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Axonal Versus Dendritic Outgrowth Is Differentially Affected by Radial Glia in Discrete Layers of the Retina

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Formation of neural cell polarity defined by oriented extension of axons and dendrites is a crucial event during the development of the nervous system. Ganglion cells of the chicken retina extend axons exclusively into the inner retina, whereas their dendrites grow into the outer retina. To analyze guidance cues for specific neurite extension, novel *in vitro* systems were established. Ganglion cells were purified by enzymatically facilitated detachment of the ganglion cell layer. A newly developed retrograde labeling technique and the expression analysis of the cell type-specific 2A1 antigen were used to monitor ganglion cell purification. In highly purified ganglion cells explanted onto retinal cryosections (cryoculture), axon formation was induced when the cells were positioned on the inner retina. In contrast, on outer layers of the developing retina dendritic outgrowth was prevalent. Because radial glia have been demonstrated to be instructive in neuritogenesis, distinct glial cell compartments located in inner and outer retina, respectively, were isolated for functional assays. Glial end feet were purified by a physical detachment technique. Glial somata were purified by complement mediated cytolysis of all nonglial cells. When ganglion cells were cultured on different glial compartments, axon formation occurred on end feet but not on glial somata. In striking contrast, on glial somata dendrites were formed. The data support the notion that ganglion cell polarity is affected by the retinal microenvironment, which in turn is possibly influenced by radial glia, being themselves polarized.

Key words: axon; cell polarity; chicken retina; cryoculture; dendrite; end feet; enzymatic delayering; ganglion cell purification; radial Müller glia

Cell polarity of neurons is a prerequisite for directed information flux within neuronal networks and, consequently, is essential for the functioning of the brain. On the molecular level neuronal polarity is reflected by the compartmentation of distinct proteins such as microtubule-associated protein protein 2 in dendrites and phosphorylated neurofilaments in axons (Craig and Banker, 1994).

The mechanisms that control the spatially restricted display of molecular components and initiate the transition from an essentially nonpolarized to a polarized cell morphology are likely to be based on a complex interplay between various factors (Sargent, 1989). Epigenetic factors appear to constitute a diverse set of extracellular matrix proteins and soluble components. Dermatan sulfate, for example, facilitates dendritic elongation of cortical neurons (Lafont et al., 1994), whereas chondroitin sulfate proteoglycan was reported to initiate axonal outgrowth of thalamic and mesencephalic neurons (Fernaud-Espinosa et al., 1994) but not of axons of ganglion cells (Brittis et al., 1992). Furthermore, distinct glycosaminoglycans (especially in solution without their protein cores) stimulate neurite extension (Brittis and Silver, 1994), whereas the same molecules are inhibitory when bound to the substratum. BMP-7 (OP-1), but not BMP-2 or BMP-4, induces the formation of sympathetic dendrites (Lein et al., 1995). In all these cases the cell interactions involved remain elusive. Because a variety of components have been discussed with controversy, detailed understanding of neuronal cell polarity formation is emerging slowly (Snow et al., 1991; Lafont et al., 1992).

One system especially suitable to approach this topic is the avascular chicken retina. The retina is characterized by an alternating sequence of plexiform and nuclear layers with distinct cell types in defined tissue layers. In addition, the topography of different types of neurites is documented in detail. The current research has been facilitated considerably by the fact that within the avascular chicken retina radial Müller glial cells are the only non-neuronal cell type present.

Morphological differentiation of retinal ganglion cells (RGCs) starts with axon formation immediately while the newborn cells approach their final destination in the ganglion cell layer. Axons are extended exclusively along the inner retinal surface but not into outer layers. In contrast, dendritic growth of RGCs is spatiotemporally separated from axonal outgrowth. Dendrites are extended specifically into the presumptive inner plexiform layer of the outer retina. (Within the context of this presentation, we define for simplicity all tissue layers of the undifferentiated retina except the optic fiber layer and ganglion cell layer as "outer retina.")

Although guidance mechanisms for these dendrites remain elusive, axon outgrowth is possibly affected by radial glia (H. Stier and B. Schlosshauer, unpublished data). Radial glia cells span nearly the entire width of the retina, with end feet at the vitreal surface and cell somata in the outer retina. Therefore, radial glia expand in both tissue zones, where distinct and exclusive neurite differentiation of ganglion cells occurs. The present results indicate that different tissue environments play an instructive role in the development of RGC polarity. In addition, our experiments show for the first time that the retinal microenvironment is

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specified by subcellular domains of radial glia with opposite effects on axonal versus dendritic development.

MATERIALS AND METHODS

All chemicals used were from Sigma (St. Louis, MO) unless stated otherwise. Only water of Millipore (Bedford, MA) quality was used. F-12 culture medium consisted of F-12 (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies), 2% heat-inactivated chicken serum (Life Technologies), 2 mM glutamine (Eurobio), 10 U/ml penicillin (Eurobio), and 10 U/ml streptomycin (Eurobio). The following primary antibodies were used: monoclonal antibody (mAb) 2A1 (Schlosshauer et al., 1990), mAb 2A10 (Schlosshauer et al., 1993), and mAb 2M6 (Schlosshauer et al., 1991). Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA): fluorescein-(DTAF-) and rhodamine-(TRITC-) conjugated goat anti-mouse IgG and IgM. All secondary antibodies were used at a dilution of 1:200.

Coverslip coating: poly-D-lysine and laminin. For enrichment of retinal ganglion cells by "enzymatic delayering" and cell culture experiments, it was essential to guarantee a high-quality coating of coverslips. For this purpose, coverslips (diameter, 12 mm; Marienfeld, Bad Mergentheim, Germany) were heat-sterilized in water. After drying of the coverslips in the air stream of a clean bench, 25 μl of a poly-D-lysine (PDL) solution was pipetted onto each coverslip and incubated (37°C, 1 hr). One hundred micrograms of PDL/ml H_2O was used for standard applications; 500 μg of PDL/ml H_2O was applied when coverslips were used to isolate glial end feet as cell culture substrata or to immobilize frozen tissue slices for cryoculture experiments (PDL500). The coverslips were washed three times with water, dried, and stored up to 6 d at 4°C. For laminin coating 25 μl of laminin solution (1 243 217; Boehringer Mannheim, Indianapolis, IN; diluted 1:20 in HBSS; Life Technologies) was spread onto each PDL100-coated coverslip and incubated (4°C, 16 hr).

Establishment of retinal cell cultures and glial substrata. Retinal cells were prepared as described (Schlosshauer et al., 1993) and seeded onto PDL100- and laminin-coated coverslips. For preparing pure radial glia monolayers, cells of embryonic day 8 (E8) were seeded in high density (250,000 cells/cm^2) in uncoated 35 mm culture dishes (Nunc, Roskilde, Denmark) to induce formation of a glial monolayer. The neurons on top of the glial monolayer were removed by a complement-mediated cell lysis using the neuron-specific mAb 2A10 (Schlosshauer et al., 1993) and rabbit serum (Life Technologies), followed by thorough washing. Glial monolayers as well as glial end feet on PDL500-coated coverslips (see below for isolation protocol) were used as substrata for coculturing with purified RGC.

Enrichment procedure for RGC: enzymatic delayering. E7 retinas (stage 30–31; Hamburger and Hamilton, 1951) from White Leghorn chicken were dissected in HBSS. Subsequently, retinas were carefully flat-mounted onto adhesive nitrocellulose filter membranes (Satorius AG; 13006-50-ACN) with photoreceptors facing the membranes. Retinas were tightly adhered to the nitrocellulose filter.

During the first step, the glial end feet layer was removed after aspirating surplus fluid. A PDL100-coated coverslip was carefully pressed onto each retina. After a 10 min incubation at 37°C, the specimen was flooded with HBSS, and the coverslips were removed with the end feet layer sticking to them (Halfter et al., 1987). During the next preparation step, the remaining tissue was trypsinized (Sigma T 8253; 1 mg/ml PBS, 12 min, 37°C) and thereafter washed three times with HBSS. The ganglion cell layer was isolated during the final step. Surplus fluid was removed from the specimen as described above, and a new PDL100-coated coverslip was carefully pressed onto the retinal tissue. The specimen was incubated for 10 min in an incubator (5% CO_2, 37°C). Afterward, coverslips were lifted off with the ganglion cell layer sticking to the surface of the coverslips and put immediately into a 35 mm culture dish with 2 ml of HBSS containing DNase (Sigma DN 25; 20 mg/ml). RGCs were carefully rinsed off the coverslips and incubated for 5 min at room temperature to fragment released DNA. The resulting cell suspension was transferred to a 10 ml centrifugation tube; 5 ml of HBSS was added, and the mixture was centrifuged (100 × *g*, 9 min). The pellet was resuspended in 1 ml of F-12 culture medium and quantified using the computer-assisted cell analysis system (CASY) I cell-analyzing unit (Schärfe Systems, Reutlingen, Germany). The cells were seeded in varying densities (15,000–100,000 cells/cm^2, as indicated in the text) onto different substrata (PDL100- and laminin-coated coverslips, retinal cryosections, glial end feet, and glial monolayers).

Determination of the enrichment factor for RGCs. To determine the enrichment factor of the enzymatic delayering procedure, two independent methods were used. For the first, a novel retrograde labeling method of RGC was devised. The second was based on an *in vitro* axon outgrowth assay using the RGC axon-specific marker mAb 2A1 (Schlosshauer et al., 1990). For retrograde labeling of RGCs, E7 chicken eyes were removed, preserving the pigmented epithelium and the proximal part of the optic nerve. The optic nerve was cut with a pair of microscissors ~0.5 mm behind the optic nerve head to produce a smooth surface. Crystals of TRITC-conjugated low molecular weight dextran (D-3308; Molecular Probes, Eugene, OR) were applied onto the trans-section site of the nerve. Subsequently, the retina still attached to the vitreous body was incubated in F-12 culture medium (37°C, 2 hr). After two washing steps with HBSS, the vitreous body was removed, and the retina was processed to gain mixed retinal cell suspensions. Alternatively, such retinas were used for enzymatic delayering to enrich the RGC as described above. The amount of TRITC-dextran positive RGCs was determined in triplicates of 200,000 cells each of (1) mixed retinal single-cell preparations or (2) enriched RGCs. Three independent series were performed and evaluated.

In a second approach the enrichment factor was determined by culturing retinal cells with or without enzymatic delayering in low density on PDL100- and laminin-coated coverslips. Cells were cultured for 30 hr, fixed with 4% paraformaldehyde (PFA) in PBS and stained with mAb 2A1 to visualize RGC axon-specific antigen expression. The number of cells, which extended a 2A1-positive axon longer than 50 μm, was determined. Each value was determined by counting the cells on two or three coverslips. Statistical analysis was based at least on three independent preparations. Data were considered significant at $p < 0.01$ (Student's *t* test).

Cryocultures of RGCs on retinal tissue sections. Cryocultures were performed as described recently (Stier and Schlosshauer, 1995). Briefly, flat-mounted retinas were frozen in liquid nitrogen without previous paraformaldehyde fixation or infiltration with sucrose. Tissue was cryosectioned in the radial axis perpendicular to retinal layers. Up to 12 20 μm tissue sections were immobilized onto individual PDL500-coated coverslips. After extensive washing over 24 hr at 4°C, RGCs were seeded onto cryosections and kept in culture for 1–3 d. Ganglion cells were visualized by fluorescence microscopy using either mAb 2A1 and secondary antibodies or TRITC-conjugated phalloidin (Sigma P 51571; 1 μg/ml, 1 hr, 22°C).

Histology. Retinal tissue was fixed in 4% PFA–PBS, infiltrated with 30% sucrose in PBS overnight, embedded in OCT compound (Miles, Elkhart, IN), and frozen in liquid nitrogen-cooled 2-methylbutane. Cryosections were cut perpendicular to retinal layers and were immobilized on adhesive glass slides (Marienfeld, Bad Mergentheim, Germany). mAb staining of cryosections or PFA-fixed cell cultures was performed as described earlier (Schlosshauer et al., 1984) and viewed with an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany). For scanning electron microscopy, specimens were glutaraldehyde-fixed, dehydrated with isopropanol, critical point-dried, sputtered with gold, and analyzed in a scanning electron microscope (Stereoscan 90; Cambridge Instruments/Leica, Bensheim, Germany).

RESULTS

In recent experiments we have demonstrated that within the retina directed axon extension of RGC is attributable to a dual mechanism, which implies inhibition in outer tissue layers and permissiveness and attraction in the inner retina (Stier and Schlosshauer, 1995). For these experiments retinal explant systems were used, which facilitated investigations on axons growing out of the explant tissue but did not allow analysis of RGC dendrite formation, being restricted to the interior of the tissue mass. To be able to perform a comparative study on both neurite types, it was essential to establish an alternative system based on isolated cells.

Enzymatic delayering of the retina

Taking advantage of the laminated structure of the retinal tissue with RGCs positioned in a discrete layer, we aimed to develop a procedure that was based on the physical separation of different retinal tissue layers after controlled protease treatment. The procedure was termed enzymatic delayering. A retina from E7

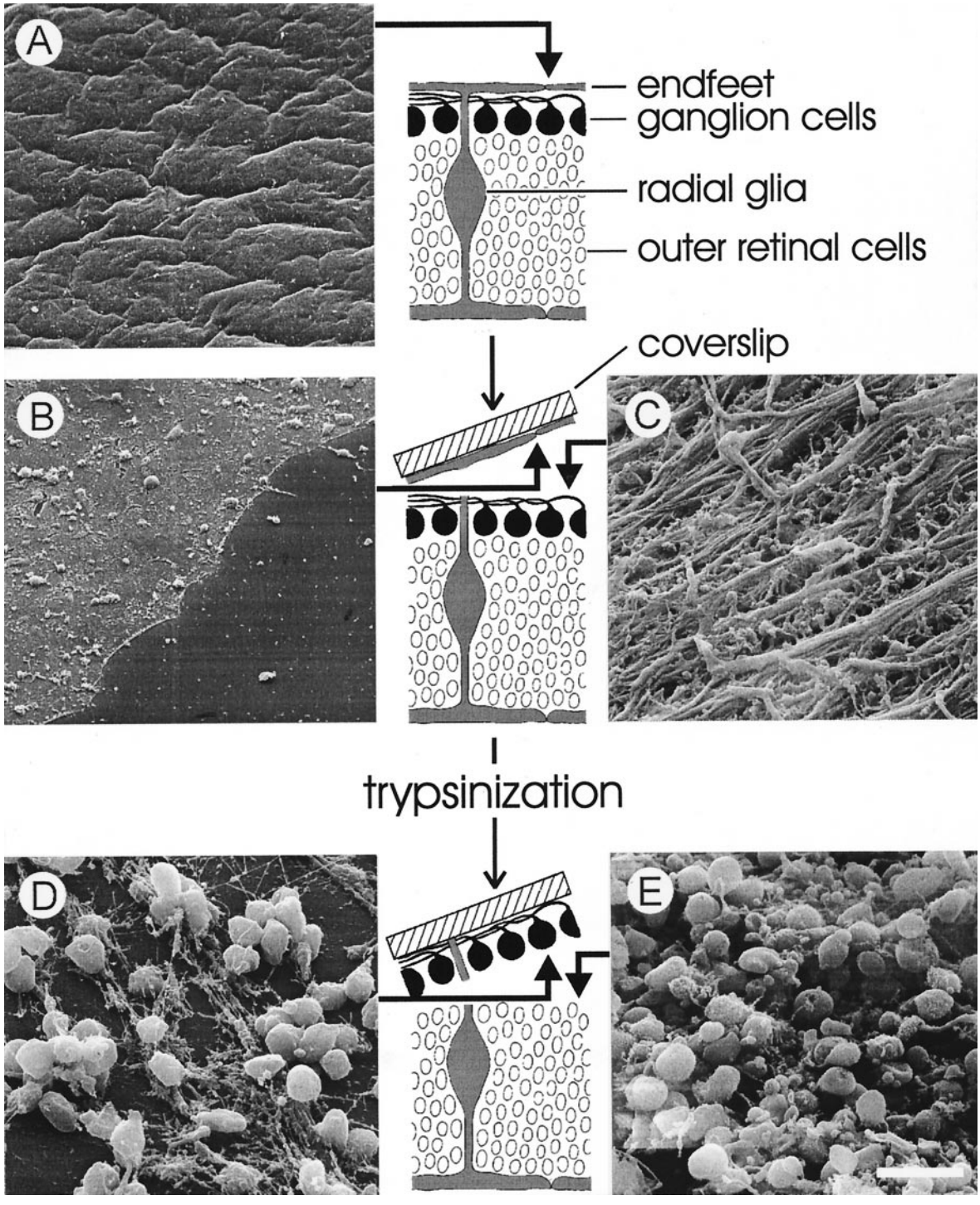

Figure 1. Enzymatic delayering. The schematic *center column* depicts the experimental layout. A chick retina from E7 was flat-mounted, and the glial end feet layer was mechanically detached using a poly-D-lysine-coated glass coverslip. After intermediate trypsin treatment of the remaining tissue, the delayering procedure was repeated. The resulting cell layer, which adhered to the coverslip, consisted of RGCs. *A–E*, Corresponding scanning electron micrographs of the different layers, as indicated by *filled arrows*. *A*, Vitreal surface of glial end feet. *B*, Retinal surface of isolated end feet. *C*, RGC axons of the optic fiber layer. *D*, Isolated RGCs with neurites. *E*, Cells of the outer retina. Scale bar (in *E* for *A–E*), 20 μm.

was dissected free from non-neuronal tissue and flat-mounted onto a nitrocellulose filter with the presumptive photoreceptor layer attached to the filter. As revealed by scanning electron microscopy, the still accessible inner surface of the retina was composed of a homogeneous layer of glial end feet, forming the inner limiting membrane (Fig. 1*A*). An adhesive poly-D-lysine-coated glass coverslip was pressed onto the planar surface of the tissue and removed again, which resulted in the detachment of glial end feet from the retina (Fig. 1*B*). After removal of the end feet, RGC axons of the optic fiber layer became exposed (Fig. 1*C*). Thereafter, a protease treatment was used to destabilize the adhesive forces between RGCs and cells of the outer retina.

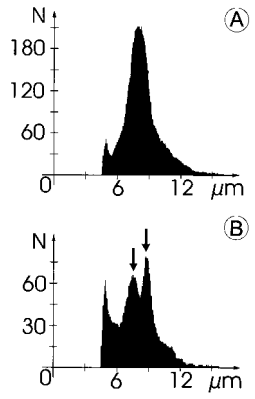

Figure 2. Histogram of purified RGCs. Retinal cell populations were characterized by a CASY. *x*-axis, Particle size; *y*-axis, particle number; peaks at ~5 μm represented cell debris. *A*, Unfractionated retinal cells showed a major peak at 8 μm. *B*, The population of purified RGCs was characterized by a double peak at 7.5 and 9 μm (*arrows*).

Subsequent isolation of RGCs was achieved by pressing another PDL-coated glass coverslip onto the tissue. RGCs together with their axons stuck to the adhesive glass surface (Fig. 1*D*). The remaining tissue surface was mainly represented by cells of variable diameter, which were essentially devoid of long neurites, as normally seen in the optic fiber layer (Fig. 1*E*).

Characterization of purified RGCs

Initial characterization of purified RGCs was performed using cell size as test parameter. Cell sizes were determined by a CASY. Unfractionated cell populations of retina E7 yielded two peaks at ~5 and ~8 μm (Fig. 2*A*). The minor peak at 5 μm represented cell debris, as judged from comparative microscopic inspection. The fraction of purified RGCs was characterized by three peaks (Fig. 2*B*). The debris peak was relatively pronounced, likely being attributable to the presence of fractured neurites of RGCs. Peaks at 7.5 and 9 μm possibly represented two major differentiation stages but not mature subtypes of RGCs, because subtypes of RGC populations with clearly different cell sizes are evident only at later stages of development (Vanselow et al., 1990). The bimodal distribution of the purified population is typical of RGCs, as has been documented for premature RGCs of the rat retina, with similar peak values at 7.5 and 8.5 μm (Lindsey and Weinreb, 1994). The average yield of purified RGCs was 0.5–1 × 10^6 cells per retina E7. Because the procedure of cell size analysis was rapid and reliable, all further preparations were analyzed this way and used only if the histogram indicated the triple-peak fingerprint.

Although the cell size analysis together with the histological characterization suggested that RGCs were enriched by enzymatic delayering, we aimed to gain direct evidence by two additional approaches. One method was based on selective *in situ* labeling of RGCs before purification. For the *in situ* approach, a novel procedure for retrograde labeling *in vitro* was developed (Fig. 3*A*). Rhodamine-conjugated dextran crystals were inserted into the nerve stump of isolated eyeballs and thereafter cultured for 2 hr to allow retrograde transport of the fluorescent dye into RGC somata. The general feasibility of the procedure was demonstrated in flat-mounted retinas. Top views of mounted retinas revealed populations of cell somata and axons, which were oriented concentrically converging at the optic nerve head, as known from RGC axons (Fig. 3*B*). Cross-sections of such retinas indicated that retrogradely labeled cells were restricted to the innermost somata layer, i.e., the ganglion cell layer (Fig. 3*C*). Cell morphologies and sizes varied among the population of labeled RGCs, although it could not be excluded that differences originated from the plane of sectioning.

However, the data were in line with CASY histograms, which revealed that purified RGCs possibly represented a population of differently sized neurons. Although only a fraction of RGCs was retrogradely labeled, the number of cells was sufficient to allow quantification. After retrograde labeling, retinas were processed as above to gain single-cell cultures either with or without previous enrichment of RGCs by enzymatic delayering. The cellular morphology (Fig. 4*A*) and the nucleus diameter (Fig. 4*B*) of freshly prepared RGCs varied considerably. Quantification of labeled cells originating from three independent experimental series was performed by fluorescence microscopy. Among 2 × 10^5 unfractionated retinal cells per glass coverslip, 50.58 ± 5.13 labeled cells were found. In contrast, enzymatic delayering yielded on average 1004.17 ± 84.87 labeled cells. This represented a 19.8-fold enrichment of labeled cells (Fig. 4*C*).

To substantiate the data of RGC purification, an additional approach for quantification was used, which made use of the expression of a differentiation antigen (axonal 2A1 antigen) during short-term cultivation on laminin (Schlosshauer et al., 1990). Under the culture conditions used, cells formed mostly unbranched neurites of 200–800 μm in length, which were 2A1-positive (Fig. 5*A,B*). Cells were seeded at low density (15,000 cells/cm^2) to allow identification of individual cells within the rapidly forming neurite network; 46.83 ± 10.60 immunoreactive cells/cm^2 were found in unfractionated populations, whereas 1002.20 ± 74.90 cells/cm^2 were identified among enriched RGCs (Fig. 5*C*). Similar as in the above approach, an enrichment factor of 21.4 was calculated. Based on the ratio of RGCs present in the E7 retina, the enzymatic delayering technique must be considered to provide an essentially pure RGC population (see below).

Antigen expression and morphological features of the purified cells were in line with the assumption that enzymatic delayering provided cells with characteristics typical of RGCs. As shown below, the cells expressed the neuronal 2A10 antigens (Schlosshauer et al., 1991) in addition to the RGC-specific 2A1 antigen (Schlosshauer et al., 1990). In addition to immunocytochemistry, affinity cytochemistry (phalloidin binding to F-actin) and phase-contrast microscopy permitted evaluation of cell morphology (see Figs. 5, 6, 8). Among the different retinal cell types, the RGCs are the only projection neurons of the retina, which rapidly extend axons that exceed their somata diameter by several orders of magnitude in the adult visual system (Ramon Y Cajal, 1933). Indeed, purified cells had the ability to extend neurites over 1000 μm/24 hr with an elongation speed of 50 μm/hr (Bauch, 1996).

The enrichment of RGCs was ~20-fold, as determined by two independent methods. On the day of enzymatic delayering (stage HH30/31; Hamburger and Hamilton, 1951) the retina has ~3 × 10^7 cells in total (Dütting et al., 1983). Depending on the type of investigation performed, at this developmental stage 1.5–3.6 × 10^6 RGCs have been shown to have migrated to the ganglion cell layer (Kahn, 1974; Prada et al., 1991; Snow and Robson, 1994). Consequently, based on even the most conservative calculation the purified RGC preparation must be considered essentially devoid of contamination, with a purity of >99%. Furthermore, contamination by displaced amacrine cells can be excluded, because this subset of cells migrates into the ganglion cell layer at

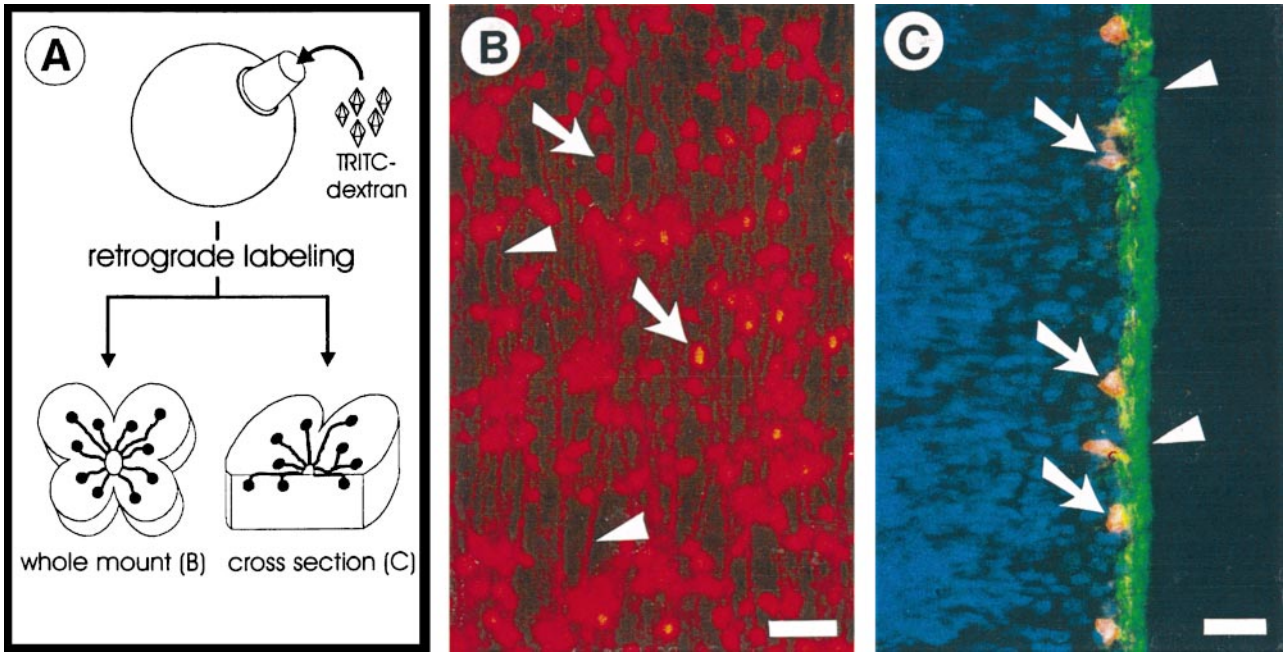

Figure 3. Retrograde labeling *in vitro*. *A*, Schematic presentation of the experimental layout. An eyeball of an embryonic chick was dissected out together with the blood–eye barrier forming pigment epithelium. Rhodamine-labeled dextran was inserted into the optic nerve stump, and the eye was incubated for 2 hr *in vitro* to allow retrograde transport into RGCs. Thereafter, the retina was processed for microscopic inspection (*B, C*). *B*, Flat-mounted retina viewed from the vitreal side. Axons (*arrowheads*) and RGC somata (*arrows*) were clearly discernible after retrograde transport of the fluorescent dye. *C*, Cryostat section of retrogradely labeled RGCs (*red, arrows*) after counterstaining with the nucleus stain DAPI (*blue*) and mAb 2A1 specific for RGC axons (*green, arrowheads*). The retrograde labeling technique specifically marks RGCs. Scale bars: *B*, 50 μm; *C*, 25 μm.

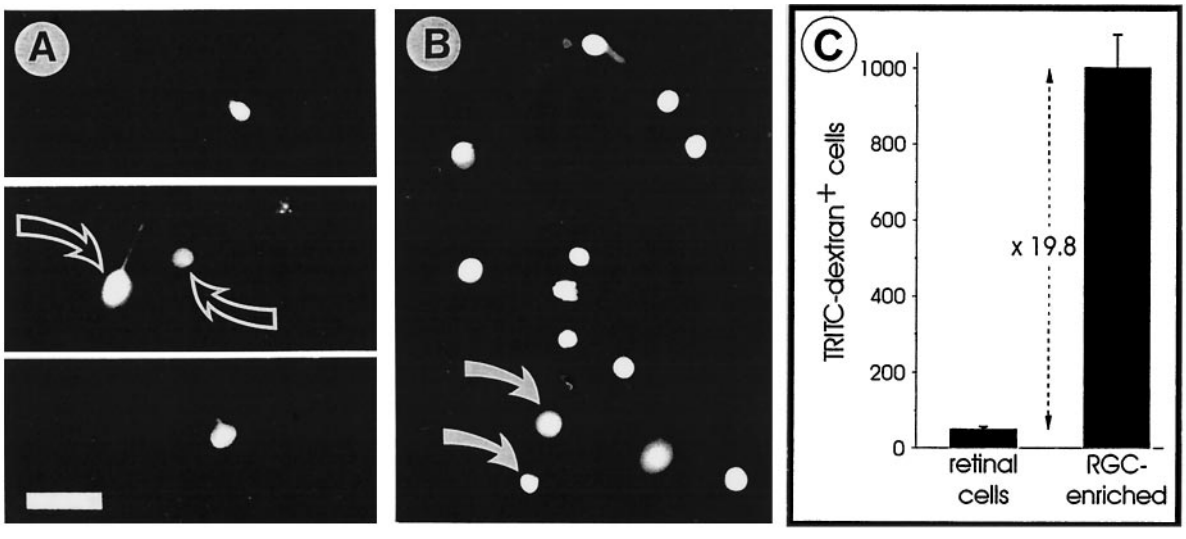

Figure 4. Quantification of purified RGCs after retrograde labeling. Retinas were retrogradely labeled by TRITC dextran, and two cell populations were investigated: unfractionated cells after dissociation without previous enyzmatic delayering (*retinal cells*) and purified RGCs (*RGC-enriched*). *A*, TRITC dextran fluorescence. Three representative images of retrogradely labeled cells are shown. *B*, DAPI nucleus fluorescence. Note the heterogeneous cell morphologies (*A, open arrows*) and diverging nucleus diameters (*B, filled arrows*). *C*, Quantification of retrogradely labeled cells processed with or without enzymatic delayering. Ganglion cells were enriched by enzymatic delayering 19.8-fold ($p < 0.001$). Scale bar (in *A* for *A*, *B*), 30 μm.

approximately E12 (Spira et al., 1987; Spence and Robson, 1989), i.e., 5 d later than the time point of enzymatic delayering.

Layer-specific differentiation of RGCs on retinal tissue sections

To address the question of whether the formation of cell polarity would be affected by the tissue microenvironment, we seeded purified RGCs on cryosections of the embryonic chicken retina (cyroculture) (Fig. 6*A*). Cryocultures have been shown to preserve the cytoarchitecture of the tissue environment to a large extent. Most notably, high-resolution analysis can be realized for transplanted cells both in native and non-native tissue microenvironments (Carpenter et al., 1994; Stier and Schlosshauer, 1995). For cryocultures, isolated eyeballs were frozen without previous fixation and cryosectioned, and the resulting tissue sections were immobilized on glass coverslips. Ganglion cells were purified by enzymatic delayering and seeded onto cryosections. After incu-

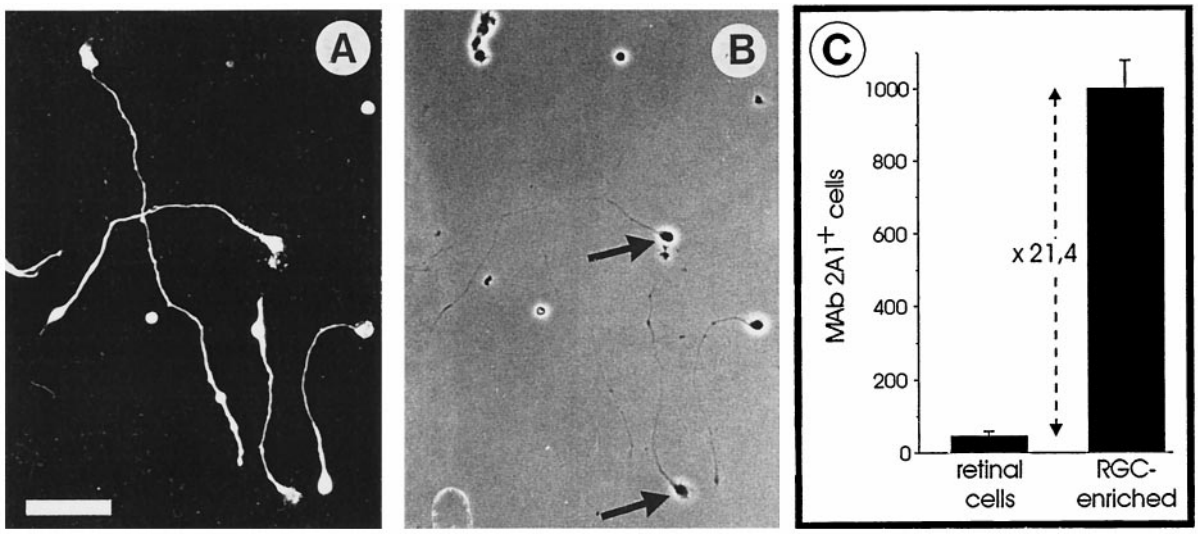

Figure 5. Quantification of purified RGCs based on antigen expression. Unfractionated retinal cells and purified RGCs (by enzymatic delayering) were cultured on PDL and laminin. After 1 *d in vitro*, cells expressing the RGC axon-specific 2A1 antigen were quantified. *A*, Epifluorescence micrograph. Retinal cells were double-labeled with DAPI and mAb 2A1. Various RGCs extended immunoreactive axons. *B*, Corresponding phase contrast; cell bodies are marked by *arrows*. *C*, Quantification of 2A1-positive cells in both fractions. Ganglion cells were enriched by enzymatic delayering 21.4-fold ($p < 0.001$). Scale bar (in *A* for *A, B*), 100 μm.

bation for 24 hr *in vitro*, cryocultures were fixed and labeled by the axon-specific mAb 2A1 and by TRITC-conjugated phalloidin, which marked F-actin present in axons and dendrites of RGCs.

Evaluation of cell polarity with regard to axon versus dendrite formation was based on morphological criteria and antigen expression. Neurites were considered axons if cell extensions were longer than five cell diameters and immunopositive for the axonal marker 2A1. Neurites were classified as dendrites if cell extensions had at least three side branches per main trunk and a tapered structure and were shorter than five diameters. In addition, in a number of experiments the absence of 2A1 antigen expression in dendritic processes was verified (Fig. 6*E*). Dendritic markers such as the microtubule-associated protein 2 were not expressed in our system and, therefore, could not be evaluated. Consequently, "dendrite" is used as an operational term.

Purified RGCs that attached to the inner layers of retinal cryosections (inner limiting membrane, optic fiber layer, and ganglion cell layer) established 2A1-positive axons. In most cases axons grew along the inner limiting membrane but, typically, did not invade outer retinal layers (presumptive inner and outer plexiform and nuclear layers) (Fig. 6*B,C*). Cells with dendrites were also situated in this region. Dendritic processes were short and did not display any orientation preference. In contrast, RGCs positioned onto outer retinal layers of cryosections were essentially devoid of axons. Instead, on outer retinal layers RGCs extended dendrites (Fig. 6*D–G*). Most cells were characterized by two to four primary dendrites, which appeared tapered and had numerous side branches that often bifurcated into several subbranches. The morphology of cell somata varied, being of circular, pyramidal, or irregular shape. Notably, the microenvironment of outer retinal layers did not inhibit 2A1 antigen expression quantitatively, although cells were essentially prevented from extending axons. In immunoreactive cells the 2A1 antigen accumulated at one cell pole (Fig. 6*F,G*), which possibly presented either the former axon hillock or a novel initiation site in the futile attempt to extend an axon. This observation is noteworthy also, because it excludes the possibility that non-RGCs preferentially adhered to the outer retinal layers, given that cell contaminations did occur (see above). Quantification of purified cells indicated that axons were formed on inner retinal layers, whereas axon extension was largely inhibited on outer retinal layers. In the outer retina, dendrite formation occurred 37.3 times more often than axon formation (Fig. 7). In summary, the data corroborated the assumption that epigenetic factors, which are locally restricted in different retina layers, specifically affect the development of RGC polarity.

Different subcellular domains of radial glia have opposite effects on RGC polarity

Recent experiments demonstrate that radial glia affect axonal outgrowth *in vitro* (Stier and Schlosshauer, unpublished data). Because the radial orientation implies that these glial cells span the functionally distinguishable inner and outer retina, we wondered whether different cell compartments of radial glia differentially affect axonal versus dendritic outgrowth. To address this question, it was necessary first to isolate two distinct glial cell compartments. Glial end feet positioned in the axon-inducing region of the inner retina were isolated by the detachment procedure as described above. The resulting sheet of end feet, which adhered to the glass coverslip, was directly used as a substratum for RGCs (Fig. 1*B*).

Employing neuron-specific antibodies for biochemical and cytochemical analysis, it has been shown that glial end feet preparations are devoid of retinal axon fragments, which could potentially transform otherwise nonpermissive end feet into a growth-promoting substratum. In addition, the previous findings support the notion that the preparation retains its functional properties *in vitro* (Halfter et al., 1987; Stier and Schlosshauer, 1995).

Glial somata could not be isolated by the same procedure. For purification of glial cells, retinal tissue was treated with protease and mechanically dissociated. For elimination of all neurons, mAb 2A10 (IgM) was applied to the heterogenous cell population in conjunction with the complement system. mAb 2A10 binds to cell surface antigens expressed on all retinal neurons. Because the

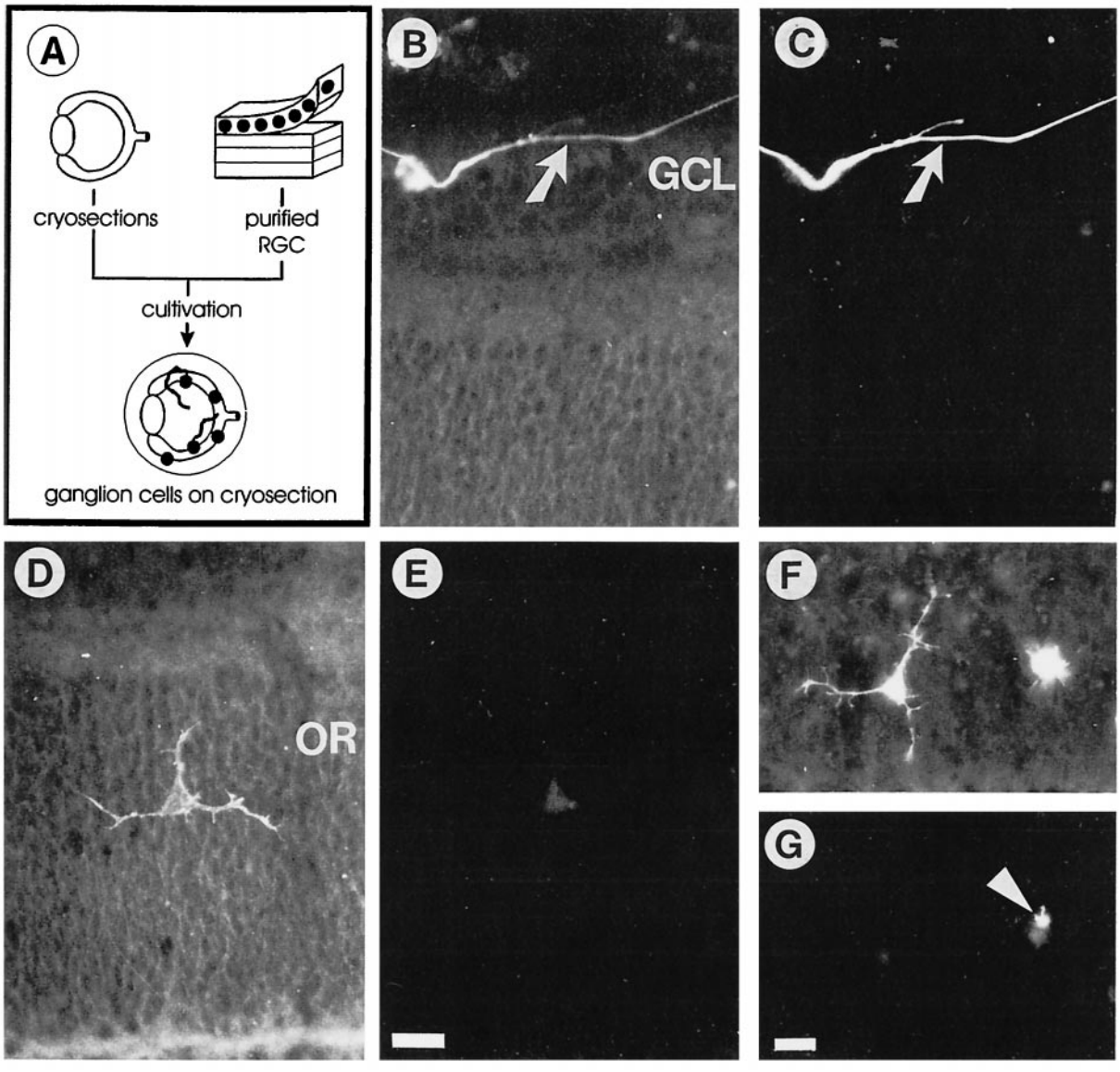

Figure 6. Cryoculture of purified RGCs on retinal tissue sections. *A*, Schematic presentation of the experimental layout. Purified RGCs were explanted onto retinal cryosections. Axonal differentiation was identified by immunolabeling with mAb 2A1; dendritic differentiation was identified by morphological evaluation after labeling cells with rhodamine-conjugated phalloidin to resolve F-actin. *B–G*, Epifluorescence micrographs. *B, D, F*, F-actin staining, which revealed the morphology of transplanted cells in addition to the structure of the retinal cryosection. *C, E, G*, Axonal staining of the cytoskeletal 2A1 antigen in transplanted RGCs. *B, C*, Ganglion cells localized on the inner retina extended axons (*arrow*). *D, E*, Most RGCs on outer retina layers extended dendrite-like neurites without detectable 2A1 antigen foci. *F, G*, Various RGCs positioned on outer retinal layers, which, although unable to form axons, still expressed an 2A1 antigen focus (*arrowhead*). *GCL*, Ganglion cell layer of retina section; *OR*, outer retina of the retinal section. Scale bars (in *E* for *B–E*, *G* for *F, G*), 20 μm.

radial glia represents the only non-neuronal cell type of the chicken retina, after complement-mediated cytolysis the resulting culture is composed only of radial glia, as demonstrated previously (Schlosshauer et al., 1993). Under the conditions used, these cells do not form processes but instead represent a pure somata preparation. The monolayer of glial somata was used as substratum for purified RGCs.

After cultivation of purified RGCs on both glial substrata, neurite formation was monitored by indirect immunofluorescence. On glial end feet the majority of RGCs extended long, mostly unbranched processes that fulfilled all axonal criteria as outlined above, including 2A1 antigen expression (Fig. 8*B*). Dendritic outgrowth was clearly restricted. The glial end feet sheet could be recognized easily by phase-contrast optics (Fig. 8*C*). Before culturing, the end feet sheet represented a homogeneous collection of end feet vesicles, which appeared as black dots (Fig. 8*C, arrowheads*). Although the preparation is likely to contain basal lamina constituents (Halfter et al., 1987), the upside-down orientation of the preparation guarantees that the retinal, not the the vitreal, surface of the end feet preparation is exposed to RGC axons. During culturing axons tended to modify the substratum, leaving behind end feet-free growth lanes.

In striking contrast to axon formation on glial end feet, dendrite-like extensions of purified neurons extended preferentially on glial somata, as was observed after labeling with the neuronal marker mAb 2A10 (Fig. 8*E*). Long unbranched and

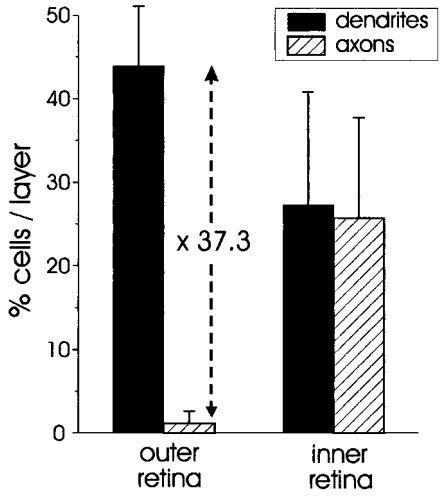

Figure 7. Quantification of neuritic differentiation of purified RGCs in cryocultures. Purified RGCs were explanted onto retinal cryosections. Axonal differentiation was identified by immunolabeling with mAb 2A1; dendritic differentiation by was identified by morphological evaluation after labeling cells with rhodamine-conjugated phalloidin to illuminate F-actin. On outer retina layers, axonal differentiation was essentially inhibited, whereas dendritic formation occurred 37.3 times more often than axonal formation ($p < 0.01$). On inner retina, axon and dendrite formation was not significantly different.

2A1-positive neurites were essentially absent. Instead, RGCs had numerous branched and tapered processes, which did not exceed five times the soma diameter. Dendritic morphologies of explanted RGCs on glial end feet differed from those on outer retina layers in cryocultures. The greater complexity and total length of dendritic-like extensions on glial monolayers might be attributable to the fact that the glial monolayer was not dried and frozen before use, as was necessary for cryosectioning. 4′,6-Diamidino-2-phenylindole (DAPI) staining revealed the presence of a confluent monolayer of glial somata with oval-shaped nuclei (Fig. 8*F*). Phase-contrast and DAPI fluorescence microscopy was used to verify that the analyzed RGCs were actually positioned on the glial somata rather than the culture dish surface.

Essentially no RGC axons were evident on somata of retinal glia. It is noteworthy that axon formation of RGCs was not inhibited by all kinds of glial somata. When RGCs were seeded on C6 glioma or purified glial somata derived from telencephalon, extensive axonal outgrowth was observed (H. Bauch and B. Schlosshauer, unpublished observations). These data suggest a specific regulatory function of retinal ganglion cells.

Quantitative evaluation of both systems substantiated our qualitative results. On glial end feet 22.88 ± 3.32% of all neurons had axons, but only 1.66 ± 1.60% bore dendrites. In this case, cells with axons outnumbered those with dendrites by a factor of 13.8 (Fig. 9). On glial somata the reverse was evident; 35.91 ± 7.56% of all neurons formed dendrites, but only 1.42 ± 0.79% developed axons. Therefore, dendritic differentiation occurred 25.3 times more often than axonal differentiation. These data were based on morphological parameters. A similar value was obtained for axons, when the immunological parameter (2A1 axon protein) was used; 29.5 ± 3.69% of RGCs developed axons on glial end feet, but only 2.06 ± 1.90% developed axons on glial somata, a 14.3-fold difference. Comparison of morphological and immunological data were not significantly different. This fact substantiated the notion that both morphology and expression of differentiation markers provided equivalent results.

In summary, the data suggest that radial glial cells are functionally polarized. End feet of radial glia support axonal outgrowth, whereas glial somata augment dendritic growth. Therefore, in the chicken retina, polarization of glial cells appears to influence the polarization of RGCs.

DISCUSSION

Relevance of the *in vitro* systems used

Stimulated by an earlier study in the rat (Shiosaka et al., 1984), we developed the procedure of enzymatic delayering for purification of chick RGCs. The isolated cells were identified as an essentially pure population of RGCs by a comprehensive set of means: (1) 2A10 neuronal marker expression, (2) 2A1 axonal antigen expression, (3) retrograde labeling, (4) cell size analysis, (5) morphological evaluation by affinity cytochemistry (phalloidin–F-actin), (6) scanning electron microscopy and immunohistochemistry of processed retinal layers, and (7) evaluation of the differentiation potential, i.e., neurite formation and speed of axonal outgrowth.

In the past different techniques have been used to purify RGCs, including fluorescence-activated cell sorting of *in vivo*-labeled cells (Armson and Benett, 1983), cell size separation by equilibrium centrifugation in a metrizamide step gradient (Cohen et al., 1989), and sequential immunopanning using Thy-1 antibodies (Barres et al., 1988). Our approach circumvents *in vivo* experimentation and costly fluorescence-activated cell-sorting instrumentation and provides a higher purity and yield than has been obtained by gradient centrifugation. Immunopanning would have been an attractive method; however, the procedure applied to chick cells suffers from major constraints. One constraint is the notoriously unsatisfactory binding and specificity of various Thy-1 antibodies during the period of cell polarity development, when Thy-1 expression is below a critical threshold (our observation; compare with the literature) (Sheppard et al., 1988). Taken together, the novel method of enzymatic delayering yields up to 1 million highly purified RGCs per retina and is therefore an excellent alternative to existing methods.

For analysis of radial glia compartments, cell somata were purified by negative selection using complement-mediated cytolysis. The resulting glial monolayer is composed of somata devoid of radial processes and end feet (Schlosshauer et al., 1993). In numerous studies it has been substantiated that cultured glial cells reflect to a large extent functional characteristics of the corresponding glial type *in vivo*. These studies comprise astrocytes (Lillien et al., 1990; Nedergaard, 1994), oligodendrocytes (Caroni and Schwab, 1988; Barres et al., 1992), Schwann cells (Ratner et al., 1988; Eldridge et al., 1989), Bergmann glia (Fishman and Hatten, 1993; Zheng et al., 1996), cerebral radial glia (Cameron and Rakic, 1994; Davenport and Thies, 1996), and retinal radial/Müller glia (Threlkeld et al., 1989; Drazba and Lemmon, 1990).

One concern about preculturing glial cells is potential dedifferentiation of cells *in vitro*, because different states of differentiation of astroglia have been shown to affect axonal and dendritic outgrowth differentially (Le Roux and Reh, 1996). To address this issue we compared 10 differentiation antigens and found no discrepancies between freshly prepared end feet and cultured somata. This agrees with findings that synthesis of neither glycosaminoglycans, including heparan sulfate, chondroitin sulfate, dermatan sulfate, and hyaluronic acid (Threlkeld et al., 1989), nor adhesion molecules such as N-cadherin and L1 (Drazba and Lemmon, 1990) are impaired *in vitro*. Furthermore, the inhibitory effect of outer retinal layers as well as the growth-promoting effect of glial end feet is observed at both embryonic and posthatched

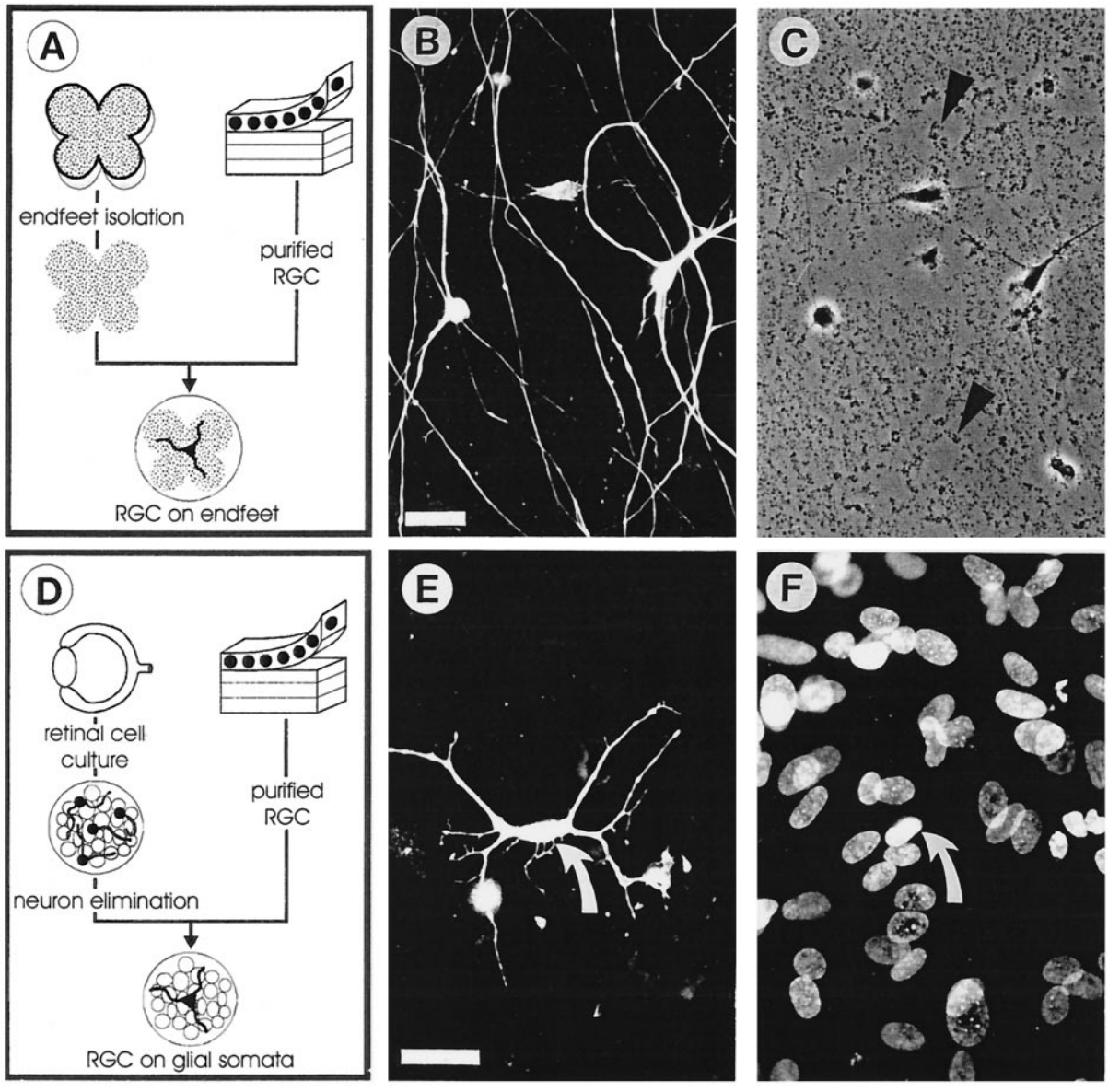

Figure 8. Differentiation of purified RGCs on glial end feet and glial somata. *A, D*, Schematic presentation of the experimental layout. Purified RGCs were explanted onto different glial subcellular domains. *A*, End feet of radial glia were purified by enzymatic delayering. *D*, Glial somata were purified by negative selection by virtue of complement-mediated cytolysis of nonglial cells. *B, E, F*, Epifluorescence micrographs. *B*, mAb 2A1-immunolabeled RGCs on glial end feet revealed extensive axonal outgrowth. *C*, Corresponding phase-contrast image. Glial end feet are marked by *arrowheads*. *E*, mAb 2A10-immunolabeled RGCs on glial somata extended dendritic processes. *F*, Corresponding staining of nuclei by DAPI revealed the presence of a confluent monolayer of glial cells. Scale bars: *B* (for *B*, *C*), 25 μm; *E* (for *E*, *F*), 30 μm.

stages (Stier and Schlosshauer, 1995). Therefore, the influence of neuronal cell polarity formation is likely to be independent of the maturation state of radial/Müller glia.

In summary, a wealth of data substantiates the biological relevance of the glial fractions and *in vitro* systems used for analysis of their impact on neuronal cell differentiation.

Development of neuronal cell polarity

When purified RGCs were explanted onto retinal cryosections, axonal outgrowth was restricted to the inner retina, whereas dendrite formation was prevalent on the outer retina. The tissue layer-specific differentiation of RGC polarity formation *in vitro* coincides with the localization of axons and dendrites *in vivo* (Ramon Y Cajal, 1933).

As deduced from dendritic outgrowth of rat somatosensory neurons and cerebral pyramidal cells of the rabbit (Globus and Scheibel, 1967), the cell pole of differential neurite initiation is possibly predetermined by the orientation of the cell axis. In RGCs, cell-intrinsic mechanisms appear also to govern the first step of axonal outgrowth. Axon initiation is always observed perpendicular to the axis of mitosis (Prada et al., 1981). Thereafter, extrinsic factors appear to regulate further neurite extension and might even override intrinsic differentiation phenotypes. Perturbation experiments of cultured hippocampal neurons demonstrate that the differentiation fate of presumptive dendrites can be changed to an axonal fate once the single axon is severed (Goslin and Banker, 1989).

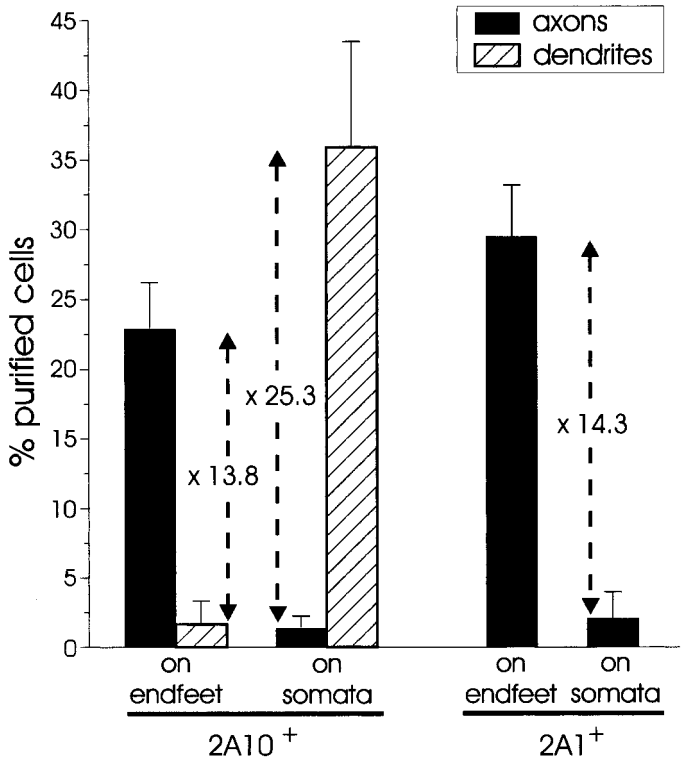

Figure 9. Quantification of neuritic differentiation on different glial compartments. Purified RGCs were explanted either on glial somata or on glial end feet. Neurite differentiation was evaluated after immunolabeling with mAb 2A1 and mAb 2A10. On glial end feet axonal extension (*black columns*) was prevalent, whereas on glial somata, only dendrites were observed (*hatched columns*). Both immunomarkers used revealed that axonal formation was essentially inhibited on glial somata ($p < 0.001$). Therefore, glial end feet and somata had differential effects on the establishment of ganglion cell polarity.

In the chick retina, axonal outgrowth is restricted to the optic fiber layer. Other retinal layers, including the presumptive inner plexiform layer where RGC dendrites are found, remain completely devoid of axons (Ramon Y Cajal, 1933). Our data obtained *in vitro* are in accord with *in situ* observations. On outer retinal layers axonal outgrowth from tissue explants (Stier and Schlosshauer, 1995) and from purified RGCs (this presentation) is essentially inhibited. Inhibition cannot be compensated for by laminin coating of retinal sections in cryocultures (Stier and Schlosshauer, 1995). Inhibition is mediated by the axon tip, because membrane fractions purified from retina induce RGC growth cones to collapse (Schlosshauer et al., 1996). Taken together, a dual mechanism based on both a supportive or attractive action and an inhibitory action guides axons vitreally.

The overall establishment of dendritic arborization is delayed in relation to axonal outgrowth (Vanselow et al., 1990), and dendritic growth is not initiated until RGC migration into the ganglion cell layer is complete (Snow and Robson, 1994). However, primitive RGC dendrites have been identified even during the process of initial axon formation shortly after cells became postmitotic (Snow and Robson, 1995). We have also observed that E7 RGCs cultured *in vitro* for 1 d extended both axons and dendrites. However, only 44% of RGCs display dendritic-like neurites on outer retina layers in cryocultures, which might be attributed to the limited potential of E7 RGCs to differentiate dendrites. In the future, it will be intriguing to evaluate in the same system whether older RGCs develop dendrites more frequently. *In vivo*, dendritogenesis occurs initially on several sides of RGC somata rather than exclusively at one pole (Prada et al., 1981), which we also observed *in vitro*. In addition, our data reveal that the initial axonal and dendritic differentiation does not depend on contact with central visual targets, as is essential for long-term survival of RGCs. Similar conclusions have been drawn from ablation experiments *in vivo* (Vanselow et al., 1990).

Formation of dendrites of rat sympathetic neurons has been shown to be induced by high molecular weight components of basement membrane extracts (Lein and Higgins, 1989). This is in agreement with enhanced dendritic elongation of cortical neurons on dermatan sulfate (Lafont et al., 1994). In addition, soluble factors such as BMP-7 (OP-1) potentiate dendritic growth (Lein and Higgins, 1989).

The molecular mechanisms of the development of RGC polarity remain elusive. Concentric axon guidance toward the optic nerve head in the plane of the optic fiber layer has been reported to be mediated by chondroitin sulfate proteoglycan (CS-PG) (Brittis et al., 1992). The developmentally regulated expression of CS-PG in the OFL and the inhibitory effect of CS-PG on RGC axons *in vitro* (Snow et al., 1991) has been interpreted as a regulatory function of CS-PG to prevent aberrant axon extension centripetally away from the nerve head. However, it is questionable whether CS-PG affects RGC polarity, because it is not expressed in the outer retina of the chick (our observation) (Snow et al., 1991), as should be hypothesized to prevent spatially incorrect axon formation.

Our data indicate that spatially restricted components in the outer and inner retina influence development of RGC polarity. The microenvironment of different retinal layers is most likely to be instructive for axonal versus dendritic growth. Whether molecular components supporting axonal formation concomitantly inhibit dendrite formation will be an intriguing aspect of future investigations.

Polarized radial glia

When purified RGCs were explanted on different radial glia compartments, axon formation was prevalent on end feet, whereas dendritic outgrowth predominated on glial somata.

These data are fully consistent with two other sets of experiments: (1) when glial end feet are removed from retinal tissue sections, RGC axons fail to orient along the inner retinal surface in cryocultures (Stier and Schlosshauer, 1995); and (2) encounter of growth cones with purified plasmic membranes from glial somata induces growth cone collapse in contrast to end feet membranes, which have no effect. This collapse-inducing effect is cell type-specific, because growth cones from dorsal root ganglia neurons are not affected by glial somata membranes (Stier and Schlosshauer, unpublished data). The accumulating body of data suggests that radial glia are possibly directly involved in differential neurite extension.

This concept is further supported by previous investigations on granular cell migration in the developing cerebellar cortex. Postmitotic granular cells use radial Bergmann glia as guide rails from the transient to the internal granular layer (Rakic, 1985). Granular cells elaborate two horizontal neurites perpendicular to the Bergmann glia axis and a third descending neurite, which grows in close contact along the radial contours of Bergmann glia. After the descending neurite, the granular cell body is translocated to the internal granular layer.

Retinal and cerebellar radial glia appear to interact in a similar

manner differentially with different neurite types and the neuronal somata. The Bergmann glia provides a permissive substratum for descending granular neurites but not for the horizontal neurites of the same neurons. In analogy to the cerebellum, postmitotic RGC bodies ride the radial trail from the germinal layer to the RGC layer, but their axons do not (Meller and Tetzlaff, 1976). Instead, axons bud directly from the vitreal process of RGCs and then grow along glial end feet *in vivo* (Halfter and Fua, 1987; Watanabe et al., 1991; Brittis et al., 1995; Snow and Robson, 1995) and *in vitro* (this paper) (Halfter et al., 1987; Stier and Schlosshauer, 1995). This contrasts with dendrites, which are well suited to extend on radial glia *in vitro* in a manner similar to that of descending fibers of granular cells *in vivo*.

The data in both systems indicate that radial glia are functionally polarized with differential effects on cell, axon, and dendrite migration. Because of the widespread existence of radial glia, it can be hypothesized that polarized glia are fundamental in structuring developing brain regions. To our knowledge, our functional assays provide for the first time insight into potential cellular mechanisms of polarity formation of RGCs. The identification of functionally relevant glial cell compartments together with a set of innovative *in vitro* systems should enable us in the future to identify the molecular components involved.

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