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Carbonic anhydrase C in the neural retina: Transition from generalized to glia-specific cell localization during embryonic development

(retinoglia/chicken embryo/glutamine synthase/immunohistochemistry/carbonate dehydratase)

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ABSTRACT **The developmental profile and cellular localization of carbonic anhydrase C (carbonate dehydratase; carbonate hydro-lyase, EC 4.2.1.1) in the neural retina of chicken embryos and adults were investigated by immunochemical and immunohistochemical methods. Carbonic anhydrase C is present in the retina by the 3rd day of embryonic development. In the undifferentiated retina, it is detectable in virtually all the cells; however, as cell specialization progresses, its level declines rapidly in the emerging neurons and increases in Müller glia cells. An exception is certain amacrine neurons that contain carbonic anhydrase C to about the 16th day of development. In the adult retina, the enzyme is confined exclusively to Müller cells (the only gliocytes in the retina). Their identification was confirmed by immunostaining for glutamine synthase, an established Müller cell "marker." The presence in the mature retina of both these enzymes in Müller cells indicates that retinal gliocytes combine functional features that, in the brain, are segregated in astrocytes and oligodendrocytes. In the embryonic retina, carbonic anhydrase C and glutamine synthase differ markedly in their developmental profiles, cellular distribution, and susceptibility to regulation by cortisol and by cell interactions. Such differences make these two enzymes an attractive "marker team" for studying developmental mechanisms in embryonic retina and specific functions of Müller cells.**

There has been growing interest in retinal glia cells—i.e., Müller cells—and their role in retina differentiation and function. Progress in this area requires more information about the developmental biochemistry of these cells, including the availability of specific molecular "markers." A well-documented marker is glutamine synthase (1–3), which is confined to Müller cells in avian (4, 5), mammalian (6), and probably other vertebrate retinas (7). Its developmental program and regulation have been studied in detail in the retina of chicken embryos (1, 2).

Recently, we examined the development and cellular localization of carbonic anhydrase (carbonate dehydratase; carbonate hydro-lyase, EC 4.2.1.1) in chicken retina, specifically the "high activity" form of the enzyme referred to as CA-C (or CA-II) and reported to be an oligodendrocyte marker in mammalian brain (8). The avian retina is particularly favorable for such a study because it is avascular and thus free of carbonic anhydrase C-containing blood cells. Carbonic anhydrase C catalyzes the hydration of metabolic CO_2 and the dehydration of bicarbonate, facilitates ion exchange, and participates in control of cellular pH and fluid secretion (9). In adult nonavian retinas, carbonic anhydrase activity has been histochemically localized in Müller cells (10). In the neural retina of mouse embryos, appearance of carbonic anhydrase activity in Müller cells was histochemically correlated with their differentiation (11). In chicken embryo retina, carbonic anhydrase activity was detected early in development (12), but its cellular localization was not investigated.

By using antiserum specific for chicken carbonic anhydrase C as a quantitative and immunohistochemical probe, we determined the developmental program of this enzyme in the neural retina of chicken embryos and investigated its cellular localization. To the best of our knowledge, this is the first attempt to study by immunological detection methods the development of carbonic anhydrase C in embryonic retina.

MATERIALS AND METHODS

Animals. White Leghorn adults and embryos were used throughout this study.

Carbonic Anhydrase C. The enzyme was purified from chicken erythrocytes by affinity chromatography (13). Purity was established by electrophoresis in $NaDodSO_4$/polyacrylamide gels and in two-dimensional gels in the presence of 9 M urea (14).

Antisera. Antiserum specific for carbonic anhydrase C was produced in albino rabbits according to a described protocol (4). Antiserum specificity was verified by immunodiffusion and immunoelectrophoresis. Antiserum specific for chicken retina glutamine synthase was produced in mice and qualified as described (4).

Carbonic Anhydrase C Quantitation. Quantitative immunoelectrophoresis was performed as described by Norgaard-Pedersen (15). Freshly isolated neural retinas (free of pigment epithelium) from adult or embryo eyes were sonicated in barbital buffer, pH 8.6/2.5 mM EDTA/0.1 mM 2-mercaptoethanol, carbamoylated by reaction with 1 M KCNO, and centrifuged. The soluble proteins were then subjected to quantitative "rocket" immunoelectrophoresis in 1% agarose gels (barbital buffer, pH 8.6; ionic strength, 0.02 M)/4% (vol/vol) antiserum. Protein was determined by the method of Lowry.

Immunohistochemistry. Immunohistochemical localization of carbonic anhydrase C was done on sections of whole chicken embryos (3 and 4 days), whole eyes (from day 5 through day 13 embryos), and isolated retinas (from day 14 embryos through adult). These were fixed for 16 hr in neutral formaldehyde, embedded in paraffin, and sectioned (4).

For simultaneous immunohistochemical detection of carbonic anhydrase C and glutamine synthase, hydrated tissue sections were sequentially treated with the following immunological probes diluted 1:50 in Tyrode's solution: rabbit antiserum to carbonic anhydrase C; rhodamine-conjugated goat anti-rabbit IgG Fab' fragment; mouse antiserum to glutamine synthase; fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG. Exposure to each probe was for 30 min at 37°C, after which the

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sections were rinsed in Tyrode's solution. Sections were mounted in FA fluid (Difco) and examined with a Zeiss ICM microscope equipped for mutually exclusive epifluorescence of rhodamine and fluorescein. Control sections were treated in the same way, except that, before use, antisera to enzymes were exposed to purified enzyme at 0.5 mg/ml to sequester specific antibodies.

For localization of carbonic anhydrase C with immunoperoxidase, tissue sections were treated with rabbit antiserum as above, rinsed, and then exposed to goat anti-rabbit IgG conjugated with horseradish peroxidase (1:20; Miles) for 30 min at 37°C. The sections were thoroughly rinsed and treated for 15 min with 0.1% H_2O_2/diaminobenzidine (0.75 mg/ml) in Tris buffer, pH 7.6. Control sections were treated similarly, except that the antiserum to carbonic anhydrase C was first exposed to purified enzyme.

Induction of Glutamine Synthase. Glutamine synthase was induced in the retina by injecting 1 mg of hydrocortisone (cortisol) phosphate in 200 μl of Tyrode's solution onto the chorioallantoic membrane of day 11 chicken embryos, which were then incubated for 48 hr (4). Glutamine synthase specific activity and protein per retina were determined as described (16).

Monolayer Cultures. Monolayer cultures were established and maintained under conditions that minimized cell reaggregation (4). Cells on glass coverslips were fixed in phosphate-buffered 4% formalin (15 min), rinsed, and immunostained like tissue sections, except that 0.02% saponin was added to permeabilize cell membranes.

RESULTS

Antiserum Specificity. The monospecificity of the carbonic anhydrase C antiserum was established by Ouchterlony double-diffusion tests against purified enzyme and against total soluble protein of embryonic and adult retinas and by standard and two-dimensional "crossed" immunoelectrophoresis of these reagents (17). In all cases, a single identical antigen was detected.

Adult Neural Retina. Immunohistochemical examination of adult neural retina detected carbonic anhydrase C only in Müller glia (Fig. 1 *A* and *B*). These cells were identified by their distinct morphology (18) and by simultaneous localization (double-label indirect immunofluorescence) in the same cells of glutamine synthase, an established Müller cell marker (Fig. 1 *C* and *D*) (4, 6). Therefore, in adult avian neural retina, immunodetectable carbonic anhydrase C is a characteristic marker for Müller cells.

The level of carbonic anhydrase C in the adult neural retina was determined by quantitative immunoelectrophoresis to be 27–32 μg/mg of protein. Since avian neural retina is free of blood elements (a source of carbonic anhydrase C), it can be estimated that this enzyme represents ≈3% of the total protein in the adult neural retina. Because the enzyme is confined to only one of the six cell types present in the neural retina—i.e., to Müller cells—their level of carbonic anhydrase C is evidently several times higher than 3%, an unusually high cellular content for an enzyme necessitated, perhaps, by absence of vascularization in the avian neural retina.

Embryonic Retina. During embryonic development of the neural retina, the level of carbonic anhydrase C undergoes striking changes, as determined by quantitative immunoelectrophoresis. Fig. 2 shows the developmental profile of carbonic anhydrase C, compared with that of glutamine synthase and with the growth pattern (protein per retina) of the neural retina. The level of carbonic anhydrase C (per mg of protein) rises sharply early in development to a peak on day 5, then declines till about day 10; by this stage, growth and cell multiplication

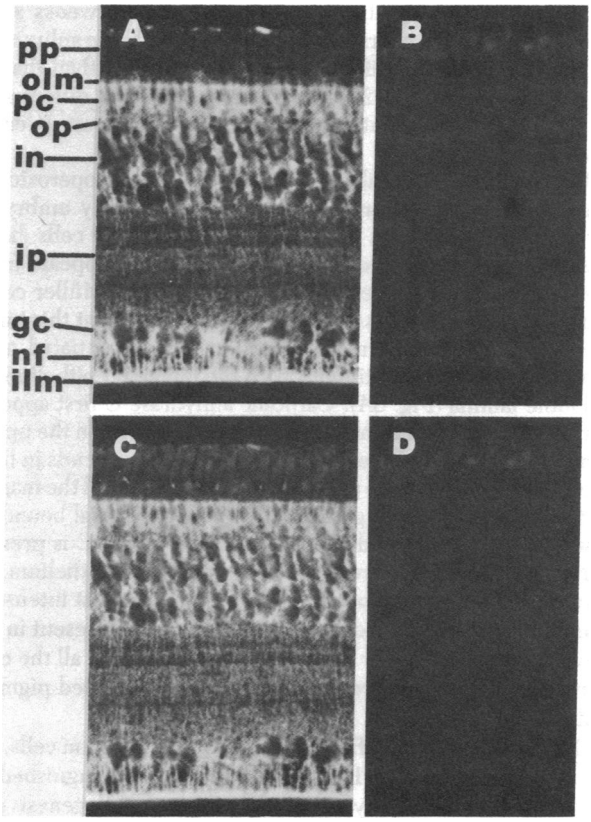

FIG. 1. Simultaneous localization of carbonic anhydrase C and glutamine synthase in Müller glia cells of adult chicken neural retina by double immunolabeling and indirect immunofluorescence of histological sections. (*A*) Rhodamine fluorescence for carbonic anhydrase C is seen only in cells that extend across the whole width of the retina; i.e., Müller glia. (*B*) Control section treated with previously absorbed carbonic anhydrase C antiserum and rhodamine; no fluorescence. (*C*) Fluorescein fluorescence for glutamine synthase in same section as in *A*, showing localization of glutamine synthase in Müller cells only. (*D*) Control section treated with previously absorbed glutamine synthase antiserum; no fluorescence. pp, Photoreceptor cell processes; olm, outer limiting membrane; pc, photoreceptor cell layer; op, outer plexiform layer; in, inner nuclear layer; ip, inner plexiform layer; gc, ganglion cell layer; nf, nerve fiber layer; ilm, inner limiting membrane. (×240.)

in the neural retina are nearly complete and cellular specialization is in progress (19, 20). Thereafter, the level of carbonic anhydrase C increases gradually as the neural retina continues

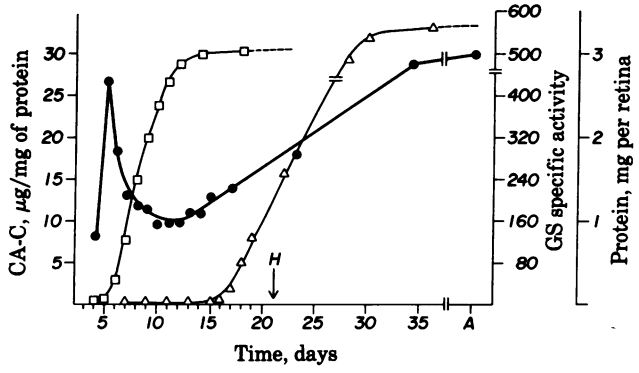

FIG. 2. Developmental profile of carbonic anhydrase C in the neural retina of chicken embryos compared with that of glutamine synthase and with growth of the retina. ●, Carbonic anhydrase C (CA-C) in retina; □, protein per retina; △, glutamine synthase (GS) specific activity. H, hatching; A, adult stage.

to differentiate and matures, and it plateaus ≈2 weeks after hatching. Thus, the overall development of carbonic anhydrase C follows a distinctly different temporal program than that of glutamine synthase (1, 2), and this is reflected in differences in the cellular localization of these enzymes during early development.

Immunohistochemical studies using the immunoperoxidase reaction showed that, in the neural retina of early embryos, carbonic anhydrase C is present in virtually all the cells; however, with progressing cytodifferentiation, it disappears from the neurons and is increasingly restricted to the Müller cells. Carbonic anhydrase C is detectable early on day 3; at this time, the prospective neural retina is still an undifferentiated neuroepithelium and is continuous with the pigment epithelium as a double lamina (Fig. 3*A*). Carbonic anhydrase C first appears at the fold of the lamina adjacent to the lens vesicle in the upper temporal quadrant of the eye (Fig. 3*A*); then, it extends in both the neural retina and the pigment epithelium around the margin of the lens and spreads gradient-like from the radial boundary toward the fundus. By day 4, carbonic anhydrase C is present throughout the neural retina and the pigment epithelium, its immunostaining in the neural retina being still most intense in the upper temporal quadrant (Fig. 3*B*). It is also present in the developing lens. By day 5, it is found in practically all the cells throughout the neural retina and the now pigmented pigment epithelium (Fig. 3*C*).

By day 6, the first definitive neurons, the ganglion cells, are present in the fundus (19, 20), and they are distinguished by absence of carbonic anhydrase C (Fig. 3*D*). As histogenesis progresses, other neurons lose their capacity to immunostain for carbonic anhydrase C. Exceptions are certain amacrine neurons that continue to stain intensely. They first appear as a double layer bordering the arising inner plexiform layer (Fig. 3*D*); as this layer expands, the perikarya of these neurons become vertically displaced, but their processes remain in contact with it and their lateral arborizations form a distinct lamina (Fig. 3 *E* and *F*). Eventually, immunostaining of these, as of all other neurons, decreases to background, but it persists long enough to permit their identification as stratified amacrine neurons of the third level and displaced amacrine neurons of the fourth level (18).

In contrast to neurons, immunostaining of Müller glia cells increases in intensity with development and spreads from their perikarya into the radial processes (Fig. 3 *E–G*). In the fundus (the most advanced region of the neural retina), carbonic anhydrase C is confined predominantly to Müller cells by day 11. However, even in the day 13 neural retina, there still are regional differences in carbonic anhydrase C localization: at the ciliary margin (the least advanced region), carbonic anhydrase C is still detectable in most of the cells, while in the fundus, it is mostly in Müller cells. By day 16, carbonic anhydrase C is confined to Müller cells almost throughout the neural retina. After hatching, it is not detectable outside of Müller cells.

Regulation of Carbonic Anhydrase C and Glutamine Synthase Levels. Cortisol can precociously induce glutamine synthase in Müller cells in neural retina of chicken embryos by differentially affecting gene expression (2, 4). We examined whether the level of carbonic anhydrase C or its cellular localization also could be influenced by this hormone. Day 11 embryos were injected with cortisol; two days later, the neural retina was isolated and the levels of both enzymes were determined. As expected, glutamine synthase specific activity was induced to a level 20–25 times higher than in untreated day 13 controls; in contrast, the level of carbonic anhydrase C was es-

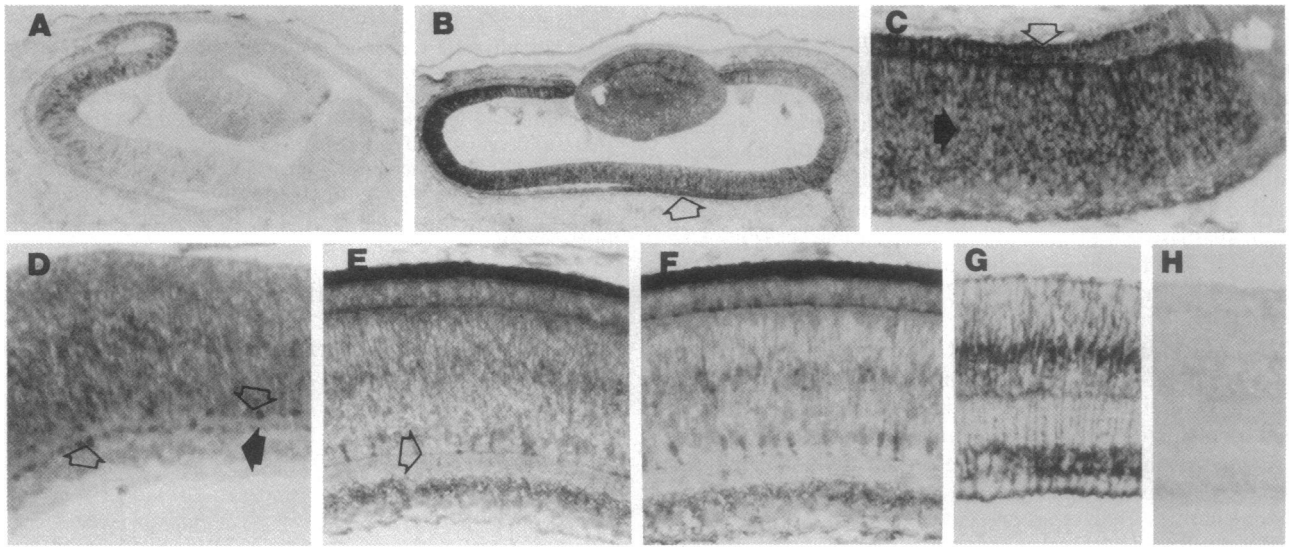

FIG. 3. Localization by immunoperoxidase reaction of carbonic anhydrase C in embryonic retina during development: progressive changes in regional and cellular compartmentalization of the antigen. (*A*) Section through the eye region of a day 3 chicken embryo showing onset of detectable immunostaining for carbonic anhydrase C in the dorsal aspect of the neural retina (NR) and the pigment epithelium (PE) near the developing lens. (×150.) (*B*) Section of eye of day 4 embryo showing staining of the NR, most intensely in the dorsal aspect, and generalized staining of the PE (⌓) and lens. (×56.) (*C*) Retina of day 5 embryo showing generalized staining for carbonic anhydrase C in most cells of the NR (▲) and PE (⌓). (×250.) (*D*) Section of day 8 NR showing reduction or absence of staining in ganglion cells (▲) and staining in double row of prospective amacrine neurons (⌓) that border the inner plexiform layer (IP). (×250.) (*E*) Day 13 embryonic NR sectioned in a region close to the ciliary margin showing immunostaining for carbonic anhydrase C in the amacrine neurons and how their lateral arborizations form a continuous line running horizontally through the IP (⌓). Note also the intensified immunostaining of Müller cell perikarya in the central region of the inner nuclear layer (IN). (×250.) (*F*) Same NR as in *E*, but sectioned closer to the fundus, showing the still intense staining of the amacrine neurons and their arborizations. Elsewhere staining is reduced, except in the perikarya of Müller cells (center of the IN) and their endings (i.e., near the PE and around the ganglion cells). (×250.) (*G*) Day 16 NR showing that staining for carbonic anhydrase C is confined to Müller cells. (×250.) (*H*) Control section for *G*, stained with previously absorbed carbonic anhydrase C antiserum. (×250.)

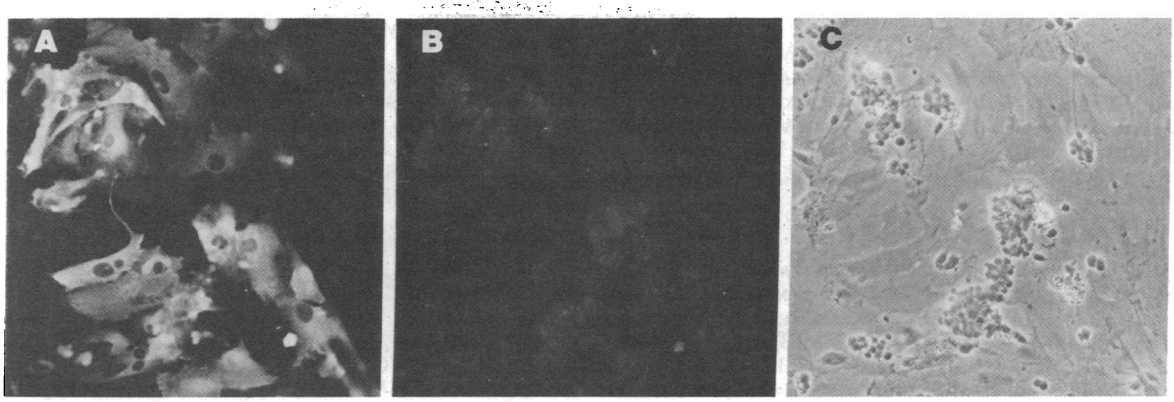

FIG. 4. Immunohistochemical examination by double-label immunofluorescence of carbonic anhydrase C and glutamine synthase in monolayer cultures derived from embryonic neural retina cells. Neural retinas were isolated from cortisol-treated day 13 embryos, the fundal region was dissociated into single cells, and the suspension was plated on glass coverslips. (*A*) Immunostaining for carbonic anhydrase C, showing intense rhodamine fluorescence in most Müller glia-derived epithelioid cells. (*B*) Simultaneous immunostaining of the same cells for glutamine synthase, showing only background fluorescein fluorescence, consistent with the absence of glutamine synthase in these cells. (*C*) Phase-contrast micrograph of same field. (×170.) Results shown are for a culture maintained for 6 days with daily medium changes.

sentially the same as in the controls. Immunohistochemical examination showed that the cellular localization of carbonic anhydrase C also was unaffected by cortisol and was typical for day 13 neural retina: in the fundus, carbonic anhydrase C was mostly in Müller cells; in the peripheral regions, it was also present in amacrine neurons. Glutamine synthase was localized only in Müller cells.

As previously reported (1, 2, 4), glutamine synthase inducibility in Müller cells and the persistence of its induced level require contact-dependent specific interactions with neurons. Previously induced glutamine synthase declines rapidly if the neural retina tissue is dissociated and the cells are maintained monodispersed in monolayer culture, regardless of the presence of cortisol in the medium (21). We examined whether the persistence of carbonic anhydrase C was similarly dependent on normal cell contacts. Day 13 neural retinas were treated with cortisol for 2 days to induce glutamine synthase, and then the fundal region was isolated and dissociated into single cells. The cells were plated as a monolayer culture and were immunohistochemically examined at intervals for carbonic anhydrase C and glutamine synthase.

After 3 hr, the cultures contained gliocytes that simultaneously immunostained for both carbonic anhydrase C and glutamine synthase. Thereafter, stainability for glutamine synthase decreased rapidly, reaching basal level by 48 hr. In contrast, carbonic anhydrase C was detectable in most of the glia-derived epithelioid cells (22) even after 6 days (Fig. 4). Therefore, at the developmental stage examined, control of carbonic anhydrase C level is apparently independent of the kind of cell interactions implicated in the regulation of glutamine synthase in the same cells.

DISCUSSION

Our immunohistochemical demonstration that, in mature avian neural retina, carbonic anhydrase C is confined to Müller glia cells agrees with the similar localization of carbonic anhydrase activity in other species (10, 11). Accordingly, we conclude that carbonic anhydrase C is a reliable glia cell marker in the mature neural retina.

The embryonic neural retina presents a different situation. Early in development, carbonic anhydrase C is immunohistochemically detectable in all the retinoblasts but, with the emergence of definitive neurons, it becomes gradually restricted to Müller cells. However, even during fetal stages, carbonic anhydrase C persists in certain amacrine neurons. These observations suggest that genes coding for carbonic anhydrase C are expressed initially in all the retinoblasts but subsequently "turned off" in developing neurons (last in amacrine neurons) and not in Müller gliocytes.

In mouse neural retina, carbonic anhydrase activity was detected first only in the late fetus and reportedly immediately in Müller cells (11); however, it also appeared transiently in "round" cells near the inner plexiform layer, and these may be homologous to the amacrine neurons described here.

Although, in the mature neural retina, both carbonic anhydrase C and glutamine synthase are Müller cell markers, their temporal and cellular differentiation programs in the embryonic neural retina are quite different (Fig. 2). Unlike carbonic anhydrase C, glutamine synthase activity increases sharply only late in development, is confined to Müller cells at all developmental stages, and is susceptible to regulation by cortisol and certain cell interactions. Some of these differences may be related to the particular functions of the two enzymes. Glutamine synthase is thought to be involved in the recycling of certain neurotransmitter molecules released by physiologically active mature neurons (23, 24). Carbonic anhydrase C has a more general role in homeostasis of neural tissues (9, 25) and therefore may be required much earlier. However, the exceptionally early appearance of carbonic anhydrase C in embryonic eye tissue suggests a more specific contribution to the development of this organ. Considering that carbonic anhydrase C functions in controlling intraoccular pressure (26) and is implicated in accumulation of intraoccular fluids, this enzyme may play an important role in morphogenesis and expansion of the embryonic eye.

In adult brain, carbonic anhydrase C is localized in oligodendroglia (8) and choroid cells (25) and glutamine synthase is present mostly in astroglia (27). In adult neural retina, both enzymes are localized in Müller cells, the only gliocytes in this tissue. Evidently, Müller cells combine functions that in the brain are relegated to disparate kinds of gliocytes. Accordingly, Müller cells may depend on a more complex balance of regulatory signals and mechanisms than their more narrowly specialized brain counterparts to effectively fulfill their developmental and physiological tasks.

We recognize David Shemin on the occasion of his 70th birthday. This work was supported by a research grant (HD01253) from the National Institute of Child Health and Human Development and by a grant (1-733) from the March of Dimes–Birth Defects Foundation.

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