowth was quantified by counting individual axons in scanning electron micrographs in experiments using preparations of glial endfeet. Axon length was given by the distance between the axon tip and the explant strip; axon number was determined by counting all axons crossing an imaginary line at a distance of 500 μm parallel to the explant strip. For scanning electron microscopy, specimens were glutaraldehyde fixed, dehydrated and sputtered with gold. Oriented axon outgrowth on cryosections was evaluated on a semiquantitative level by reference pictures according to the method of Walter et al. (1987b). Reference pictures corresponded to values ranging from 1 to 3 as depicted in Fig. 8.

RESULTS

To be able to analyse guiding cues in the retina during axonal outgrowth it was essential to devise a paradigm that allowed, (a) access of ganglion cell axons to all retinal layers, (b) experimental manipulation of axon growth and (c) direct visual inspection of neurite extension. So called cryocultures of the chicken retina were found to be a powerful technique meeting these demands. Embryonic retinae were frozen without fixation, cryosectioned perpendicular to the tissue layers, and mounted onto poly-lysine coated glass coverslips. On top of these sections, viable retina strips were explanted and the axonal outgrowth on the different tissue layers of cryosections was monitored after 2 days in culture (Fig. 1A). Explanting of chicken retina strips leads to a pronounced and specific outgrowth of ganglion cell axons (Halfter et al., 1983). Outgrowing axons were visualized by fluorescent labeling, using either a vital stain (rhodamine isothiocyanate) or an axon specific monoclonal antibody (mAb 2A1). Axons present in retinal cryosections remained essentially unstained, presumably due to partial denaturation of the corresponding antigen (Schlosshauer et al., 1990) during freezing of the nonfixed tissue.

Inner retina layer permissive for outgrowing axons

Axons originating from retina explants grew either randomly on the poly-lysine-coated glass surface or in an oriented fashion on the cryosection. On cryosections, outgrowing axons revealed a clear preference for the innermost retina layer (Fig. 1B). Axonal growth along the filter used as a mechanical support, or the pigment epithelium at the outer edge of cryosections was limited. The specific growth along the inner layer could be observed even in those cases where section segments

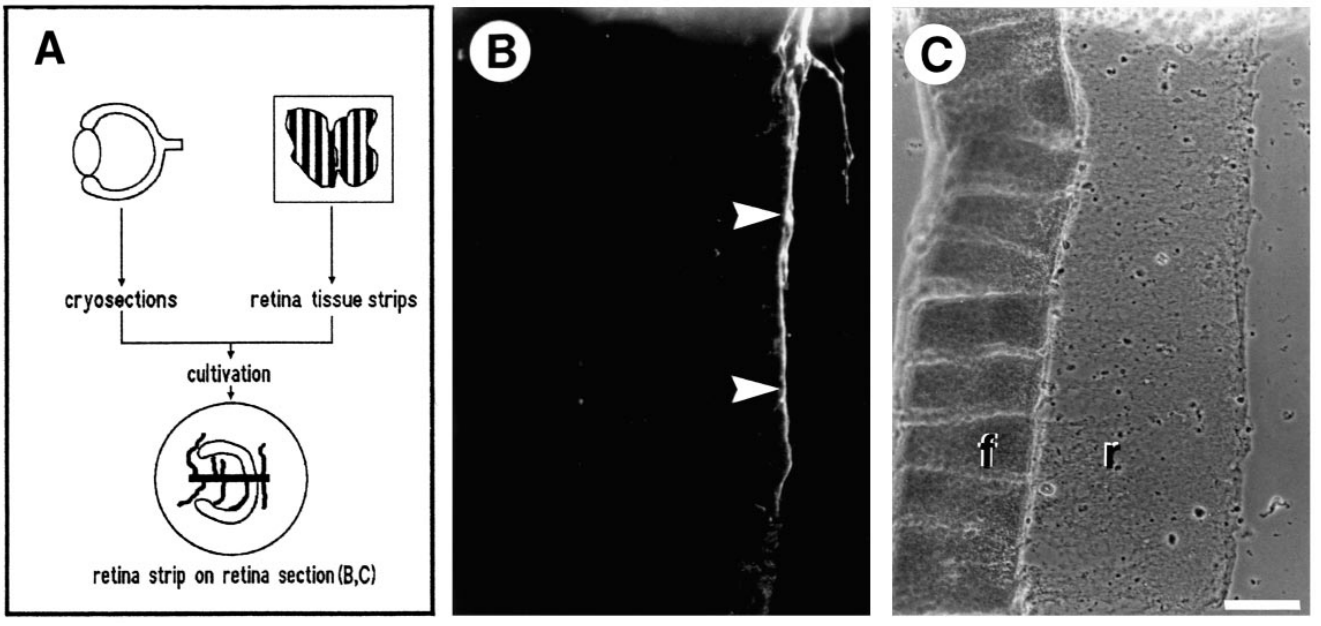

Fig. 1. The inner retina is permissive for axonal outgrowth. (A) Schematic presentation of the experimental layout. Cryostat sections of frozen retinae of chicken embryos were used as substrata for outgrowing axons from retina explant strips. (B) Immunofluorescence staining of outgrowing axons (white fibers) on a retinal cryosection. Axons originated from explant tissue visible at the top of the images. (C) Corresponding phase contrast image. Axons grew preferentially on the innermost retina layer (arrowheads in B) and avoid outer retina layers apposed to the filter support. f, mechanical filter support; r, retinal cryosection. Bar, 70 μm.

had been removed by prior thorough washing, leaving only an imprint of the original cryosection on the glass coverslip. In these cases phase contrast images did not reveal any tissue remnants, whereas immunostaining with MAb 2A10 indicated retinal antigens bound to the glass surface (data not shown). Most notable is the fact that hardly any axons innervated outer retina layers.

These results indicate that the cryoculture system reflects the in vivo situation with regard to the specific growth of ganglion cell axons along the inner retina surface, which represents a permissive tissue zone.

Mechanical guidance is not effective

Although axons appeared to grow actually on the innermost retina layer, it could not be completely excluded that they might have been guided mechanically along the edges of cryosections, representing potentially a physical barrier. In order to investigate the impact of such a stereotropism, retinal tissue was explanted onto microstructured silicon chips covered with oxide, which were made permissive by coating with laminin (Fig. 2A). The U-shaped microstructures (Fig. 2B) had a defined height similar to that of cryosections (10 μm). The inclination was 54.7˚ at the long sides and 50˚-90˚ at the narrow sides. Neither edge represented a true barrier or a guiding cue for the vast majority of ganglion cell axons (Fig. 2C). Therefore, it appeared unlikely that outgrowing axons were oriented in cryocultures simply by mechanical means.

Laminin as a guiding molecule?

Since axons of retinal explants were preferentially extended on the innermost retina layer, which included the laminin rich inner limiting membrane, it was possible that laminin holds the key for oriented outgrowth. In a perturbation experiment laminin was inactivated by UV irradiation of cryosection (Fig. 3A). The effectiveness of this treatment was verified in a specific stripe assay. Glass coverslips were homogenously coated with laminin and covered with a mask made up of approximately 200 μm wide plastic stripes. Subsequent UV irradiation resulted in inactivation of every other strip of laminin that had not been shielded by the nontranslucent plastic. Inactivation was evaluated with the aid of antibodies specific for UV labile epitopes of laminin. Indirect immunolabeling, using a fluorescently labeled secondary antibody, revealed that the procedure did indeed yield alternating active (light) and inactive (dark) laminin stripes (Fig. 3D). When outgrowing axons from retina explants were exposed to the patterned laminin substratum, inactive laminin was nearly completely avoided (Fig. 3C).

Having demonstrated the validity of the procedure, laminin in cryosections was inactivated by UV irradiation under the same conditions and the sections subsequently used for cryocultures. Surprisingly, the specificity of axonal outgrowth on the innermost retina layer was not compromised by irradiation (Fig. 3B), suggesting that laminin did not provide the directing cue in our culture system.

Guidance by axonal fasciculation?

The inner retina is formed by the axon rich OFL, which is separated from the nonneuronal vitreous body by endfeet of (immature) radial Müller glia cells. As indicated above, laminin produced by radial cells did not affect directed axon extension in vitro. Alternatively, axons originating from retina explants could possibly fasciculate with ganglion cell axons present in cryosections. This could result in the distinct growth pattern observed in cryocultures.

In order to analyse in more detail which cell interactions guide axons in the permissive zone, ganglion cell axons present in the tissue section were eliminated prior to culturing (Fig.

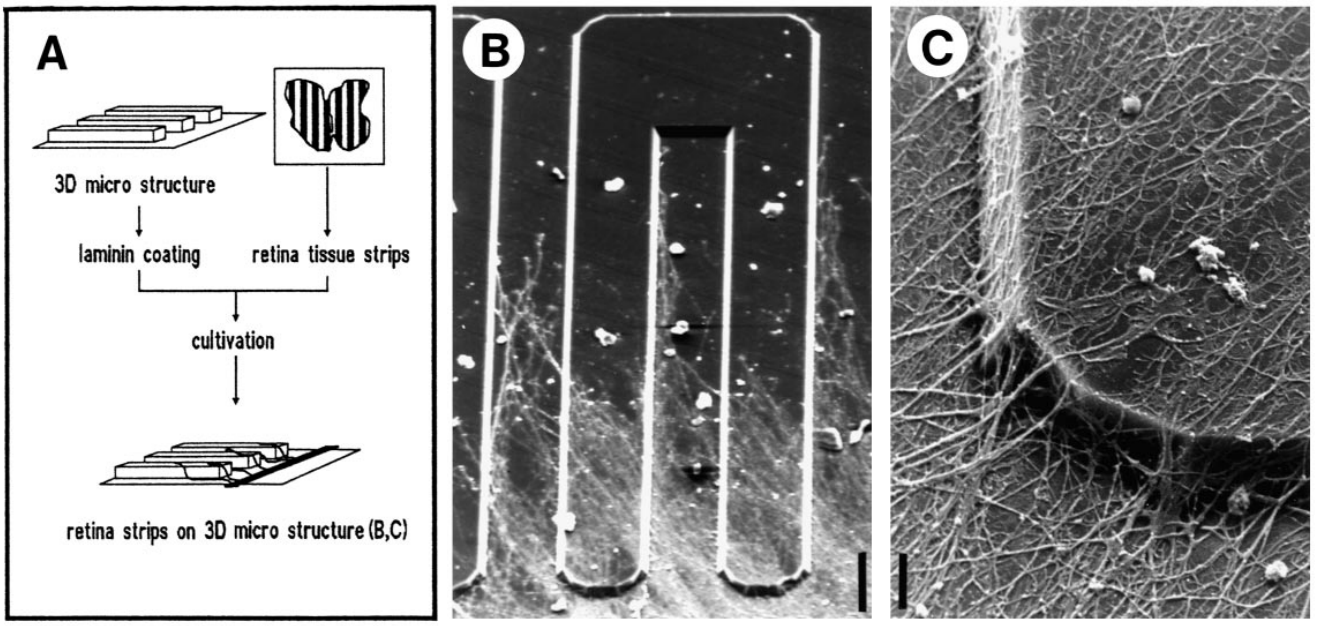

Fig. 2. Axonal outgrowth on microstructured silicon chips. (A) Schematic presentation of the experimental layout. Retinal tissue strips were explanted onto 3-dimensionally structured, laminin-coated silicon chips in order to analyse mechanical guidance. (B,C) Scanning electron microscopic images. (B) Overview of the U-shaped silicon structure. Note outgrowing axons (faint white fibers) at the bottom of the image. (C) Axons essentially ignore the physical barrier formed by the 10 μm high microstructure. Bars, 75 μm (B), 10 μm (C).

4A). To do this, presumptive optic nerves of day 4 chicken embryos were transected in vivo through a small window cut in the egg shell. The operation resulted in degeneration of ganglion cells and their axons and consequently the OFL disappeared. Ganglion cell axons in tissue sections were identified by staining with monoclonal antibody C4, specific for a NgCAM/G4-like protein in control specimens (Fig. 4C). In retinae of nerve-transected embryos, C4 immunoreactivity was absent (Fig. 4D). Correspondingly, phase contrast images revealed retinae without OFL and GCL (compare Fig. 4E,F).

Cryosections made from specimens without OFL provided an equally appropriate substratum in culture: axons from retina explants grew on the innermost retina layer, although the sections used were devoid of ganglion cell axons (Fig. 4B). Therefore, fasciculation between outgrowing explant axons and those normally present in retinal cryosections did not play a major role as a guiding cue.

Permissive zone due to endfeet of radial cells

In the OFL only distal radial glia processes and ganglion cell axons are found. Other cellular elements such as blood vessels are absent in the chicken retina. Since ganglion cell axons did not affect directed outgrowth, radial glia cell processes were expected to be responsible for the permissive character of the inner retina layer.

Two different approaches were taken to elucidate the potential relevance of glial endfeet for axonal guidance. In the first set of experiments retinae were flat mounted and the glial endfeet-forming inner limiting membrane were detached by carefully pressing an adhesive glass coverslip onto the uppermost retina layer. Retinae devoid of glial endfeet were processed for cryocultures in an identical way to untreated specimens (Fig. 5A). Fig. 5B,C show a sandwich of cryosections of a manipulated retina without glial endfeet (top) and one with endfeet (bottom). The presence or absence of the inner limiting membrane was revealed by specific immunolabeling (Fig. 5C). Axons growing out from retina explants displayed a strict preference for the specimen containing glial endfeet, whereas the retina without endfeet was avoided by axons. This type of growth specificity was evident in all samples investigated (n=40).

In the second set of experiments the substratum properties of radial glial endfeet were investigated directly. Glial endfeet were isolated by the above detachment procedure and used as a substratum in explant cultures (Fig. 5D). The isolation procedure yielded a membranous sheet formed by

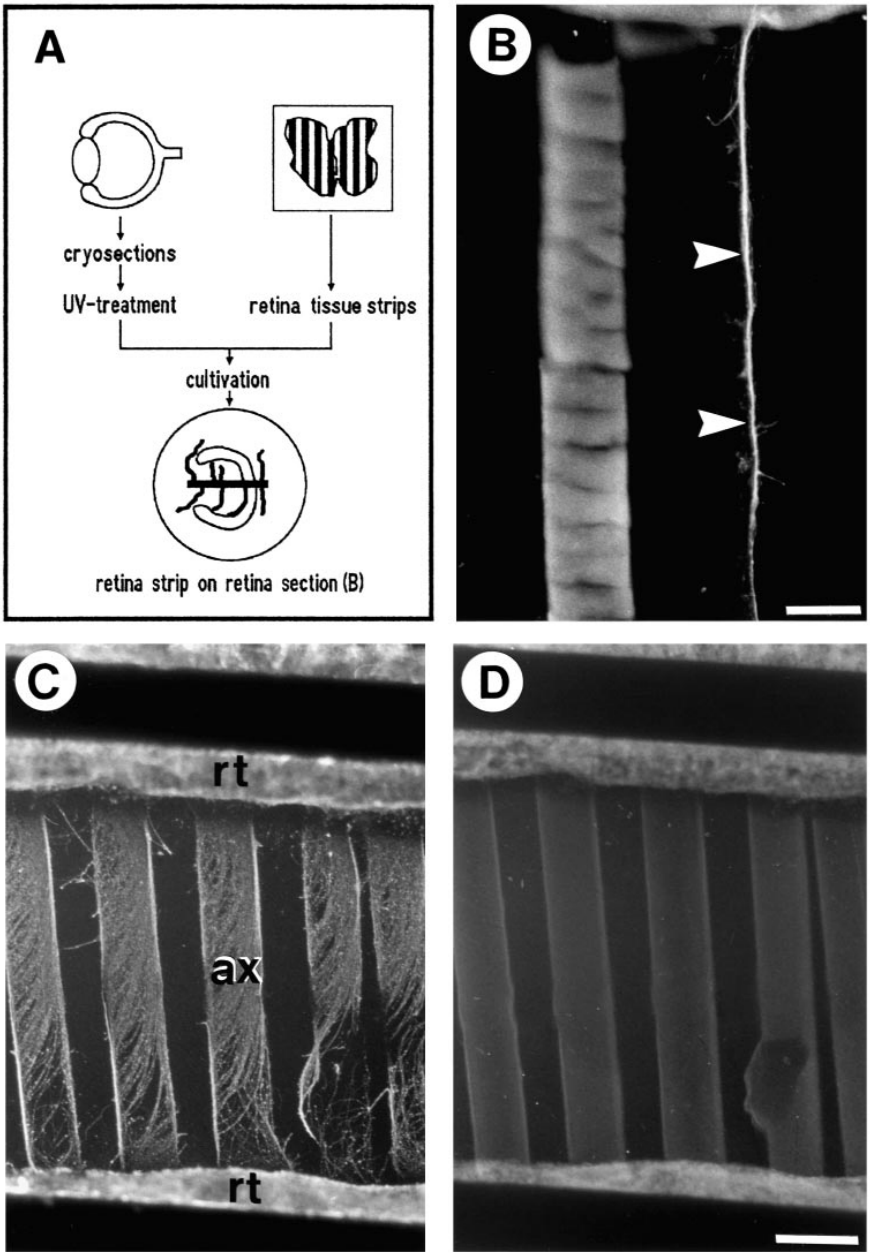

Fig. 3. Laminin and axonal guidance. (A) Schematic presentation of the experimental layout. (A) Retinal cryosections were UV-irradiated to inactivate endogenous laminin and subsequently used in cyrocultures. (B-D) Immunofluorescence images. (B) Retinal axons (white fibers) growing on irradiated cryosection. Despite the absence of active laminin, outgrowing axons remained restricted essentially to the innermost retina layer (arrowheads). On the left hand side the nitrocellulose filter used as mechanical support appears as a broad white lane. (C,D) UV inactivation of laminin was validated in a stripe assay. Homogenously coated glass coverslips were exposed to UV light using a mask of nontranslucent PVC stripes. Retinal explants were positioned perpendicular to laminin stripes and axonal outgrowth was monitored by immunolabeling. (C) Axons grew nearly exclusively on nonirradiated laminin. (D) Nonirradiated laminin (light stripes) was visualized by antibodies specific for a UV labile epitope of laminin. Consequently, inactivated laminin stripes appeared dark. Horizonal dark lanes at the top and bottom are filter supports necessary to handle the explant tissue. ax, outgrowing axons; rt, retinal tissue. Bars, 120 μm (B), 300 μm (C and D).

interconnected vesicles whose identity was revealed by the radial glia specific monoclonal antibody 2M6 (Fig. 5E). Control staining with the monoclonal antibody C4, specific for axons, indicated that the endfeet preparation was completely free of axon fragments that could have been a potential contamination.

Subsequently, we evaluated whether the endfeet preparation represented a permissive substratum under the in vitro conditions used in cryocultures by explanting retina strips on such preparations. The axonal outgrowth from standard explants (E6) on glial endfeet was very pronounced (Fig. 5F, Table 1). These results confirmed that glial endfeet were indeed an excellent substratum for neurite extension. Intrestingly, prior UV irradiation of isolated endfeet did not affect the superb substratum properties, suggesting that also in this culture system laminin was not a crucial component for axonal elongation (data not shown).

In summary, compelling evidence indicated that axons oriented themselves along glial endfeet rather than along axons, laminin or physical barriers.

Developmental regulation

Glial endfeet sheets were isolated from retinae of different developmental stages (E4, E7, E12, P10) and used as substrata in explant cultures. Quantitative evaluation of axonal outgrowth by scanning electron microscopy revealed that the number and length of outgrowing axons was essentially independent of the developmental age of glial endfeet (Table 1). On average >840 axons per explant unit, with a length of 870 μm, were counted (10 randomly selected explant areas in 3 individual cultures per developmental stage).

In a similar way retinal cryosections from chick embryos of the same developmental interval were tested qualitatively for their specificity in guiding explant axons into the innermost

Table 1. Growth promoting potential of endfeed during development

EF age	Average (μm)	Maximum (μm)	Number of axons
E4	n.d.	n.d.	916±19
E7	920±47	1079±113	917±366
E12	872±111	1071±147	674±16
P10	810±138	1048±139	872±305

Retinal explants were cultured on glial endfeet isolated as homogenous sheet (inner limiting membrane, ILM) from the developmental stages E4, E7, E12 and P10. Axonal outgrowth was quantified by counting the number and the length of axons in scanning electron micrographs from different explant cultures (N=6 per age). Axon length on E4 speciment could not be evaluated, because the dimensions of explant and outgowing axons exceeded the dimensions of the small ILM. Axon length is depicted both as average value and as value of the longest axons observed. Glial endfeet from all developmental stages provided excellent growth substrates. The differences in axon lengths and numbers are not statistically significantly)Welch test: P=0.1-0.9, with the exception of axon numbers E4/E12 P=0.0002).

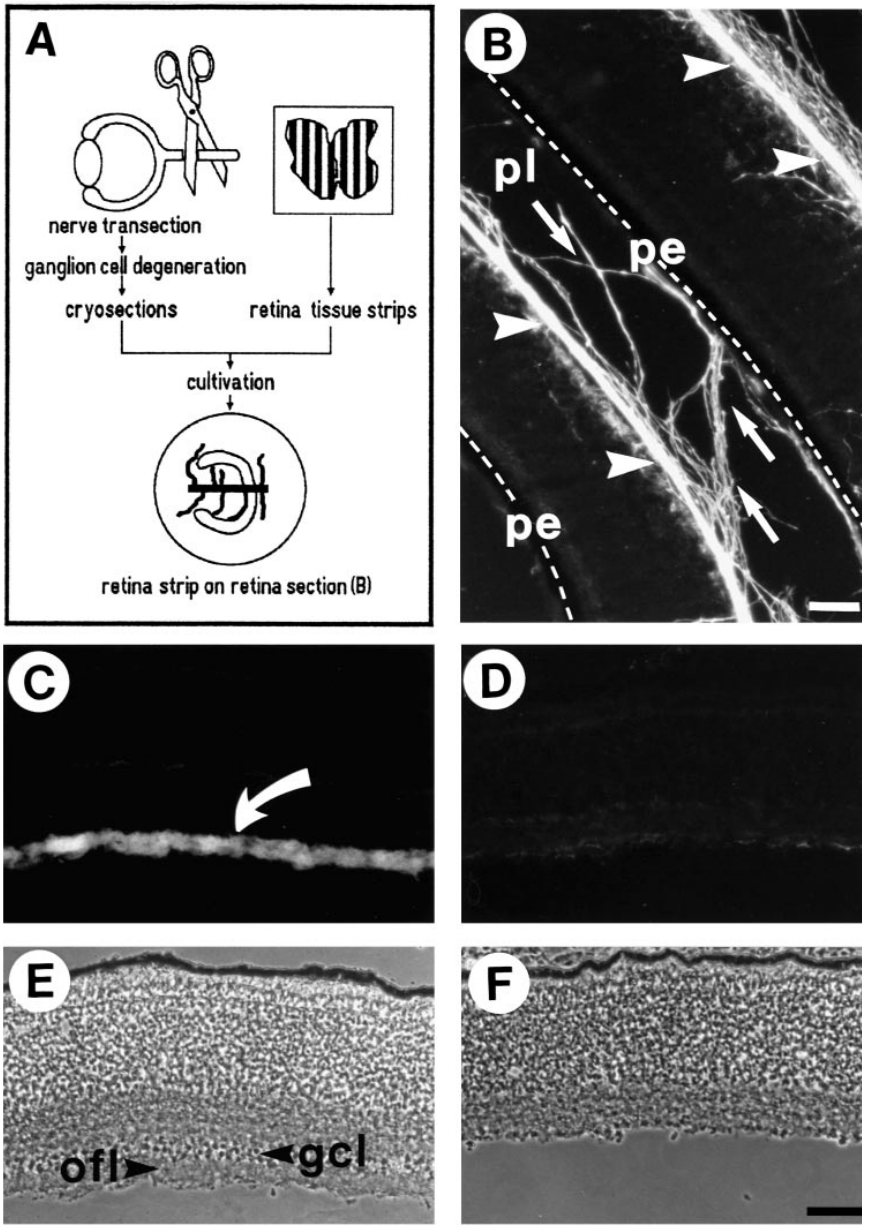

Fig. 4. No guidance by axonal fasciculation. (A) Schematic presentation of the experimental layout. Cryosections of frozen retinae of optic nerve-transected chicken embryos were used as substrata for outgrowing retinal axons. (B) Immunofluorescence staining of outgrowing axons on two neighboring cryosection of embryonic retinae after optic nerve transection. For orientation the boundary of the pigment epithelium is indicated by dashed lines. Axons grew on the poly-lysine coated glass surface (arrows) and preferentially on the innermost retina layer (arrowheads) despite the absence of ganglion cell axons in the retinal tissue section. (C) Control section stained with mAb C4 to reveal endogenous ganglion cell axons (arrow). (D) After optic nerve transection in vivo, retrograde degeneration leads to the loss of the ganglion cell layer, the optic fiber layer and consequently C4 immunoreactivity. (E,F) Corresponding phase contrast images for C and D, respectively. gcl, ganglion cell layer; ofl, optic fiber layer; pe, pigment epithelium; pl, poly-lysine-coated glass surface. Bars, 50 μm (for B-F).

retina layer. Also in this case the guiding was found to be active at all stages investigated. Therefore, both test systems showed temporally independent characteristics.

Outer retina layers are nonpermissive for outgrowing axons

In principle, the oriented growth of ganglion cell axons along the innermost retina layer could be caused by an opposing microenvironment in different tissue areas: a permissive/inductive and a nonpermissive/inhibitory one.

Axons, which navigated initially on the poly-lysine coated glass surface, typically continued to grow specifically on the inner retina layer when contacting the cryosection. This suggested that the inner retina layer provided a permissive substratum for axons.

However, this interpretation did not necessarily explain why

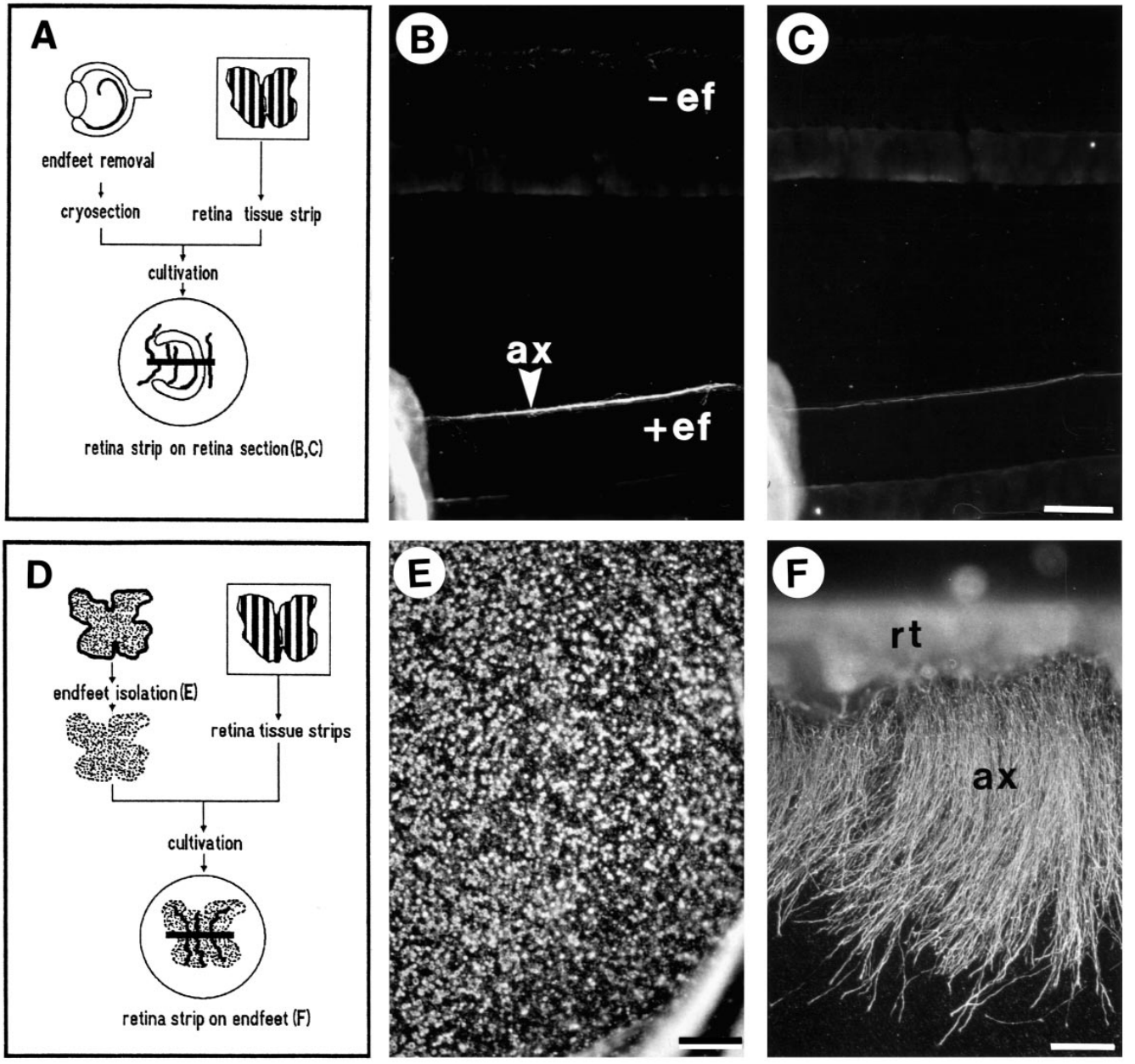

Fig. 5. The permissive retinal zone is due to endfeet of radial glia. (A,D) Schematic presentations of the experimental layout. (B,C,E,F) Immunofluorescence images. (A) Retinae were flat mounted, the inner limiting membrane (glial endfeet plus ECM molecules like laminin) mechanically detached or left intact and processed for cryocultures. (B,C) Double immunolabeling of axons and laminin of a sandwich of cryosections with and without glial endfeet. (B) Axonal outgrowth from retina explants was restricted to retinal cryosections with endfeet (bottom). On sections devoid of endfeet, axon growth was completely abolished (top). (C) The presence or absence of endfeet was indirectly demonstrated by laminin staining. (D) Isolated endfeet were used as the substratum in retinal explant cultures in order to evaluate directly their growth promoting potential. (E) The glial endfeet preparation isolated as sheet-like structure is composed of small vesicles which were identified with the antibody 2M6, specific for radial glia cells. (F) Axons originating from retina explants show pronounced outgrowth on glial endfeet. ax, outgrowing axons; –ef and +ef, without and with glial endfeet; rt, retinal explant tissue. Bars, 175 μm (B,C); 30 μm (E); 135 μm (F).

outer retina layers were avoided by ganglion cell axons. Therefore, we investigated the substratum properties of outer retina layers in more detail. Nonpermissiveness could have been due to the absence of an appropriate substratum to which neurites could attach. Consequently, we overlaid retinal cryosections with laminin (Fig. 6A). Binding of laminin to cryosections was monitored using a laminin specific antibody (Fig. 6B). Even in the presence of laminin, innervation of outer retina layers was very sparse, revealing no obvious difference in comparison to untreated control specimens (Fig. 6C).

A potential artefact of cryocultures could arise from cytoplasmic components released from broken cells of cryosections. These components, though possibly of no guiding relevance, could be functionally inhibitory and obscure the

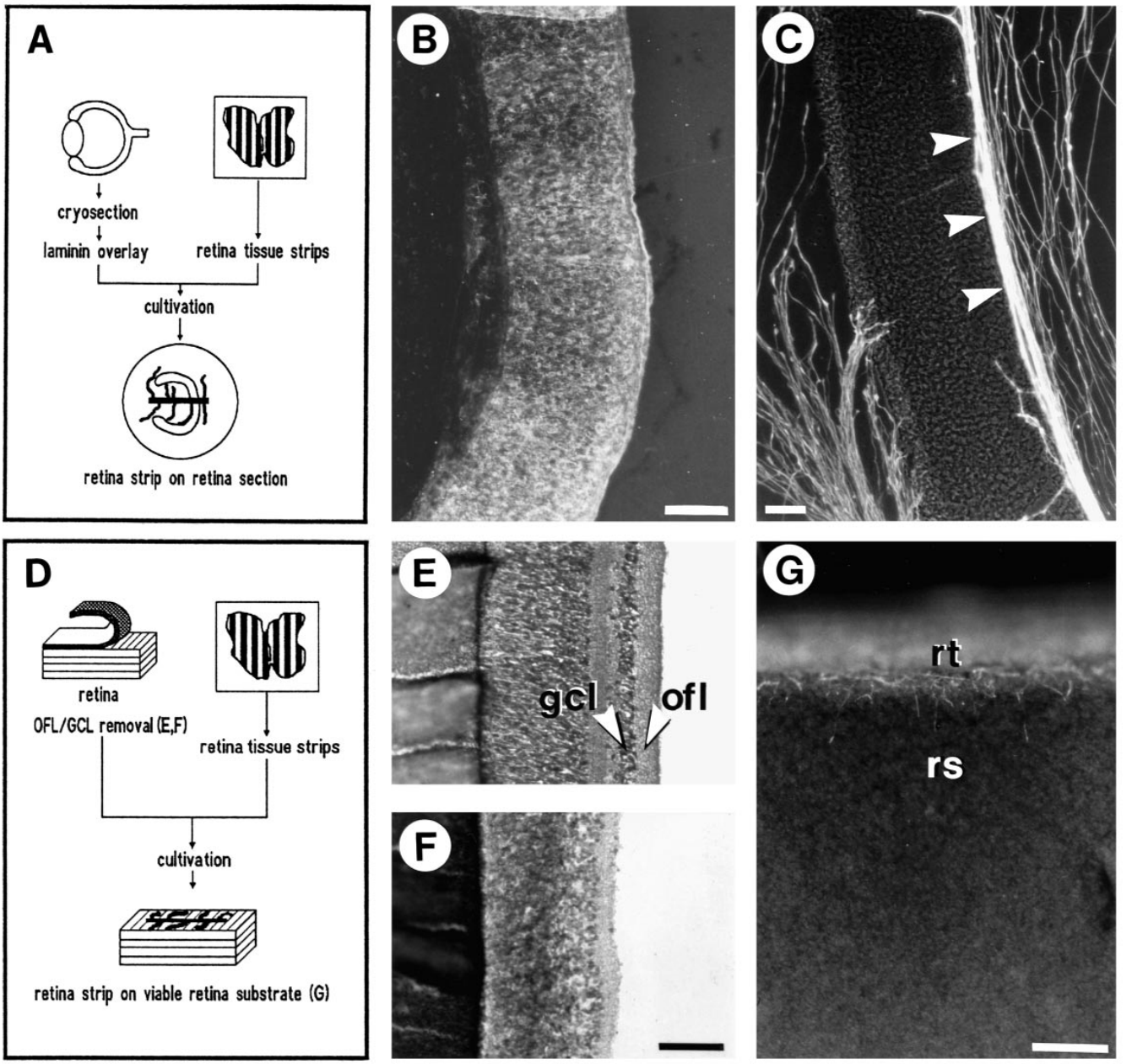

Fig. 6. Inhibitory outer retina layers. (A,D) Schematic drawings of the experimental layout. (A) Retinal cryosections were coated with laminin and thereafter used as substrata for retinal explant cultures. (B) Immunofluorescence image of exogenous laminin applied to retinal cryosections. (C) Fluorescence-phase contrast double image of immunolabeled axons growing preferentially along the innermost retina layer (arrowheads) and on the coated glass surface. Despite laminin also being present on the outer retina layers, only a minority of axons are extended in this tissue region. (D) In order to evaluate whether axonal inhibition also occurs on viable cells of the outer retina, retinae were flat mounted, the OFL and GCL mechanically detached and the resulting tissue used as substratum for retinal explant cultures. (E,F) Toluidine blue stained retina sections of control (E) and experimental specimens devoid of OFL and GCL (F). Both layers are marked by arrowheads in E. (G) Retinal tissue strip explanted onto a flat mounted retina devoid of OFL and GCL. Viable tissue of the outer retina was essentially inhibitory – axonal outgrowth (white fibers) from the explant was very sparse. rs, viable retina substratum devoid of OFL and GCL; rt, retinal tissue strip. Bars, 100 μm (B); 50 μm (C); 75 μm (D,F); 150 μm (G).

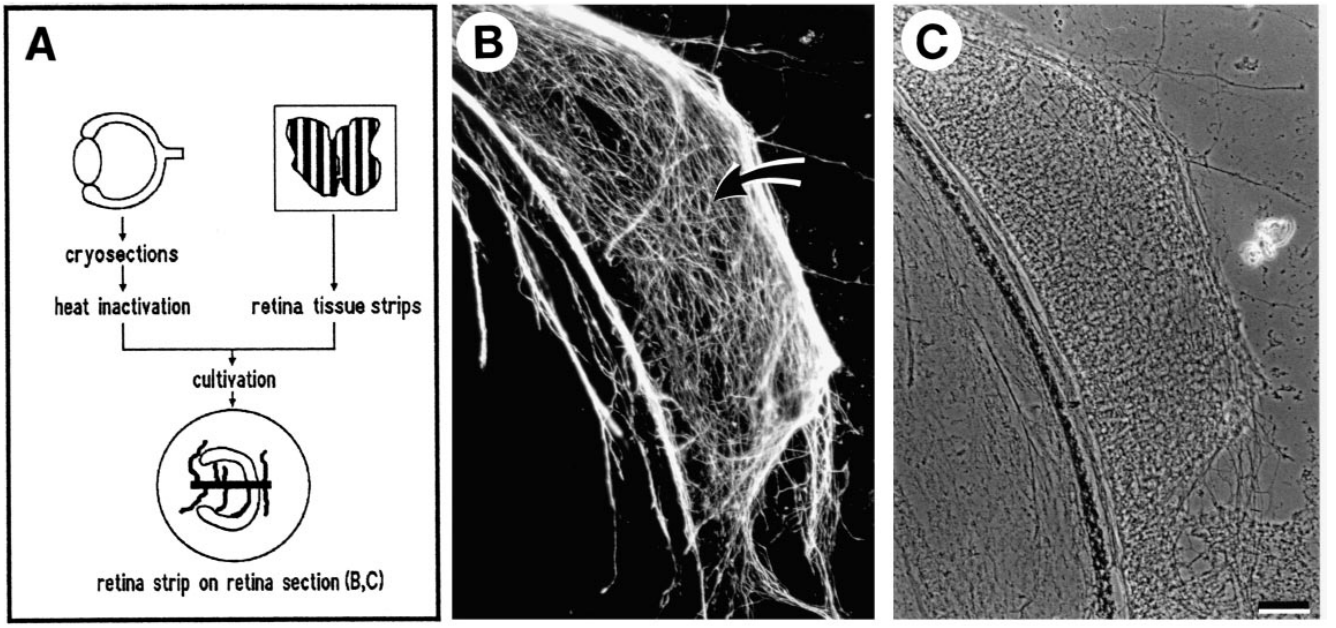

Fig. 7. Inactivation of inhibitory outer retina layers. (A) Schematic presentation of the experimental layout. Cryosections of frozen retinae were heat treated (70˚C) and used as substrata for outgrowing axons from explant cultures. (B) Immunofluorescence image. Heat treatment of cryosections inactivated inhibitory activity resulting in significant axonal outgrowth on inner and outer retina layers (arrow). (C) Corresponding phase contrast image. Bar, 50 μm.

actual guidance mechanism. To circumvent these potential problems, axonal outgrowth was also monitored on viable cells of the outer retina (Fig. 6D).

With the aid of an iterative detachment procedure, ganglion cells and their axons were eliminated from flat mounted retinae. Cross sections of such specimen revealed that the detachment of individual tissue layers, starting from the innermost retina surface, resulted in retinae devoid of OFL and GCL (compare Fig. 6E,F). Consequently, the cells of the outer retina layers became exposed and could be directly employed as a substratum. Retinae of nerve-transected embryos were inappropriate for this approach, because of glial endfeet covering the surface of the IPL. When retina explants were cultured directly on outer retina layers, axonal outgrowth was found to be essentially inhibited. Only a small number of short axons were extended (Fig. 6G; compare with Fig. 5F). Therefore, the inhibitory activity of outer retina layers turned out to be an inherent feature of cells independent of their cellular integrity.

In a third approach we postulated a possible inhibitory factor present in outer retina layers. One approach widely applied to demonstrate inhibitory activity is to inactivate the inhibition and consequently reverse an initial experimental phenomenon (Walter et al., 1987a; Lichtman, 1994; Carpenter et al., 1994; Wang et al., 1994). We employed a number of physical, chemical and biochemical means in order to convert outer retina layers into a permissive substratum. Prior to culturing, cryosections were treated with heat, organic solvent, acid or base, detergent and a protease (Fig. 8). Heat inactivation at 70˚C for 30 minutes clearly reduced the nonpermissive character of outer retina layers, whereas the physical integrity of the tissue remained unaffected as judged from microscopic images (n=9) (Fig. 7B). However, the permissive activity of the innermost retina layer appeared to be only partially affected, because axons still showed a certain preference for this region. A partial inactivation could also be produced by pretreatment of cryosections with trypsin; essentially no effect was observed with 0.1 N HCl, methanol and Triton X-100. Application of 0.01 N NaOH induced complete detachment of the cryosection from the coverslip. Based on camera lucida drawings, reference drawings of cryocultures were made to qualify how the different treatments affected the inhibition of axons on outer retina layers (Fig. 8).

In summary, the findings corroborate the hypothesis that outer retina layers form an inhibitory environment for outgrowing ganglion cell axons.

DISCUSSION

The presented data suggest that guidance of retinal axons could rely on a dual mechanism, possibly based on the formation of an inhibitory and a permissive zone. This concept is supported by experimental observations made in the retinotectal pathway of *Xenopus*, where a similar mechanism has been discussed. Retinal ganglion cell axons grow selectively along permissive endfeet of radial neuroepithelial cells, avoiding other substructures in the optic pathway (Holt, 1989). Only distortion of the developmentally regulated microenvironment by heterochronic transplantation of eye primordia induces aberrant axonal growth (Cornel and Holt, 1992).

Permissive endfeet

Axonal outgrowth in cryocultures was observed along the innermost retina layer, suggesting that endfeet of radial glia provided guiding cues. Eliminating glial endfeet in retinal cryosections prior to culturing completely prevented axonal outgrowth on the innermost tissue layer. Monitoring the axon

Axon growth on treated cryosections

Treatment of cryosection	Endfeet (EF)	Outer layer	Inhibition abolished
Control	yes	no	-
Laminin	yes	no	-
70°C 30'	yes	yes	+
CH_3OH	poor	no	-
HCl	poor	no	-
NaOH	n.d.	n.d.	n.d
Triton	yes	no	-
Trypsin	yes	yes	+/-

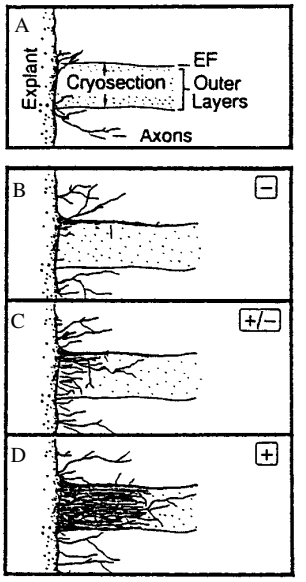

Fig. 8. Axonal outgrowth on pretreated retinal cryosections. Retinal cryosections were pretreated as indicated on the left hand side and described in Materials and Methods, before retinal explants were placed onto the sections. The axonal extension on the innermost glial endfeet-containing layer and on the outer retina layer of cryosections was classified as 'yes' and 'no'. Whether the growth inhibition on outer retina layers was modified by any type of pretreatment was scored according to figures based on camera lucida drawings of different cryocultures. (A) Cartoon to illustrate details of growth patterns. (B-D) Reference drawings to show to what degree the inhibition was abolished. (B) −, not abolished; (C) +/−, partially abolished; (D) +, abolished. Axonal growth on outer retina layers can be induced especially by heat inactivation. Each treatment was repeated at least 5 times. EF, endfeet containing innermost retina layer; n.d., not determinable due to detachment of the cryosection from the glass support.

growth on endfeet preparations of different developmental stages (E4-P10) revealed equally superb substratum characteristics, which is in agreement with results obtained with E8 preparations (Halfter et al., 1987).

An accumulating body of data substantiates the assumption that endfeet of radial glia/neuroepithelial cells are the natural substratum for growing axons. In situ growth cones of ganglion cell axons are specifically situated in close apposition to endfeet of radial glia/neuroepithelial in different vertebrate retinae such as frog (Holt, 1989), chicken (Halfter and Fua, 1987), mouse (Hinds and Hinds, 1974) and monkey (Williams and Rakic, 1985). In addition, it is notable that axons extend exclusively along the endfeet surface facing the retina tissue and not along the one exposed to the vitreous body. Therefore, laminin at the interface between radial endfeet and the nonneural environment of the vitreous body does not represent the natural substratum for axons in vivo (Holt, 1989; Hinds and Hinds, 1974; Williams and Rakic, 1985).

This is in agreement with our perturbation experiments in vitro. Denaturation of laminin by UV-irradiation does not affect the preferential growth of ganglion cell axons on the innermost layer of retinal cryosections. Similarly, denaturation of laminin had no effect on the superb substratum properties of glial endfeet (data not shown). Whether other extracellular matrix molecules deposited at glial endfeet serve as substrata in vitro and in vivo remains to be investigated.

Of the different cell adhesion molecules possibly involved in glia-axon interactions, preliminary data revealed that glial endfeet express only N-CAM in significant amounts, as do radial neuroepithelial cells in the optic stalk (Silver and Ruitshauser, 1984). In contrast, NgCAM/G4/C4 antigen (Rathjen et al., 1987b; Schlosshauer and Dütting, 1991), F11 (Rathjen et al., 1987b), F6/neurofascin (Rathjen et al., 1987a) and axonin-1 (Ruegg et al., 1989) were not detected on glial endfeet (our unpublished observation). N-CAM, also being present on ganglion cell axons (Schlosshauer et al., 1984), could facilitate axonal growth along glial endfeet via a homophilic binding mechanism (Hoffman and Edelman, 1983). The hypothesis of N-CAM being the sole guidance cue, however, is challenged by the observation that it is also expressed in all retina layers not innervated by ganglion cell axons (Daniloff et al., 1986).

Alternative mechanisms

The oriented axon growth in cryocultures could possibly have been based on fasciculation, i.e. explant axons could have been extended along preexisting ganglion cell axons already present in retinal cryosections. This is unlikely, because even in the absence of axons in cryosections due to prior optic nerve transection the growth specificity was preserved. Consistent with this observation is the relatively weak expression of axonal cell adhesion molecules during the period of axonal growth. At E8 only half the amount of NgCAM at E12 was measured in the OFL (Daniloff et al., 1986). Qualitative data suggest that at E6 NgCAM-like immunoreactivity is even lower (Schlosshauer and Dütting, 1991). In agreement with these data is the limited experimental success in inducing axonal growth into incorrect retina layers by blocking NgCAM and the C4 epitope in vitro (Daniloff et al., 1986; Schlosshauer and Dütting, 1991). Therefore, only a minor role can be attributed to fasciculation during oriented axonal extension in the OFL of the retina.

Stereotropism as a guidance mechanism for neurites along physical barriers such as the tissue section in cryocultures is unlikly to play a major role for a number of reasons. (a) Axonal growth was particularly pronounced at the inner side (OFL) and only limited at the outer side of cryosections although both sides must be assumed to represent similar physical constraints. (b) Heat inactivation and protease digestion induced innervation of all retina layers, although tissue sections appeared structurally unaffected. (c) Partial removal of section segments, e.g. by application of detergent, leaving only an imprint of the cryosection segment, preserved axonal specificity for the innermost retina layer despite the absence of any physical barrier. (d) Most notable is that in vitro ganglion cell

axons do not orient themselves on microstructured silicon chips representing similar physical constraints as tissue sections.

Nonpermissive zone

Innervation of outer retina layers by ganglion cell axons could not be induced by coating cryosections with laminin. In addition, axonal outgrowth was essentially inhibited on freshly isolated tissue layers of the outer retina. Heat and protease treatment of cryosections, however, lead to aberrant axonal growth in otherwise 'forbidden' zones. These results suggested an inhibitory activity – a guidance mechanism that has been recognized only recently (for review see Walter et al., 1990; Patterson, 1988). The alternative interpretation, that heat treatment demasked a permissive constituent in outer retina layers, instead of inactivating an inhibitory component, is challenged by the observation that exogenous laminin did not improve the substratum properties of outer retina layers.

Since heat and protease pretreatment, but not detergent, organic solvent and extensive washing abolished the inhibitory activity, the molecular component in question is presumably a membrane bound- or extracellular matrix protein. Recently, an inhibitory protein present in the posterior tectum of the chicken has been identified (Walter et al., 1987a). This phospholipase C sensitive glycoprotein specifically repulses temporal but not nasal ganglion cell axons in vitro (Stahl et al., 1990). It is reasonable to assume that this protein prevents formation of an incorrect topographic map in vivo, since temporal retinal axons are destined to project exclusively to the anterior tectum. To date attempts to inactivate the hypothetical inhibitory component in our cryocultures by phospholipase C treatment have failed (Stier and Schlosshauer, unpublished). An additional reason to conclude that the anticipated component in outer retina layers is probably different from the tectal inhibitor is the observation that we have no indication so far that ganglion cell axons from temporal and nasal axons show different growth specificity in control and heat-treated cryocultures.

As long as the active molecular components in our cryocultures have not been identified the term 'inhibition' will be used tentatively. In the most recent classification system three different classes of 'negative' guidance factors have been distinguished: inhibitory, outgrowth suppressing and repulsive (Baier and Bonhoeffer, 1994). In contrast to inhibitory and outgrowth suppressing factors, repulsive factors have a negative effect on axonal outgrowth only if these factors form concentration gradients. Within these gradients axons would be directed towards the lower concentration. Within a field of a homogenous concentration, however, neither the rate nor the orientation of axonal growth would be affected. The latter one does not apply to phenomena observed in our assay systems. Axonal outgrowth was blocked on outer retina layers in our cryo- and 'delayered' tissue cultures, even if axons had no chance to select an alternative substratum. Therefore the observed activity in our systems is likely to be based on inhibitory or outgrowth suppressing rather than on repulsive factors.

Chondroitin sulfate proteoglycan (CS-PG) has been reported to be instrumental in guiding ganglion cell axons within the OFL of the rat retina towards the optic fissure (Brittis et al., 1992). Since in vitro CS-PG hampers axonal extension, the developmentally regulated presence of CS-PG in peripheral retina is considered to prevent aberrant growth in the peripheral direction within the OFL (Snow et al., 1991). Despite the fact that CS-PG is absent from outer layers of the chick retina (Snow et al., 1991; our own observation), other proteoglycans might act as guidance cues for outgrowing axons.

Our data indicate that axonal navigation within the retina is based on the presence of a permissive and an inhibitory zone. Compelling evidence suggests that the permissive zone is due to cellular components of endfeet of radial glia, whose cell bodies are located in the inhibitory zone. Future investigations will be focused on determining whether radial glia express growth promoting cell surface molecules in a polarized fashion, as has been shown for the expression of NCAM isoforms on different ganglion cell compartments (Schlosshauer et al., 1984). Similarly it will be intriguing to reveal whether proximal processes and cell bodies of radial glia provide inhibitory signals for outgrowing axons. With further advanced in vitro systems at hand to dissect out different glial and neuronal cell compartments, it should be possible to elucidate the molecular components responsible for polarized cell interactions.

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REFERENCES

Autillo-Touati, A., Chamak, B., Araud, D., Vuillet, J., Seite, R. and Prochiantz, A. (1988). Region-specific neuro-astroglial interactions: ultrastructural study of the in vitro expression of neuronal polarity. *J. Neurosci. Res.* **19**, 326-342.

Baier, H. and Bonhoeffer, F. (1994). Attractive axon guidance molecules. *Science* **265**, 1541-1542.

Bhattacharjee, J. and Sanyal, S. (1975). Developmental origin and early differentiation of retinal Müller cells in mice. *J. Anat.* **120**, 367-372.

Bonhoeffer, F. and Huf, J. (1980). Recognition of cell types by axonal growth cones in vitro. *Nature* **288**, 162-164.

Brittis, P. A., Canning, D. R. and Silver, J. (1992). Chondroitin sulfate as a regulator of neuronal pattering in the retina. *Science* **255**, 733-736.

Carpenter, M. K., Hassinger, T. D., Whalen, L. R. and Kater, S. B. (1994). CNS white matter can be altered to support neuronal outgrowth. *J. Neurosci. Res.* **37**, 1-14.

Chiquet, M. (1989). Tenascin/J1/Cytotactin: the potential function of hexabrachion proteins in neural development. *Dev. Neurosci.* **11**, 266-275.

Cornel, E. and Holt, C. (1992). Precocious pathfinding: retinal axons can navigate in an axonless brain. *Neuron* **9**, 1001-1011.

Daniloff, J. K., Chuong, C. -M., Levi, G. and Edelman, G. M. (1986). Differential distribution of cell adhesion molecules during histogenesis of the chicken nervous system. *J. Neurosci*. **6**, 739-758.

Edelman, G. M. (1992). Mediation and inhibition of cell adhesion by morphoregulatory molecules. *Cold Spring Harbor Symp. Quant. Biol.* **57**, 317-325.

Globus, A. and Scheibel, A. B. (1967). Pattern and field in cortical structure: the rabbit. *J. Comp. Neurol.* **131**, 55-72.

Gray, G. E. and Sanes, J. R. (1992). Lineage of radial glia in the chicken optic tectum. *Development* **114**, 271-283.

Halfter, W., Newgreen, D. F., Sauter, J. and Schwarz, U. (1983). Oriented axon outgrowth from avian embryonic retinae in culture. *Dev. Biol.* **95**, 56-64.

Halfter, W., Reckhaus, W. and Kröger, S. (1987). Nondirected axonal

growth on basal lamina from avian embryonic neural retina. *J. Neurosci.* **7**, 3712-3722.

Halfter, W. and Fua, C. S. (1987). Immunohistochemical localization of laminin, neural cell adhesion molecule, collagen type IV and T-61 antigen in the embryonic retina of the Japanese quail by in vivo injection of antibodies. *Cell Tissue Res.* **249**, 487-496.

Hammarback, J. A., Palm, S. L., Furcht, L. T. and Letourneau, P. C. (1985). Guidance of neurite outgtrowth by pathways of substratum-absorbed laminin. *J. Neurosci. Res.* **13**, 213-220.

Hinds, J. W. and Hinds, P. L. (1974). Early ganglion cell differentation in the mouse retina: an electron microscopic analysis utilzing serial sections. *Dev. Biol.* **37**, 381-416.

Hoffman, S. and Edelman, G. M. (1983). Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. *Proc. Natl. Acad. Sci. USA* **80**, 5762-5766.

Holt, C. E. (1989). A single-cell analysis of early retinal ganglion cell differentiation in Xenopus: from soma to axon tip. *J. Neurosci.* **9**, 3123-3145.

Lafont, F., Rouget, M., Triller, A., Prochiantz, A. and Rousselet, A. (1992). In vitro control of neuronal polarity by glycosaminoglycans. *Development* **114**, 17-29.

Lichtman, J. W. (1994). Konfokale Mikroskopie. *Spektrum der Wissenschaft* **10**, 78-87.

Lochter, A. and Schachner, M. (1993). Tenascin and extracellular matrix glycoproteins: from promotion to polarization of neurite growth in vitro. *J. Neurosci.* **13**, 3986-4000.

Meller, K. and Tetzlaff, W. (1976). Scanning electron microscopic studies on the development of the chick retina. *Cell Tissue Res.* **170**, 145-159.

Oohira, A., Matsui, F. and Katoh-Semba, R. (1991). Inhibitory effects of brain chondroitin sulfate proteoglycans on neurite outgrowth from PC 12D cells. *J. Neurosci.* **11**, 822-827.

Patterson, P. H. (1988). On the importance of being inhibited, or saying no to growth cones. *Neuron* **1**, 263-267.

Prada, C., Puelles, L. and Génis-Gálvez, J. M. (1981). A Golgi study on the early sequence of differentiation of ganglion cells in the chick embryo retina. *Anat. Embryol.* **161**, 305-317.

Prada, C., Puga, J., Pérez-Méndez, L., López, R. and Ramirez, G. (1991). Spatial and temporal patterns of neurogenesis in the chick retina. *Eur. J. Neurosci.* **3**, 559-569.

Prada, F. A., Magalhaes, M. M., Coimbra, A. and Genis-Galvez, J. M. (1989). Morphological differentiation of the Müller cell: golgi and electron microscopy study in the chick retina. *J. Morphol.* **201**, 11-22.

Qian, J., Bull, M. S. and Levitt, P. (1992). Target-derived astroglia regulate axonal outgrowth in a region-Specific manner. *Dev. Biol.* **149**, 278-294.

Ramon Y Cajal, S. (1933). The retina of birds. In *The Structure of the Retina*, (ed. Thorpe, S. A. and Glickstein, M.).pp. 76-92. Springield, Ill: Charles C. Thomas.

Rathjen, F. G., Wolff, J. M., Chang, S., Bonhoeffer, F. and Raper, J. A. (1987a). Neurofascin: a novel chick cell surface glycoprotein involved in neurite-neurite interactions. *Cell* **51**, 841-849.

Rathjen, F. G., Wolff, J. M., Frank, R., Bonhoeffer, F. and Ruitshauser, U. (1987b). Membrane glycoproteins involved in Neurite fasciculation. *J. Cell Biol.* **104**, 343-353.

Rousselet, A., Fetler, L., Chamak, B. and Prochiantz, A. (1988). Rat mesencephalic neurons in culture exhibit different morphological traits in the presence of media conditioned on mesencephalic or striatal astroglia. *Dev. Biol.* **129**, 495-504.

Ruegg, M. A., Stoeckli, E. T., Kuhn, T. B., Heller, M., Zuellig, R. and Sonderegger, P. (1989). Purification of axonin-1, a protein that is secreted from axons during neurogenesis. *EMBO J.* **8**, 55-63.

Schlosshauer, B., Schwarz, U. and Rutishauser, U. (1984). Topological distribution of different forms of neural cell adhesion molecule in the developing chick visual system. *Nature* **310**, 141-143.

Schlosshauer, B. (1989). Purification of neuronal cell surface proteins and generation of epitope-specific monoclonal antibodies against cell adhesion molecules. *J. Neurochem.* **52**, 82-92.

Schlosshauer, B., Dütting, D. and Wild, M. (1990). Target-independent regulation of a novel growth associated protein in the visual system of the chicken. *Development* **109**, 395-409.

Schlosshauer, B., Grauer, D., Dütting, D. and Vanselow, J. (1991). Expression of a novel Müller glia specific antigen during development and after optic nerve lesion. *Development* **111**, 789-799.

Schlosshauer, B., Stier, H. and Egert, U. (1993). Graded distribution of the neural 2A10 antigen in the developing chicken retina. *Dev. Brain Res.* **76**, 13-22.

Schlosshauer, B. and Dütting, D. (1991). Intraretinal pathfinding of ganglion cell axons is perturbed by a monoclonal antibody specific for a G4/Ng-CAM-like cell adhesion molecule. *Dev. Brain Res.* **63**, 181-190.

Schlosshauer, B. and Herzog, K.-H. (1990). Neurothelin: an inducible cell surface glycoprotein of blood-brain barrier-specific endothelial cells and distinct neurons. *J. Cell Biol.* **110**, 1261-1274.

Seidel, H., Csepregi, L., Heuberger, A. and Baumgärtner, H. (1995). Anisotrophic etching of crystalline silicon in alkaline solution. *J. Elektronchem. Soc.* **137**, 3612-3626.

Silver, J. and Ruitshauser, U. (1984). Guidance of optic axons in vivo by a performed adhesive pathway on neuroepithelial endfeet. *Dev. Biol.* **106**, 485-499.

Snow, D. M., Watanabe, M., Letourneau, P. C. and Silver, J. (1991). A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth. *Development* **113**, 1473-1485.

Snow, D. M. and Letourneau, P. C. (1992). Neurite outgrowth on a step gradient of chondrointin sulfate proteoglycan (CS-PG). *J. Neurobiol.* **23**, 322-336.

Spence, S. G. and Robson, J. A. (1989). An autoradiographic analysis of neurogenesis in the chick retina in vitro and in vivo. *Neuroscience* **32**, 801-812.

Stahl, B., Müller, B., Boxberg, Y. von, Cox, E. C. and Bonhoeffer, F. (1990). Biochemical characterization of a putative axonal guidance molecule of the chick visual system. *Neuron* **5**, 735-743.

Turner, D. L. and Cepko, C. L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. *Nature* **328**, 131-136.

Valverde, F., Facal-Valverde, M. V., Santacana, M. and Heredia, M. (1989). Development and differentiation of early generated cells of sublayer VIb in the somatosensory cortex of the rat: A correlated Golgi and autoradiographic study. *J. Comp. Neurol.* **290**, 118-140.

Walter, J., Henke-Fahle, S. and Bonhoeffer, F. (1987a). Avoidence of posterior tectal membranes by temporal retinal axons. *Development* **101**, 909-913.

Walter, J., Kern-Veits, B., Huf, J., Stolze, B. and Bonhoeffer, F. (1987b). Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. *Development* **101**, 685-696.

Walter, J., Allsopp, T. E. and Bonhoeffer, F. (1990). A common denominator of growth cone guidance and collapse? *Trends Neurosci.* **13**, 447-452.

Wang, L. C., Baird, D. H., Hatten, M. E. and Mason, C. A. (1994). Astroglial differentiation is required for support of neurite outgrowth. *J. Neurosci.* **14**, 3195-3207.

Wetts, R. and Fraser, E. (1988). Multipotent precursors can give rise to all major types of the frog retina. *Science* **239**, 1142-1145.

Williams, R. W. and Rakic, P. (1985). Dispersion of growing axons within the optic nerve of the embryonic monkey. *Proc. Natl. Acad. Sci. USA* **82**, 3906-3910.

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