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

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**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 $^3$H-$\gamma$-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.\(^1\) 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 $\gamma$-aminobutyric acid (GABA) at a time when retinal circuitry is still immature.\(^6\) 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-
GABAergic Phenotype in Retinal Cultures

GABAmergic neurons in a chemically defined medium. Expression of GABAergic markers, occurs by postnatal day 5 in many, but not all, ACs. However, unlike HCs, the expression of GABAergic markers appears evenly distributed throughout the retina. The mechanism for the downregulation of GABA-immunoreactivity and 3H-GABA uptake in HC 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. Experiments offer several advantages over cultures of dissociated retinal neurons: The organization of the retina and morphology of various cell types is not compromised.
mised by enzymatic dissociation; the retina is isolated and accessible to pharmacologic manipulation; and developmental pathways that are mediated by cell–cell contact are minimally perturbed.

MATERIALS AND METHODS

Culture

This study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All salts and reagents were of cell culture grade and were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted. New Zealand White rabbits pups (<24 hours old) were obtained from a local supplier and were killed on the day of arrival. The eyes were enucleated and immersed in sterile oxygenated Krebs–Ringer bicarbonate buffer (KRB), pH 7.4 (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4, 25 mM NaHCO_3, 1.2 mM KH_2PO_4, 2.5 mM CaCl_2, 11.1 mM glucose, all final concentrations) that had been supplemented with a 1% mixture of antibiotics (per milliliter: 10,000 U penicillin, 10,000 µg streptomycin, 25 µg fungizone; JRH Biosciences, Lenexa, KS) and filtered through a 0.25-µm filter unit (Nalge, Rochester, NY).

The anterior portion of the eye was removed under aseptic conditions by an encircling incision just dorsal of the ora serrata. After removal of the lens and vitreous, the tissue was flattened with radial cuts through the retina and sclera. Six uniform pieces of retina (two central, two inferior, two superior) were made with a 2-mm diameter dermatologic biopsy punch. A gentle stream of filtered KRB was used to float the punches of neural retina off the sclera and pigmented epithelium. The punches were transferred to a petri dish and were mounted photoreceptor-side up on sterile cellulose acetate filters (Micron Separations, Westborough, MA) that had been coated with a thick emulsion of Matrigel (Collaborative Biomedical Products–Becton Dickinson Labware; Bedford, MA). Explants (N = 3; two rabbits per N) were harvested after 1, 3, 5, or 7 days DIV and rinsed in phosphate-buffered saline (PBS) containing 1.5 mg/ml bovine serum albumin, 1 mM CaCl_2, and 1 mM MgCl_2. 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 epifluorescence and the appropriate excitation filter.

Immunocytochemistry

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 CaCl_2, and 1 mM MgCl_2. The explants were incubated in antibody that had been diluted 1:1000 in buffer, and the fluorescence was viewed on a Zeiss microscope equipped with epifluorescence and the appropriate excitation filter.

Secondary antibodies and avidin–biotin complex (ABC) reagent were obtained from Vector Laboratories. Anti-mouse and anti-rat biotinylated secondary 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% CO_2, and fed with supplemented DMEM–F-12 after a conditioning period of 48 hours.
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). 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 GCL were pale in the presence of Nissl compared to those in the ONL. Almost all were located in a row that lay just proximal to the IPL in the INL compare 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 AGs. In addition to AGs 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 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.
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 micro. 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 \(^3\)H-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.\(^{31-33}\) 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 retinas.

**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.

**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.10

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,17 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.28,34

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.35–38 In pioneering studies, Adler39 described that monopolar cells...
FIGURE 6. Autoradiography of $^{3}$H-$\gamma$-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 μM.

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 microenvironment influence the development of neurochemical phenotypes in culture. Lohrke 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.28 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 Fletcher41 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 retinal cells 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-immunoreactive cell types diminish 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.42

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 3H-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, γ-aminobutyric acid (GABA), histochemistry, horizontal cells, immunocytochemistry, retinal cell culture

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**References**


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