Expression and Downregulation of the GABAergic Phenotype in Explants of Cultured Rabbit Retina

Cheryl Rowe-Rendleman, Cheryl K. Mitchell, Michael Haberecht, and Dianna A. Redburn

Purpose. To study the morphologic and neurochemical development of the rabbit retina in explant culture.

Methods. Explants of retina from newborn rabbits were cultured in defined medium in the absence of serum or soluble growth factors. The morphology of the explant and the neurochemical development of the GABAergic system were examined by light microscopy, autoradiography, and immunohistochemistry for 7 days and compared to those of the postnatal rabbit retina in vivo.

Results. Cultured explants from newborn rabbit retina develop and maintain well-defined plexiform and cellular layers up to 7 days. Exogenous 3H-γ-aminobutyric acid (GABA) and antibodies against GABA labeled a population of horizontal, amacrine, and displaced amacrine cells in the ganglion cell layer during the first 3 days in culture. After 4 days in culture, the extent of uptake and immunolabeling was diminished among all three cell types, but labeled horizontal cells were markedly rare. At 7 days in culture, uptake and GABA-like immunoreactivity could not be detected in horizontal cells, but antibodies to calbindin-D reacted with horizontal and amacrine cells in the appropriate retinal layers. Peanut agglutinin lectin binding studies revealed a mosaic of cone photoreceptor inner segments indistinguishable from that of neonatal retina in vivo.

Conclusions. The experiments show that the maturation of cellular layers and the developmental expression of the GABAergic phenotype can be observed in retinal explants cultured under chemically defined conditions. Histochemical evidence is presented that indicates cultured explants of newborn rabbit retinas express markers of the GABAergic phenotype in a manner consistent with that observed in vivo. The authors show that horizontal cells continue to survive in culture after the diminution in GABA immunoreactivity. Invest Ophthalmol Vis Sci. 1996;37:1074–1083.

Classical studies of neurogenesis in the vertebrate retina demonstrate that cone photoreceptors, horizontal cells (HCs), ganglion cells (GCs), and a few subclasses of amacrine cells (ACs) become postmitotic, migrate to their characteristic positions, and begin terminal differentiation before birth.1–5 In the rabbit, immunocytochemical and autoradiographic evidence indicates that pioneering subclasses of HCs, ACs, GCs, and interplexiform cells (IPCs) synthesize, accumulate, and maintain endogenous stores of γ-aminobutyric acid (GABA) at a time when retinal circuitry is still immature.6–9 As development proceeds, the relative number of cells and cell types that possess GABAergic markers diminishes. The presence of an intact GABAergic system in the neonate presents the possibility that GABA may have a role in the development of the retina. To examine some of the factors that control the expression of the GABAergic phenotype, we have developed an organotypic tissue culture in which the morphologic and neurochemical development of the retina resembles that of intact tissue. A preliminary report of these findings has been presented.10

Compelling evidence from cultured neurons sug-
gests that GABA has a neurotrophic function in the developing central nervous system. In the mature central nervous system, activation of GABA<sub>A</sub> receptors produces membrane hyperpolarization by gating a picrotoxin and bicuculline-sensitive Cl<sup>-</sup> channel. Pharmacologic experiments with isolated cerebellar granule cells prompted Schousboe and coworkers<sup>3</sup> to concur that in developing neurons, the effects of GABA are mediated by high-affinity, picrotoxin-sensitive Cl<sup>-</sup> channels that hyperpolarize the membrane. In contrast, electrophysiological experiments by Cherubini and colleagues<sup>12</sup> indicated that GABA induces a bicuculline-sensitive and Cl<sup>-</sup>-dependent depolarization of CA3 hippocampal neurons from 1-week-old rats. In addition to its effects on membrane polarization, GABA plays a unique role in the metabolism of immature neurons. In cultured mouse neuroblastoma or immature hippocampal cells, exogenous GABA promotes the development of synapses and increases the production of intracellular vesicles.<sup>11,13</sup> Moreover, Ca<sup>2+</sup> imaging studies indicate that the K<sup>+</sup>-stimulated and Na<sup>+</sup>-independent release of GABA elicits Ca<sup>2+</sup> fluxes in neonatal, but not in adult, cerebellar, neocortical, and retinal neurons.<sup>14-16</sup> When considered together, these data suggest that GABA may have more than one mechanism of action in the developing central nervous system.

Results of experiments in the rabbit retina suggest that GABA is involved in the development, maintenance, or both of cellular mosaics in the outer retina. Although the subunit composition of the GABA<sub>A</sub> receptor is not understood fully, immunocytochemical studies in frozen sections of adult and neonatal rabbit retinas indicate that the α1, β2, and β3 subunits co-localize with structures in the outer and inner plexiform layers (OPL and IPL).<sup>17,18</sup> This result is meaningful because the coexpression of recombinant α and β subunits in Xenopus oocytes is sufficient for the formation of benzodiazepine-insensitive receptors with GABA-activated anion channels, suggesting that the subunits in developing retina may form GABA-sensitive ion channels.<sup>18</sup> This hypothesis is supported in part by experiments in which a single intraocular injection of picrotoxin, a noncompetitive inhibitor of the GABA<sub>A</sub> receptor, disrupts the subsequent development of the cone photoreceptor mosaic in newborn rabbits (<24 hours old).<sup>20</sup> Presumably, GABA<sub>A</sub> receptors in the outer retina mediate the effects of picrotoxin on photoreceptors. A similar injection at postnatal day 5 does not have any effect on the organization of photoreceptor mosaics.

In retinas of several mammalian species, the expression of the GABAergic phenotype is under strict regional and temporal control.<sup>19,21</sup> Developmental studies show that GABA-accumulating and immunoreactive cells appear in the inner retina before they do in the outer retina. Lam and coworkers<sup>22</sup> reported that cells in the nascent amacrine and ganglion cell layers accumulate exogenously supplied <sup>3</sup>H-GABA at embryonic day 22 (gestation lasts 31 days). In newborn rabbit retinas, HCs, ACs, IPCs, and presumably displaced ACs in the ganglion cell layer (GCL) incorporate <sup>3</sup>H-GABA. The intensity of radiolabeling increases in these cell types by postnatal day 3. By postnatal day 5, the diversity of neuronal cell types that accumulate <sup>3</sup>H-GABA uptake diminishes. In the GCL, radiolabeling that had been observed in nearly every cell at postnatal day 1 becomes scattered among a few displaced ACs. In the inner nuclear layer (INL), uptake becomes restricted to IPCs and to the row of ACs closest to the IPL. In the outer retina, the labeled profiles of HCs could not be detected.<sup>23,24</sup> In contrast, the uptake of <sup>3</sup>H-GABA increases in Müller glia as the retina matures. The pattern of labeling clearly reveals Müller glia end feet and thin ascending processes in the inner retina.

The pattern of immunoreactivity to GABA is similar to the distribution of reduced silver in <sup>3</sup>H-GABA-accumulating cells in the embryonic and early postnatal retina. Using extremely high-titer antibodies, Pow and Wong<sup>25</sup> show evidence of GABA immunoreactivity in putative ACs and GCs embryonic day 25. Their studies corroborate and extend others that indicate the number and type of immunoreactive cell types increases through postnatal day 3.

We previously reported little or no GABA immunoreactivity in the outer retina of 5-day-old rabbits.<sup>7</sup> Other laboratories also have reported that GABA-like immunoreactivity in adult rabbits is limited to local circuit neurons in the inner retina. However, in our most recent studies,<sup>17</sup> we have observed some GABA-like immunoreactivity among HCs localized within the paracentral region at postnatal day 5. Horizontal cells in the peripheral retina were not immunoreactive with the antibody to GABA.<sup>26</sup>

Downregulation, or the gradual reduction in the expression of GABAergic markers, occurs by postnatal day 5 in many, but not all, ACs. However, unlike HCs, the labeling of the remaining population of cells in the amacrine and ganglion cell layers is robust and appears evenly distributed throughout the retina. The mechanism for the downregulation of GABA-immunoreactivity and <sup>3</sup>H-GABA uptake in HCs is unknown. Newborn rabbit retina was cultured to study this developmental phenomenon in vitro.

Explant cultures of retinal punches offer a versatile tool for the study of development in vitro. This approach permits the examination of developing GABAergic neurons in a chemically defined medium. Explants offer several advantages over cultures of dissociated retinal neurons: The organization of the retina and morphology of various cell types is not compro-
Fixed and stained sections from three cultured measuring approximately 400 μm². Pyknotic cellular nuclei and the soma in the GCL were counted in a field punches on each type of filter were examined at 16X, had been coated with either polylysine or Matrigel. Approximately 20% ± 2% of the cells in the GCL had dark pyknotic nuclei. After 4 days in vitro (DIV), the relative number of pyknotic nuclei in cultures grown on Matrigel was still 20%. In contrast, in cultures that had been grown on polylysine, approximately 50% of the cellular profiles in the GCL were pyknotic (data not shown). Results were consistent, and only tissue that remained on filters was used in the following experimental procedures. Explants that did not stay on the Matrigel-coated filters were discarded.

Explants were maintained in 1 ml of medium for as long as 1, 3, 5, or 7 days in Dulbecco's modified Eagle's medium containing F-12 nutrient mixture (DMEM–F-12; Gibco Laboratories; Grand Island, NY) and supplemented with 1.5 mg/ml transferrin, 0.25 mg/ml insulin, 9.6 mg/ml putrescine, 0.03 mg/ml selenium, 0.1 U/ml progesterone (all from Sigma Chemical) and the antibiotic mixture. Cultures were incubated at 37°C in a humidified chamber, equilibrated with 5% CO₂, and fed with supplemented DMEM–F-12 after a conditioning period of 48 hours.

**Peanut Agglutinin Cytochemistry**

To visualize the photoreceptor mosaic, harvested explants at 5 DIV were pretreated for 15 minutes with phosphate-buffered saline (PBS) containing 1.5 mg/ml bovine serum albumin, 1 mM CaCl₂, and 1 mM MgCl₂. Our earlier work showed that there was no difference in peanut agglutinin (PNA) labeling in fresh or fixed tissue. The tissue was exposed for 15 minutes to fluorescein isothiocyanate-tagged PNA (Vector Laboratories, Burlingame, CA) that had been diluted 1:10 in PBS as previously described. The fluorescence was viewed on a Zeiss microscope equipped with epi-fluorescence and the appropriate excitation filter.

**Immunocytochemistry**

The explants (N = 3; two rabbits per N) were harvested after 1, 3, 5, or 7 days DIV and rinsed in phosphate buffer, pH 7.2. Explants for GABA immunocytochemistry were fixed for 24 hours at 4°C in 4% paraformaldehyde and 0.1% glutaraldehyde (Ted Pella; Redding, CA) in 0.1 M phosphate buffer, pH 7.2. Explants for calbindin immunocytochemistry were fixed in 4% paraformaldehyde under the same conditions. After rinsing, the explants were incubated in antibody that had been diluted in PBS, pH 7.2, containing 0.2% Triton X-100 and either 0.1% normal rabbit serum for rat anti-GABA (Chemicon, Temecula, CA) immunocytochemistry or 0.1% normal horse serum for mouse anti-calbindin-D 28K (CaBP-28 kDa; Sigma Chemical). Each antibody was diluted 1:1000 in buffer, and the explants were exposed for at least 7 days at 4°C. Background was reduced substantially by immersing the explants in PBS for a minimum of 24 hours before incubation with secondary antibody.

Secondary antibodies and avidin–biotin complex (ABC) reagent were obtained from Vector Laboratories. Anti-mouse and anti-rat biotinylated secondary
antibodies were diluted 1:200 in PBS. Explants were incubated for 24 hours and rinsed in PBS. This was followed by incubation overnight in ABC (Vector). Immunoreactivity was visualized with 3,3'-diaminobenzidine tetrahydrochloride (Polysciences; Warrington, PA) in 0.05 M Tris–HCl, pH 7.2, containing 0.1 M imidazole and 0.01% H2O2.

The tissue was dehydrated in a graded series of alcohols and embedded in epoxy resin. To view immunoreactivity, plastic sections (5 μm) were prepared on a Zeiss sliding microtome. One-micrometer sections were counterstained with cresyl violet (Nissl) for morphologic analysis.

**Incorporation of 3H-GABA**

Freshly harvested explants were incubated in KRB containing 50 μCi/ml 3H-GABA (76.2 Ci/mmol, specific activity; Amersham, Arlington Heights, IL) as previously described. The sections were fixed overnight in 0.05 M cacodylate buffer containing 2.5% glutaraldehyde and postfixed for 1 hour in 1% osmium tetroxide (EM Sciences; Gibbstown, NJ). The tissue was embedded in epoxy resin and blocks, were sectioned at 1 μm for light microscopy. The sections were coated in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) and exposed for 6 weeks. (N = 3; 1 rabbit per N.)

**RESULTS**

**Gross Morphology**

Uniform circular explants containing all layers of the neural retina were removed from newborn rabbit. To prevent mechanical shearing or tearing that ultimately could distort the morphology of the cultured explant, pieces of retina were floated free of the sclera and pigmented epithelium with a gentle stream of filtered KRB (Fig. 1).

Gross analysis of the explant after 1 DIV revealed a trilaminar structure with a clear separation of the cell layers by the outer and inner plexiform layers (Fig. 2a). The cells in the outer and inner nuclear layers showed distinctive shapes and staining characteristics. Elongated neuroblasts in the developing outer nuclear layer (ONL) appeared dark in the presence of the Nissl stain. The outer border of explant was distinguished by a darkly stained continuous line suggestive of an outer limiting membrane (OLM). The outer border of explant was distinguished by a darkly stained continuous line suggestive of an outer limiting membrane (OLM). Some of the larger profiles at this border contained mitotic figures (data not shown). The zone that separates the ONL from the INL was demarcated by a row of pale, crescent-shaped cells (arrowheads) with processes in the OPL. The lighter staining of the cell nuclei suggests that the chromatin in these cells is dispersed. Somata were larger than the surrounding

ones in the ONL and INL. Their shapes and positions were consistent with those of HCs in normal retina. Approximately one third of the soma in the INL and CCL were pale in the presence of Nissl compared to those in the ONL. Almost all were located in a row that lay on either side of the IPL. The IPL was discrete and contained numerous pale processes in several lamina. Smaller cells with round somata that lay just proximal to the IPL in the INL compared favorably in size and position to ACs. At least two sizes of cells were observed in the GCL of cultured explants. Many of the large somata on the vitreal side of the IPL appeared mature and were probably GCs (asterisk). The smaller, more numerous somata were probably displaced ACs. In addition to ACs and GCs, immunofluorescent labeling with GABA antibodies indicates that some subtypes of IPCs are also established at birth. It is possible that IPCs comprise some of the mature cell types in the cultured explant. Pyknotic profiles (long arrows) were observed in the explants at each day of culture. At 1 DIV, the majority of these opaque cells appear limited to the inner retina.

After 3 DIV (Fig. 2b), most of the cell bodies in the ONL were elongated and immature, whereas many of those in the INL were pale, rounded, and mature. The density of cell bodies adjacent to the OPL was greater than that of cells at the OLM. Many of the cells that bordered the OLM tended to elaborate tiny protruberances that resembled photoreceptor inner segments. The development of rudimentary inner segments with well-developed cilia has been demonstrated in dissociated cultures from 1- to 2-day-old Long Evans rat and organotypic slice cultures from 6-day-old albino rat reti-
In those studies, the cells that elaborated these inner segments often were oriented toward each other in circular patches called rosettes or retinoids. Rosettes were rare in explants that remained attached to the Matrigel support throughout the culture period. In flat explants, the presumptive inner segments were parallel and produced a regular array across the the surface of the explant.

At 5 DIV (Fig. 2c), the arrangement of the somata in the outer retina was more columnar as it was in intact retina. In the tips of the putative inner segments, swelling developed that was comparable to the cytoplasmic swelling that gives rise to the elongated cilium of the inner segments.27

At this level of study, distinctions between rod and cone photoreceptors could not be made. However, in normal development, cone photoreceptor processes migrate to the OPL from large somata in the ONL at the border with the OLM. The somata of rod photoreceptors are generally smaller and closer to the border with the OPL. The arrangement of cell bodies in the ONL looked like that of the mature retina. The thickness of the ONL was nine cell somata deep, and compaction near the center of the layer was 60 to 100 cells/mm². The thickening of the OPL suggests that the neurites from photoreceptors, HCs, and IPCs entered the OPL at this stage. The larger HC cell bodies (arrowheads) were detected easily and formed a single row across the section.

The GCL was noticeably thinner at 5 DIV than at 1 DIV. The number of larger, presumably GC somas were fewer than the smaller, presumably displaced AC somas. Pyknotic profiles in the GCL increased as the explants aged. This is not unexpected because the central nervous system targets of GCs are not present in the culture. The thinning of the GCL could reflect necrosis and/or programmed cell-death among cells in the GCL.

At 7 DIV, the explants became thinner and more fragile (Fig. 2d). Rosettes were apparent only in sections of explants that did not adhere to the Matrigel during the culture period. To promote adhesion, cultured explants were disturbed as little as possible.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933418/)
FIGURE 3. Peanut agglutinin (PNA) histochemistry in 5 days in vitro culture shown in wholemount. Fluorescence-tagged PNA selectively labels galactosyl (β-1,3) N-acetylgalactosamine, a glycoconjugate associated with the cone interphotoreceptor matrix in intact tissue. As shown here, the plane of focus is on the outer surface of the retina. Circular labeling in the explant corresponds to the membranes on inner segment-like processes in the culture. Bar = 15 μM.

ther thinning and loss of the nerve fiber layer and cells in the GCL was expected and is consistent with the severance of central connections for the GC fibers. There were a few surviving cell somata in the GCL (asterisk), and these were probably displaced ACs. No distinguishable difference in morphology was observed in explants from central, inferior, or superior retina at any experimental time point.

Peanut Agglutinin Histochemistry

Peanut agglutinin binds to glycoconjugates in the inner segments of cone photoreceptors. This lectin has a binding affinity for galactosyl (β-1,3) N-acetylgalactosamine, which is associated with the interphotoreceptor matrix surrounding the cone photoreceptor cells.29,30 At 5 DIV, PNA binding was most pronounced in the peripheral membrane of the cellular elements in the developing ONL (Fig. 3). The fluorescent labeling was concentrated at the retina’s most distal surface. The lectin revealed a regular mosaic of circular profiles that correspond in size and position to the cone photoreceptor array micron. The pattern of PNA histofluorescence was the same as that obtained from retina of a 5-day-old rabbit, suggesting that the normal array of cone photoreceptors was maintained in these 5-day-old cultures.16

Calbindin Immunocytochemistry

To determine whether HCs survive in cultured explants when GABA-immunoreactivity and 3H-GABA uptake cannot be detected, sections were probed for immunoreactivity to cell-specific proteins expressed throughout development. In developing and adult rabbit retina, a monoclonal antibody to calbindin, a calcium-binding protein, reacts predominantly with A-type, and to a lesser extent B-type, HCs.21-35 After 3 DIV (Fig. 4a), robust calbindin immunoreactivity was detected in HCs (short arrows). Some of the short, stubby neurites in the OPL were labeled as well, but it was not possible to distinguish A- from B-type HCs in the sections. After 7 DIV (Fig. 4b), calbindin immunoreactivity increased in HC somas (short arrows) and neurites in the OPL. Cells were evenly dispersed in the sections, and the neurites appeared to have grown thicker and longer. The antibody also labeled cells (arrowheads) on either side of the IPL. Calbindin immunoreactivity in HCs was dispersed evenly in all re-

FIGURE 4. Calbindin immunoreactivity in the cultured retina. Various cell types in the inner nuclear layer and ganglion cell layer from 3 and 7 days in vitro (DIV) were immunoreactive to calbindin antibodies. At 3 DIV (a), horizontal cells (HCs, short arrows) are the predominant immunoreactive cell type in the retina. It is not possible to distinguish A- from B-type HCs in the vertical section. At 7 DIV (b), staining in HCs was retained, and staining of cells in the inner retina (arrowheads) became more prominent. Bar = 20 μM.
regions of the retina, which was the pattern we observed in normal development.

**GABA Immunocytochemistry**

At 1 DIV, antibodies to GABA reacted with the somata of HCs (arrowheads) and cells in the inner retina (Fig. 5a). Immunoreactivity that was detected in HCs at 3 DIV (data not shown) was noticeably diminished at 5 DIV (Fig. 5b). The pattern of staining in the OPL was irregular, and a single immunoreactive HC was detected in approximately 50 sections obtained from the eyes of six animals. Immunoreactive ACs (short arrows) appear evenly dispersed in a single layer just proximal to the IPL at 1 DIV. At 5 DIV, the staining was more pronounced in the inner retina than at 1 DIV. The immunoreactive soma of putative ACs were small (short arrows) and appeared on either side of the IPL. Although we have shown regional differences in GABA immunoreactivity during normal development, this was not observed in culture.

**Incorporation of $^3$H-GABA**

After 1 DIV, the incorporation of $^3$H-GABA was found in the HC, AC, and GC layers of the explant (Fig. 6a). The label appeared in HC soma (arrowheads) that were identified by their position in the INL and characteristic crescent shape. The density of silver grains was greatest over the putative AC and GC somas (arrows). Occasionally, silver grains decorated profiles in the ONL near the OLM. This type of labeling also was observed in vivo, but the nature of the cells is unclear. It is possible that these were postmitotic cells in the process of migrating out of the ventricular zone toward the INL.

At 3 DIV, the proportion of cells in the inner retina that accumulated exogenous $^3$H-GABA increased relative to that of HCs in the outer retina. In the labeled HCs (arrowheads), the density of silver grains was greatest in the soma but included processes that extended laterally into the OPL (Fig. 6b). In the inner retina, ACs (arrows) just proximal to the INL were labeled heavily. These cells extended processes to two sublamina in the IPL.

After 5 DIV (Fig. 6c), there was no specific incorporation of $^3$H-GABA in the outer retina. Longer exposure of the sections (up to 8 weeks) did not result in the detection of any new cell types nor in an increase in the number of labeled HC soma. In contrast, labeling in the inner retina remained robust in the cell bodies (arrows) and in the two sublamina within the IPL. Explants from central, inferior, and superior retina showed the same pattern of $^3$H-GABA uptake.

**DISCUSSION**

The overall goal of this study was to develop an experimental system to examine the maturation of GABAergic neurons in vitro. In the current study, we show that markers of the GABAergic phenotype are expressed in cultured neural retina in a manner that is temporally and spatially consistent with that observed during development in normal retina. This is significant because several investigators have developed longer surviving cultures and have reported the presence of neurotransmitters, but the temporal expression of these neurochemical markers was altered.

Growth factors, cellular interactions, and age of the donor at the time of culture affect the development of various cell types. Growing evidence suggests that adjuvants, such as bovine serum albumin, cellular adhesion proteins, and growth factors from immortalized cell lines, extend the life and promote the development of various cell types in culture. In pioneering studies, Adler described that monopolar cells...
FIGURE 6. Autoradiography of $^3$H-7-aminobutyric acid uptake in neonatal explants after 1 (a), 3 (b), and 5 (c) days in culture. The sections are unstained. Reduced silver grains are observed within cellular profiles corresponding to a population of HCs (arrowheads) after 1 and 3 days in culture. The density of grains over horizontal cell profiles is reduced after 3 days and absent after 5 days in culture. In contrast, grains associated with cells in the amacrine and ganglion cell layers (arrows) are seen throughout the culture period. Bar = 20 $\mu$M.

from dissociated embryonic chick retina develop cone photoreceptor inner but not outer segments in cultures supplemented with serum. Hollyfield and colleagues argued that the close juxtaposition of glycosylated elements in pigment epithelium with nascent photoreceptor inner segments promotes the differentiation of outer segments and the upregulation of opsin proteins in dissociated Xenopus photoreceptors. Turner and colleagues showed that retinal pigment epithelium-conditioned medium and a diffusible factor from glia enhanced cell survival and promoted expansion of the apical membrane in photoreceptors that were isolated from 1- to 2-day-old rats. Thus, it appears that certain specialized structures in the retina require nonneuronal or extraretinal factors, or both, for differentiation.

In our cultures, 1-day-old rabbit retinas were cultured in a chemically defined medium in the absence of pigment epithelium, serum, or soluble growth factors. Punches of neural retina were grown in the presence of Matrigel, which is a mixture of basement membrane proteins, particularly laminin, collagen IV, heparin sulfate proteoglycans, and entactin. We postulate that this complex provides extracellular matrix proteins that enhance the probability that cells will remain attached to the substrate. The laminar structure of the retina is minimally perturbed by the transfer of the explant to the culture dish. Neurites in the inner and outer retina can grow toward their appropriate targets in the plexiform layers in the presence of appropriate gradients of adhesion molecules and neurotransmitters. The plexiform and nuclear layers are still present after 7 days in vitro. The binding of PNA by structures in the photoreceptor layer of the explant at 5 DIV indicates that inner segment glycoproteins are expressed in vitro. The presence of a regular mosaic of fluorescent profiles suggests that cone photoreceptors elaborate inner segment proteins in the absence of elements from the pigmented epithelium. In normal rabbits, outer segments appear at day 10; thus, the expression of outer segment proteins (e.g., guanylate cyclase or phosphodiesterase) was not investigated.

To examine the maturation of the GABAergic phenotype in vitro, we evaluated the results of other experimental systems comprised of dissociated cells and organotypic slices of retina. The data show that elements of the microenvironmment influence the development of neurochemical phenotypes in culture. Lührke and colleagues maintained dissociated HCs from 3-day-old rabbit retinas in sandwich cultures for up to 21 days. The neurons had stout primary dendrites that resembled those of A- and B-type HCs. GABA-like immunoreactivity and $^3$H-GABA uptake were detected in these neurons, but the GABAergic markers did not show the same developmental time course as that seen in intact retina. These results
suggest that the pattern of neurochemical maturation in horizontal cells may rely on interactions between the neurons and their targets in the OPL during the period of synaptogenesis in the outer retina.

Previous studies have shown that organotypic cultures express a variety of cell-specific markers. Retinas from 6-day-old rats were cultured on clotted chicken plasma. The slices survived for more than 4 weeks and showed well-defined lamination. Not unexpectedly, the proportions and thickness of the different layers were distorted in the older cultures. At 16 DIV (almost equivalent to postnatal day 22), antibodies against GABA labeled ACs but not HCs. Interpretations of a different study suggest that the GABAergic phenotype is downregulated after postnatal day 12 in rat HCs. Kalloniatis and Fletcher demonstrated that antibodies to GABA label HCs in 8- to 12-day-old rats. Because the HCs in the organotypic cultures were examined after the peak period of expression of GABA-like immunoreactivity in the intact rat retinas, it is possible that GABA immunoreactivity in the outer retina was missed.

We examined calbindin immunoreactivity in cultured explants of rabbit retina to determine whether the diminution of GABAergic markers in HCs coincides with cell death or dysfunction. Calbindin immunoreactivity was detected in HCs of 3 DIV punch cultures. It was not possible to distinguish between A- and B-type HCs in the transverse sections used. After 7 DIV, the pattern of calbindin immunoreactivity resembled that of intact neonatal rabbit retina. The presence of calbindin immunoreactivity in HCs suggests that the metabolic capacity of these cells has not been compromised by culturing the retina. Further, it can be confirmed that the diminution of GABAergic markers in explant cultures at 5 DIV is not the result of selective HC death. The role of calbindin in cells has not been elucidated fully. Its calcium binding properties suggest that it may be involved in regulating intracellular ionic calcium concentrations. Calbindin-like immunoreactivity has been observed in retina from several species. During development in rats, calbindin immunoreactivity was observed as early as embryonic day 17 in the outer and inner thirds of the neuroblastic layer. Peak immunoreactivity was observed between postnatal days 2 and 4 and was located in HCs, ACs, and GCs.

Our preliminary characterizations indicate that explanted rabbit neural retina possess neurochemical similarities with intact retina, particularly with respect to the regulation of GABAergic markers. Cells in the horizontal, amacrine, and ganglion cell layers of the explant react with antibodies to GABA and accumulate $^3$H-GABA in a manner consistent with that observed in intact postnatal retina. The labeling of putative ACs, displaced ACs, and HCs becomes more robust at 3 DIV. These events indicate that HCs, ACs, and displaced ACs maintain endogenous stores of GABA and possess a GABA transport system in early cultures. At 5 DIV, the number of radiolabeled and immunoreactive cell types diminishes, with the most striking decrease occurring in the HC population. Accumulating evidence indicates that HCs play an important role in the integration of neurotrophic signals in the OPL. We propose that chemical signals, cellular interactions, or both promote the downregulation of the GABAergic phenotype, especially in HCs. We speculate that GABA itself may be one of the regulatory signals involved in this change. Ongoing studies will examine the effect of growth factors on the expression of other neurochemical markers in retinal punch cultures.

Key Words
development, $\gamma$-aminobutyric acid (GABA), histochemistry, horizontal cells, immunocytochemistry, retinal cell culture

Acknowledgments
The authors thank Cindy Koutz and Lilly Krosby for technical assistance, and they thank Dr. Tom Reh for helpful discussions that led to the development of this project.

References
7. Messersmith EK, Redburn DA. $\gamma$-Aminobutyric acid immunoreactivity in multiple cell types of the developing rabbit retina. *Vis Neurosci.* 1992;8:201–211.


