

# rd1 Photoreceptor Degeneration: Photoreceptor Rescue and Role of Metalloproteases in Retinal Degeneration

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# Mouse retina explants after long-term culture in serum free medium

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**Abstract**

The neonatal mouse retina remains viable as an explant in serum-supplemented growth media for more than 4 weeks. Interpretation of drug effects on this tissue is compromised by the enigmatic composition of the serum. We sought to remove this ambiguity by culturing neonatal as well as late postnatal mouse retina in serum-free nutrient medium. In this study three important observations were made, (1) there is histotypic development of neonatal as well as preservation of late postnatal mouse retinal structure during long-term culture in serum-free medium, although the late postnatal tissue tends to show some loss of cells in the outer nuclear layer. (2) Protein expression in explant photoreceptor cells was similar to that in the litter-matched ones, except for green cone opsin and interphotoreceptor retinoid-binding protein, although mRNA of the latter is present at similar amounts as in age-matched in vivo controls. (3) Cells of the inner retina stained by antibodies to calcium-binding proteins display some novel sprouting of processes. The results show that the mouse retina can be cultured as an explant for more than 4 weeks in a serum-free medium. This represents an important step forward because, (1) the possibility of interference of drug effects by unknown serum factors has been eliminated; and (2) the spent culture medium can be analyzed to investigate biomolecules released by the retina in vitro. 

*Keywords:* Serum-free culture; Retinal cell survival; Photoreceptor-specific protein; Calcium-binding protein

## 1. Introduction

We are focusing our efforts on developing a mouse retinal explant culture system that will be able to perform the functional dynamics of the organ beyond those of the dissociated cells. The principle advantage is that organotypic in vitro systems are likely to add methods for more advanced and detailed analysis, which is not possible in various in vivo situations that remain difficult to interpret due to their complexity. At present, mouse neonatal retinal explants can be maintained in vitro for more than 4 weeks (Caffé et al., 1989; Caffé and Sanyal, 1991; Söderpalm et al., 1994; Mosinger Ogilvie et al., 1999) i.e. the time span in which major developmental and degenerative processes in the normal and retinal degeneration (rd) retina take place (e.g. Sanyal and Hawkins, 1981). Morphological and molecular analyses of the tissue demonstrated that, while in culture, most of the histotypic and neurochemical characteristics develop at roughly equivalent ages when compared with the in vivo littermates. This system was then exploited for investigations into retinal apoptotic mechanisms (Söderpalm et al., 1999), as well as for screening of potential photoreceptor rescue agents. We, and others, reported that combinations of survival factors, viz., NGF + FGF2 or CNTF + BDNF, is a much more efficient strategy to protect photoreceptor cells from degeneration than when these compounds are used separately (Mosinger Ogilvie et al., 2000; Caffé et al., 2001).

The primary aim of this report is to convey if the mouse retinal explant survives under serum-free condi-

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tions. The use of serum in the culture medium creates an obstacle for straightforward interpretation of results. Serum is a complex emulsion of proteins (e.g. globulins, albumin), polypeptides (trophic factors and growth inhibitors), lipids and additional organic and inorganic molecules, some of which remain unknown. From batch to batch the serum components may differ quantitatively and qualitatively. This limits the validity of assays applied to identify proteins in the spent medium, whereas the presence of unknown substances introduces uncertainty that any observed effect is solely due to the compound added by the experimenter. Such complications can be avoided by using a serum-free culture system (e.g. Bottenstein and Sato, 1979; Koper et al., 1984; Romijn, 1988). In the past reports on short term maintenance of dissociated retinal cells in serum-free medium have appeared (e.g. Politi and Adler, 1988; Spoerri et al., 1988; Altshuler and Cepko, 1992; Sheedlo and Turner, 1995). More recently several variations of retinal explant cultures in serum-containing growth medium were published (Germer et al., 1997; Mosinger Ogilvie et al., 1999; Johansson et al., 2000; Pinzon-Duarte et al., 2000). However, data about long-term serum-free culture of the whole mouse retina are not available.

To achieve our aim histology, immunohistochemistry and number of photoreceptors of neonatal and late postnatal mouse retinal explants that were kept in serum-free medium for a long-term were analyzed. For the qualitative and quantitative assessment of the photoreceptor population, three antibodies recognizing rhodopsin, blue- and green cone visual pigments (Szél et al., 1986, 1996) were used. These are photoreceptor-specific markers for which the temporal and spatial characteristics are known in detail (Szél et al., 1993), serving as a good template for comparative purposes. The inner retina profiles were qualitatively assessed by staining with antibodies which selectively bind three calcium-binding proteins i.e. calbindin, parvalbumin and calretinin. These markers were selected because they visualize broad spectra of cellular elements, i.e. horizontal, bipolar, amacrine and ganglion cells, in the mouse retina and a detailed report on their in vivo distribution appeared recently (Haverkamp and Wässle, 2000). We consider these data not comprehensive but sufficient to evaluate the status of the retinal explant in culture.

## 2. Material and methods

### 2.1. Animals and tissue dissection

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the European Communities Council Directive (86/609/EEC). In addition, the Swedish National Animal Care and Ethics Committee approved experiments. Handling of animals and tissue was described in details previously (Caffé et al., 1989; Caffé and Sanyal, 1991). For the current experiments this basic protocol was altered only slightly. Briefly, pigmented (+/+) C3H mice were sacrificed at postnatal day 2 (PN2) or PN7 by decapitation and at PN11 or PN21 by cervical dislocation. The removed heads were wiped clean with tissue soaked in 70% ethanol. Wrapped in this way the heads were transported into the culture room to a laminar flow cabinet from which point onwards all handling was performed aseptically. The eyes were enucleated and incubated in a basal medium supplemented with 1.2% proteinase K (Sigma Chemical Co, St. Louis, MO) at 37 °C for 15 min. To inhibit the proteinase K activity the eyes were thoroughly (3–5 times) rinsed with R16 nutrient medium. A brief treatment with 2% serum prior to thorough rinsing was also tested, but no difference in final outcome between the two treatments was observed after which the rinse-only option was used routinely. The anterior segment, vitreous body, and sclera were then removed and the retina flat-mounted with the photoreceptor-side down on a cellulose filter attached to a polyamide grid. Explants with retinal pigment epithelium (RPE) attached were cultured in 6-well culture dishes.

### 2.2. Tissue culture

The complete list of chemicals making up the originally developed R16 nutrient medium for brain tissue (GIBCO BRL, Gaithersburg, MD) was published before (Romijn, 1988; Romijn et al., 1988). However, since the R16 nutrient medium takes central stage in the current study, we briefly re-describe the composition of this medium. The R16 powder is composed of 41 ingredients that can arbitrarily be divided into three groups. Group 1 consists of salts. Group 2 includes the amino acids except for the potentially neurotoxic amino acids glutamate and aspartate. Group 3 includes sugars and vitamins. Chemicals for the R16 stock solution are dissolved in Millipore water to which $NaHCO_3$, trace elements, biotin, and ethanolamine are added. The original R16 medium is prepared by adding 19 supplements, composed of BSA, hormones and vitamins, to the stock solution (Romijn, 1988). For the retinal culture cytidine 5′-diphospho-ethanolamine and cytidine 5′-diphosphocholine are added (Caffé et al., 1989). The retinal explants were cultured in 1.6 ml of serum-free R16 nutrient medium. Further culture conditions and the frequency of medium replacements remained the same as published before (Caffé and Sanyal, 1991; Caffé et al., 1993; Söderpalm et al., 1994, 1999, 2000). For the present study all explants were cultured up to a

postnatal age of 28 days, meaning that the PN2 tissue was maintained for 26 days in vitro (PN2 + div26) whereas PN21 tissue was cultured for 7 days (PN21 + div7). From here onwards age-matched mice were sacrificed and sections from in vivo eyes were processed in parallel. The tissue was then fixed in 4% paraformaldehyde, infiltrated with 25% sucrose in Sörensen's phosphate buffer, cryosectioned (10 μm) and stored cold until further processing.

### 2.3. Tissue analysis

For general histology hematoxylin and eosin-stained sections were used. For determining number of rows in the outer nuclear layer (ONL) of the explants a vertical column in the center of every fourth section was selected without preconditions, except that rosettes were avoided. Counts were statistically processed using one way analysis of variance (ANOVA) at 5% significance level, followed by Fisher's protected least significant difference post-hoc comparisons. The identity of the explant photoreceptors was determined immunohistochemically. Three opsin antibodies were used: (1) a polyclonal to rhodopsin (AO, 1:10 000); (2) a monoclonal to green cone visual pigment (COS-1, 1:500); and (3) a monoclonal to blue cone visual pigment (OS-2, 1:2 000). All these primary antibodies were kindly provided by Professor A. Szél (Szél et al., 1986). Furthermore, polyclonal antibodies to arrestin (1:5000) and to interphotoreceptor retinoid-binding protein (IRBP, 1:500) were used. Both these antibodies were a gift from Dr. G.J. Chader and Dr. I. Gery (NIH, Bethesda, MD) and were described before (Van Veen et al., 1986a,b). Secondary antibodies coupled to biotin, which were reacted with avidin-horseradish peroxidase (avidin-HRP) and di-aminobenzidine (DAB), revealed the specific immune reactions. To evaluate the inner retina, the sections were immunostained by using three antibodies directed against calcium-binding proteins, (1) a monoclonal to calbindin (mouse anti-calbindin-D; MAB clone CL300, 1:200, Sigma Chemical Company). This bound antibody was detected either with the DAB method or goat anti-mouse antibodies conjugated to FITC. (2) a monoclonal to parvalbumin (mouse anti-parvalbumin; MAB1572, 1:100, Chemicon International, Inc.). Goat anti-mouse secondary antibody conjugated with Cy-2, FITC or Texas red was used as secondary antibody. (3) a polyclonal to calretinin (goat anti-calretinin; PAB1550, 1:100, Chemicon International, Inc). To visualize the reaction donkey anti-sheep or donkey anti-goat secondary antibodies conjugated with either FITC or Texas red were used. As controls either the primary or the secondary antibody was omitted, which did not show labeling. Immunolabeled sections of retinal explants were compared with those of the parallel stained litter-matched retina. Overnight incubation with the primary antibodies and 1 h incubation with secondary antibodies was carried out at room temperature. Between and after the immunoreactions the sections were washed 3 × in PBS (pH 7,0). All histochemical and immunohistochemical reactions were examined at the light microscopic level and photographed with an Axiophot photomicroscope (Zeiss, Oberkochen, Germany). Adobe Photoshop was used for image processing. Only contrast and brightness of the images were adjusted.

### 2.4. RT-PCR techniques

RT-PCR was used for the detection of IRBP mRNA in total RNA (10 μg) isolated from cultured retinal tissue. Retinal RNA was isolated from mice sacrificed on PN2 and the retina cultured for 12 days (PN2 + div12). As controls we used total RNA isolated from PN14 retina and adult whole eye (Clontech, USA) (42–48 days of age).

For PCR detection of IBRP mRNA we used primers previously shown to recognize IBRP sequences in mouse, amplifying a IRBP fragment of 131 base pairs (bp) (Kutty et al., 1993). For RT-PCR, and the first strand cDNA synthesis enriching IRBP sequences, we used the following antisense primer, 5′-ATG TGG CCT TTT TTT TTT TTT TTT T.

PCR analyses were performed using the following pairs of primers, 5′-CAG AGG ATG CCA AAG ACC GA (forward) and 5′-GAA TCT CAA GTA GCC AAT GT (reverse). After an initial hot start at 94 °C for 10 min the following PCR-conditions were used: denaturation 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, using Ampli Taq (Applied Biosystems, the Netherlands) in standard buffer and performing 35, 25, 20 or 15 cycles. Products were analyzed on 1% agarose gels.

## 3. Results

### 3.1. Observations in hematoxylin and eosin-stained sections

Whole mouse retinas were dissected out at PN2, PN7, PN11 and PN21 and maintained for 26 (PN2 + div26), 21 (PN7 + div21), 17 (PN11 + div17) and 7 (PN21 + div7) days in vitro (div), respectively. Rosette formation was a very rare incident.

For reasons of comparison hematoxylin and eosin stained and the three photoreceptor-specific immuno-labeled images are presented in one figure plate but the results are described separately in the text. The statistics regarding number of rows of photoreceptors in the ONL are given in Table 1. The corresponding images are displayed in panels a, e, i and m of Fig. 1. At PN28

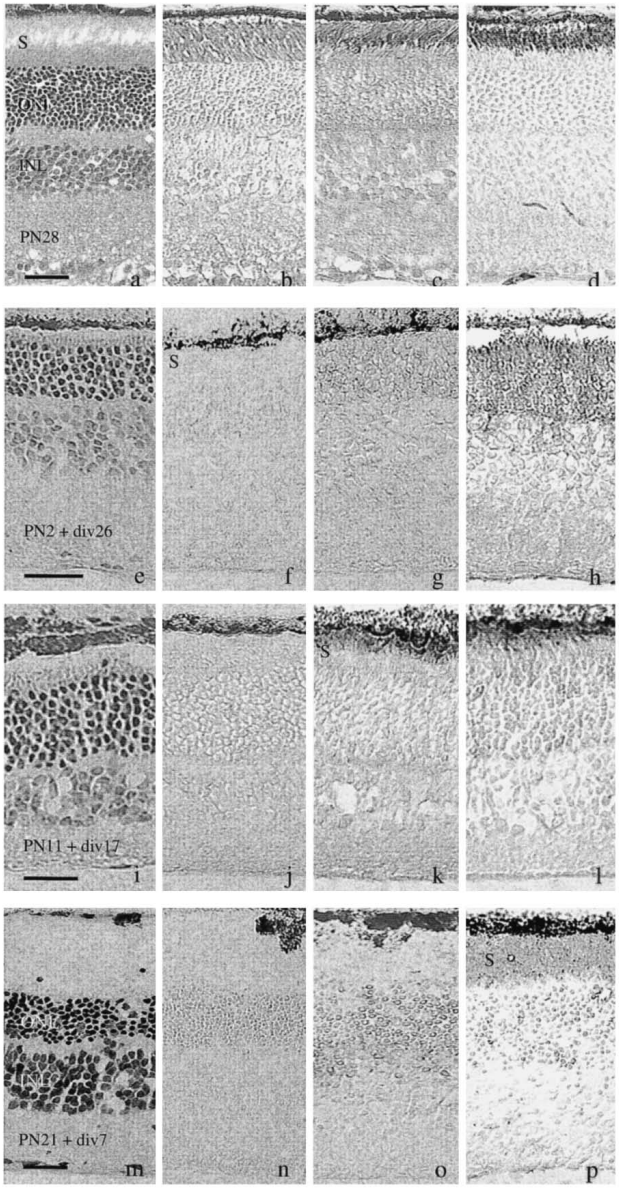


Fig. 1. (*Continued*)

Table 1
Mean ± S.E.M. of the numbers of rows in the outer nuclear layer (ONL) of PN28 litter-matched retina and retinal explants isolated at postnatal day n (PNn) and cultured for a number of days (n) in vitro (divn) in serum-free R16 nutrient medium

| | In vivo PN28 | PN2+div26 | PN7+div21 | PN11+div17 | PN21+div7 |
|---|---|---|---|---|---|
| Number of rows in ONL | 13.6 ± 0.2 | 7.9 ± 0.2 | 8.1 ± 0.3 | 8.0 ± 0.3 | 7.4 ± 0.3 |

Whereas the number of rows in the ONL is significantly higher ($P<0.0001$) in the litter-matched retina than in the retinal explants, there is no difference between the latter.

in vivo mouse retina (panel 1a) contains 13.6 ± 0.2 (mean ± S.E.M) rows of cell bodies in the ONL. The PN2 + div26 (panels 1 e–h) and PN7 + div21 (not shown) retinal explants cultured in serum-free medium acquire the characteristic histotypic lamination and maintain 7.9 ± 0.2 and 8.1 ± 0.3 rows of nuclei in the ONL, respectively. PN11 + div17 (panels 1 i–l) explants show good preservation of retinal architecture with 8.0 ± 0.2 rows of nuclei in the ONL. PN21 + div7 (panels 1 m–p) explants display 7.4 ± 0.3 rows in the ONL. The number of ONL rows in the PN28 tissue was significantly higher ($P < 0.0001$) than those in the explants of all ages, but there is no statistically significant difference between explants. Some other observations were made in the hematoxylin and eosin sections at the end of the culture period. First, recognizable elongated outer segment-like structures are often expressed by PN2 + div26 to PN11 + div17 photoreceptors whereas these structures are degenerating and form a debris zone in the PN21 + div7 explants. Second, clumps of pigment-laden cells (panel 1 n), not observed in PN2 + div26 to PN11 + div17 tissue, are traversing the subretinal space in PN21 + div7 explants.

### 3.2. Observations in immuno-labeled outer retina

Expression patterns of the three photoreceptor-specific proteins by the retina in vivo or in vitro are also presented in Fig. 1. The in vivo labeled IRBP is found exclusively in the inter-photoreceptor matrix (IPM; panel 1 b). In the explants, IRBP expression is completely different. PN2 + div26 (panel 1 f) and PN11 + div17 (panel 1 j) cultured retina did not show immunoreactivity for IRBP in the subretinal space and IRBP labeling is also totally absent from the IPM debris zone in PN21 + div7 explants (panel 1 n). However, RT-PCR (Fig. 2), performed on cultured retina and age-matched control showed no or little difference in IRBP gene expression.

In vivo, arrestin labeling can involve the outer and inner segments, photoreceptor somata as well as punctate labeling in the outer plexiform layer (OPL). However, with the staining protocol used in the current series of experiments only the photoreceptor segments are clearly labeled by the arrestin antibody (panel 1 c). When the outer segment-like profiles are present, the arrestin antibody produced similar staining patterns in PN2 + div26 to PN11 + div17 (panel 1 k) explants. We were unable to obtain appreciable staining of arrestin in the PN21 + div7 explants (panel 1 o). Strong rhodopsin labeling is limited to the rod outer segments of the PN28 littermates (panel 1 d). Sections derived from PN2 + div26 (panel 1 h) and PN7 + div21 (not shown) explants, however, show this labeling in rod somata and, when present, the outer segment-like structures. In PN11 + div17 (panel 1 l) sections, rhodopsin labeling is present in the outer segment which corresponds to litter-matched retina. Similarly to arrestin, opsin labeling in PN21 + div7 explants (panel 1 p) was very weak, if present.

Fig. 1. Mouse retinal explants cultured in serum-free medium; morphology and expression of photoreceptor-specific proteins. Photomicrographs of sections through the retina of the litter control PN28 (a–d) and retinal explants of PN2 + div26 (e–h), PN11 + div17 (i–l) and PN21 + div7 (m–p) cultured in serum-free nutrient medium. All explants acquire or maintain typical organotypic lamination throughout the culture period. In panel i (PN11 + div17) characteristic segment-like structures are evident. In PN21 + div7 explants (m) photoreceptor segments have formed a debris zone during the culture period. Panels in the second column display series of IRBP labeling in litter-matched retina (b), PN2 + div26 (f), PN11 + div17 (j), and PN21 + div7 (n) retina. IRBP labeling is found only in the photoreceptor segment layer of the litter matched animal (b). IRBP immunoreactivity is very weak or absent from the culture material at all ages (PN2 + div26 to PN21 + div7). In panel n pigment-laden cells traversing the subretinal space are evident. Panels in the third column display series of arrestin labeling in litter-matched retina (c), PN2 + div26 (g), PN11 + div17 (k), and PN21 + div7 (o) retinal explants. With the presently used protocol, arrestin labeling is confined to the photoreceptor segments in the litter-matched retina (c). Labeling by this antibody is also present only in the photoreceptor segments, when present, viz., PN11 + div17 (k). Panels in the fourth column display series of rhodopsin labeling in litter-matched retina (d), PN2 + div26 (h), PN11 + div17 (l), and PN21 + div7 (p) retinal explants. In the litter-matched retina, rhodopsin labeling is limited to the photoreceptor outer segment (d). In PN2 + div26 material (h) the immunoreactivity remained in the cell bodies of the ONL since characteristic outer segments are absent. PN11 + div17 explants show rhodopsin staining comparable to the litter-matched retina. The rhodopsin immunoreactivity in the PN21 + div7 photoreceptor segment debris zone is very weak, if at all present. Abbreviations, s; photoreceptor segment zone, onl; outer nuclear layer, inl; inner nuclear layer. Scale bars applying to each row are 50 μm.

### 3.3. Observations on immuno-labeled cone photoreceptors

It is established that blue but not green cones are present both in mouse explants and transplants. Explants used for this investigation were sectioned and labeled in their entirety so that both the blue (ventral S-field) and the green cone zones (dorsal M-field) were included in the sections. Only the results concerning the green cones are presented in this report, as those of the blue cones are not unique. Fig. 3 summarizes the results. Litter-matched retina displayed a normal M-field (panel 3 a). In PN2 + div26 explants (panel 3 b) green cones are completely absent. PN11 + div17 explants (panel 3 c) show a few green cones whereas in PN21 + div7 explants (panel 3 d) many immunostained elements remain in the subretinal debris zone containing the dorsal M-field. Control staining of PN21 + div7 tissue only displayed sporadic pigment-laden profiles in the subretinal space, confirming that the immunopositive labeling was true green cone labeling. The dotted-like appearance of green cone elements, however, indicated degenerating outer segments and confirmed the observations made after the hematoxylin and eosin staining.

### 3.4. Observations in the immuno-labeled inner retina

For this report no statistics were performed for elements of the inner retina. We focussed on qualitative parameters like proper location of somata, and, where possible, the shape of neural profiles. Fig. 4 displays images used for this qualitative assessment employing antibodies directed against three calcium-binding proteins. Calbindin is presented and discussed mainly as a marker of the horizontal cells and their processes in the OPL, whereas parvalbumin and calretinin are presented and discussed in relation to profiles centered around the IPL. Panel 4a shows the calbindin positive profiles in the in vivo (PN28) retina. Neurons in the ganglion cell layer and a mixed population of inner plexiform layer (INL) somata are labeled. They form three bands of processes in the IPL. Apart from this, typical horizontal cells are strongly calbindin immunoreactive. In the OPL a dense network of horizontal cell neurites is confined within the boundaries of this structure. In all retinal explants, an example of which is illustrated in panel 4b, the horizontal cell bodies occupy a normal location and are of the same shape as encountered in vivo. Most of their neurites are located, as they should be, within the boundaries of the OPL in the cultured tissue. However, some aberrant sprouting, most evidently into the ONL, is also present. Some of these aberrant processes even traverse the entire ONL to reach the external limiting membrane. It can also be seen that, in the age-matched in vivo retina, the calbindin immunopositive neurons at the inner part of the INL are greater in number than those in the retinal explants. The parvalbumin antibody strongly reacts with INL cells lining the IPL and a great number of

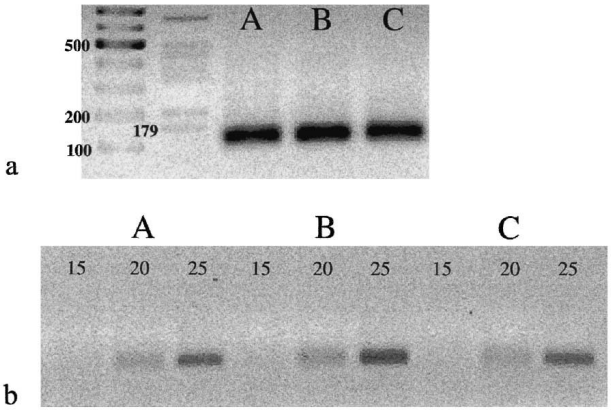


Fig. 2. IRBP RT-PCR. Expression of the IRBP transcript in (A) cultured retina (B) age-matched control and (C) adult control eye. The different tissues were analyzed by RT-PCR using primers for IBRP and run for 35 cycles. PN2 + div12 (Lane A) showed expression of an IBRP fragment, represented by a band of around 150 bp, corresponding to non-cultured tissue PN14 (Lane B) and adult control eye (Lane C). Numbers associated with ladders represent size in base pairs (fig. a). Similar relative amount of amplified fragments indicate a corresponding rate of IRBP expression in PN2 + div12 (A), PN14 (B) and adult eye PN42–48 (C) (fig.b).

cells in the ganglion cell layer of the PN28 littermate mouse retina (panel 4c). Horizontal cells are not stained. In the IPL a dense network of processes is present, but clearly distinct layers are not produced. The OPL is not labeled. In retinal explants the staining pattern of parvalbumin is qualitatively the same (panel 4d). However, although no quantitative analysis was performed, the impression is that the number of profiles is reduced both in the INL and ganglion cell layer of the retinal explants. There is no evidence of parvalbumin immunoreactive neurites beyond the normal location. In vivo, calretinin (panel 4e) labels numerous neurons of various types and sizes in the ganglion cell layer and very many cells in the inner part of the INL. Also cells located in the position of bipolar cells are stained. Three bands of calretinin immuno-positive processes are produced in the IPL. The age-matched in vitro image is shown in panel 4f. This demonstrates the maintenance of a normal calretinin organization. It can be seen, however, that the number of stained cells in the INL and ganglion cell layer of the retinal explants is reduced. The staining for calcium-binding proteins did not demonstrate clear and consistent differences between PN2 + div26, PN7 + div21, PN11 + div17 and PN21 + div7 retinal explants.

## 4. Discussion

### 4.1. Retina culture systems

Three main outcomes have been presented in this report, (1) the neonatal as well as late postnatal mouse retina can be cultured for at least 26 days in serum-free medium; (2) with some caution, the distribution pattern of photoreceptor-specific proteins in explants corresponds to litter-matched retina, except for soluble proteins like IRBP; and (3) the calcium-binding protein markers expressed by the inner retina cellular elements are equivalent to the in vivo retina, but some aberrant sprouting of neurites may occur in the former.

Our aim is to achieve a retina organ culture system in where the tissue corresponds to the in situ counterpart in such a way that the explant can be accepted as an excellent model to assess retinal phenomena. During the early stages of research and development of this

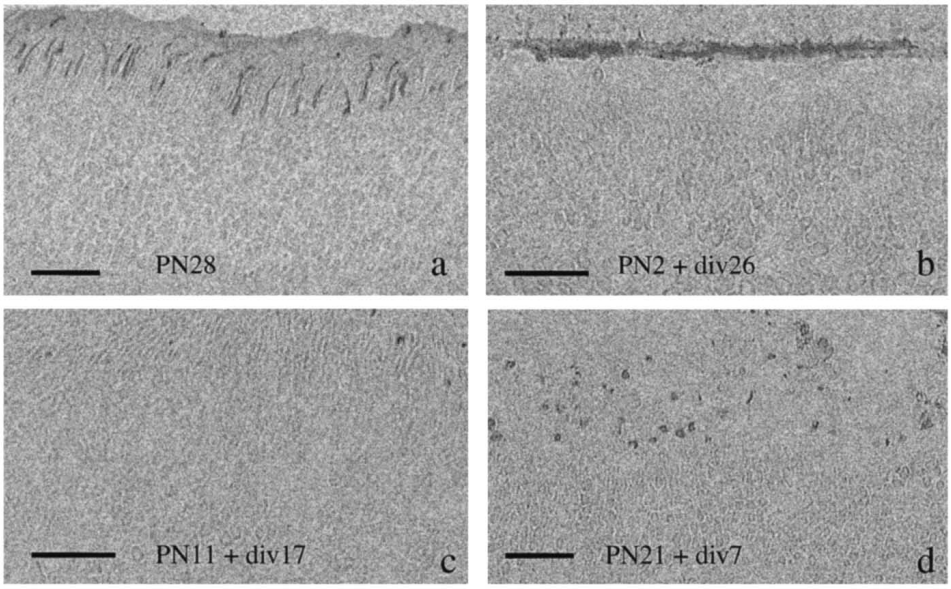


Fig. 3. Expression of green cones by normal mouse retina and mouse retinal explants. (a) green cone immunoreactivity in litter-matched (PN28) C3H +/+ retina. Many labeled profiles are present in the tissue. Sections taken from retinal explants at PN2 + div26 (b) do not display green cones. Sporadic green cones are present in PN11 + div17 explants (c), whereas PN21 + div7 explants (d) show green cone labeling which resembles the litter-matched (PN28) mouse retina. Scale bars are 50 μm.

culture technique, a growth medium supplemented with high concentrations of serum was used since the nutrient requirement of individual cells of this complex tissue is unknown. Indeed, there are indications that some retina cells may rapidly degenerate in serum-free culture conditions (Turner, 1985). However, neural tissue could also be cultured successfully in conditions where serum was replaced by commercially purified bovine serum albumin, with trace amounts of contaminating globulins (Romijn, 1988; Kivell et al., 2000). In addition we noticed that our mouse retinal explants survive equally well in 2 and 10% serum supplemented medium (Caffé et al., 2001). The successful use of the present serum-free medium marks a significant advance in retinal explant culture. Adler and colleagues (Politi and Adler, 1988; Politi et al., 1988; Abrams et al., 1989; Politi et al., 1989) have extensively studied isolated neurons of mouse retina from different ages cultured in serum-free medium. They have reported that under these circumstances PN2 photoreceptors survive for roughly 2 weeks in vitro, whereas those from PN5 and PN7 retina could be kept viable only for approximately 7–8 and 4–5 days, respectively. Thus, in our hands the whole retina, even when obtained from the late postnatal mouse, can be kept viable much longer than previously reported for isolated cells. For this reason the 7-days culture period for the PN21 retinal explants is also called long-term. However, the definitions for a long-term culture of late postnatal mouse retinal explant will likely change in the near future. Another feature about the serum-free system concerns the novel prospects of in-depth analytical evaluation. For instance, we aim for better outer segment differentiation and maintenance of the cultured tissue. The serum-free system now provides the opportunity for systematic medium modifications that will likely promote achievement of these goals.

### *4.2. Neurochemistry of retinal explant photoreceptor cells*

As far as investigated, no difference exists in the distribution of rhodopsin, arrestin, and IRBP immunoreactivity between retinal explants cultured either in serum-supplemented (Caffé et al., 1993, 2001) or serum-free medium. Thus, serum appears not to influence the metabolic parameters and transport of these photoreceptor-specific proteins in vitro. When the issue of in vitro versus in vivo is considered, PN11 + div17 cultures display rhodopsin and arrestin expression limited to the outer segments, exactly as in vivo. Therefore, culturing does not adversely affect production and transport of these proteins, provided outer segments are present in the tissue. This suggests that photoreceptor metabolic processes in the explants are apparently normal. Which is also shown by RT-PCR in that both cultured and normal control retinas express similar amounts of IRBP message. It is established that even isolated photoreceptor cells produce IRBP (Politi et al., 1989). However, the IRBP immunostaining in retinal explants is much weaker than that in litter-matched retina in spite of the observation that the amount of IRBP mRNA is comparable to that of age-matched controls. Since a metabolic defect is not considered there should be another reason for this phenomenon. Normally IRBP binds to matrix proteoglycans in the IPM indicated by the observations that in rodents IRBP redistributes simultaneously with insoluble matrix components, i.e. the proteoglycans, in the subretinal space (Uehara et al., 1990; Mieziewska et al., 1994; Mieziewska, 1996). The failure to detect IRBP in the subretinal compartment of retinal explants might, therefore, be due to a problem with the IPM components. It also could be that the IRBP, being a soluble secreted protein, is washed out by the relative large amount of culture medium. To address this issue the synthesis of matrix proteoglycans by the retinal explants and measuring the total amount of IRBP in the used culture medium is in progress.

#### *4.2.1. The issue of retinal cone identity*

The mouse strain that was used in the present experiments carries a retina divided into a dorsal half with blue and green cones (M-field) and a ventral half with exclusively blue cones (S-field) (Szél et al., 1992, 1993, 1994). In the mouse there is also a temporal difference in the expression of the two cone types; blue cones can be detected from PN4 while green cones start to appear only from PN11 (reviewed by Szél et al., 1996). Blue visual pigment is believed to be the cone default pathway. For instance, they appear to differentiate normally even in retinal explants (Söderpalm et al., 1994). Wikler et al. (1996) have addressed the issue of molecular signals involved in green cone pigment expression, using retinal explants similar to those employed in this report. They observed that green cones are only expressed in vitro when dorsal retinal explants are harvested from PN3 or older pups but concluded that both the blue and green cone identities are determined prenatal. Our results confirm that blue identity appears to be a matter of early intrinsic processes in the cone but rejects that the absence of green cones in retinal explants is due to some intrinsic cone metabolic error. For determination of green cone identity, instead, we postulate an important role by an external regulatory signal during the late postnatal period (Röhlich et al., 1994; Szél et al., 1994). Thyroid (T3) hormone and receptors are strong candidates for mediating the external signals (Ng et al., 2001). That these signals continue acting during the late postnatal period follows from our observations that further green visual pigment biosyn-

thesis is halted after the retina has been isolated. An in-depth discussion about the retinal cellular elements that might convey the green cone molecular signals falls beyond the scope of this paper (see e.g. Cepko et al., 1996; Belliveau et al., 2000; Cepko, 2000). The cognate thyroid receptors are present in the chick retina ONL (Sjöberg et al., 1992); therefore, the photoreceptor cell bodies themselves might be the only elements involved. However, the spatial and temporal characteristics of retinal aldehyde dehydrogenase expression are reminiscent of that of the asymmetrical cone fields (McCaffery et al., 1992, 1999). Aldehyde dehydrogenase is produced by Muller cells, which create isolated retinal columns. Therefore, we suspect that Muller cells may also be involved in the production of green cone visual pigment.

### 4.3. Neurochemistry of explant inner retinal cells

It is well established that $Ca^{2+}$ plays a crucial role in many cellular processes. Calcium-binding proteins, ex-

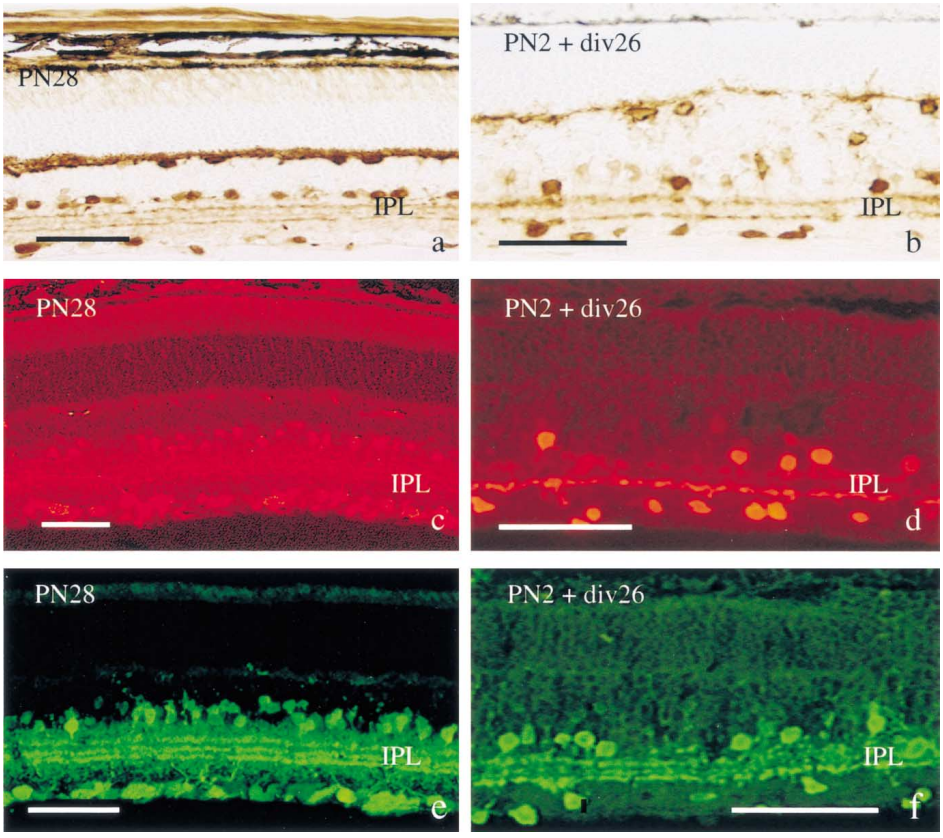


Fig. 4. Immunofluorescence of calcium-binding proteins in mouse retinal explant. (a) calbindin immunoreactivity in litter-matched (PN28) retina. Strong labeling is present in cells present in the ganglion cell layer and inner part of the inner nuclear layer. Three lamina formed by neurites occur in the inner plexiform layer (IPL). Furthermore, heavy labeling is displayed by horizontal cells, which extend their processes into and within the outer plexiform layer. Although similar elements are stained in the cultured retina, see for example a section from a PN2 + div26 retinal explant (b), the immunoreactivity is less organized after culturing. Sprouting of calbindin stained profiles into the ONL of retinal explants is present. Parvalbumin staining of the litter matched (PN28) mouse retina (c) and retinal explants (d) shows labeled cells on both sides of the IPL. The number of stained profiles appears to be smaller, but no obvious sprouting can be observed. Panels e and f represent calretinin immunoreactivity in the litter control (PN28) (e) and retinal explant (PN2 + div26) (f). There appears to be a reduced number of stained profiles in the explant. The panels displaying the retinal explants are enlarged for a better appreciation of the cellular organization. Scale bars are 50 μm.

pressed in a cell-specific manner, help to regulate the bioavailability $Ca^{2+}$ ions. Haverkamp and Wässle (2000) have published an extensive report on the cell-type specificity of biomolecules in the normal mouse retina, including the neuronal markers calbindin, parvalbumin and calretinin. The results we have achieved in the normal retina are identical as those previously reported. A comparison between in vivo and cultured tissue demonstrated a qualitatively normal calbindin organization in the retinal explant, except for some sprouting of processes from the OPL into the ONL. Although the numbers of labeled cells was not verified statistically in the tissue investigated for this report, knowledge of the normal distributions permitted us to conclude that the number of immunostained elements by all three markers is reduced in the mouse retinal explants. The calcium-binding protein containing mouse retinal elements are not alone in their reactive capabilities. Johansson and colleagues (2000) studied the development of cholinergic and nitrergic transmitter systems in rat retinal explant after 2–3 weeks in vitro and also found an altered expression in the tissue. To conclude, not only Muller glia cell (Caffé et al., 1993), but the entire inner retina circuitry seems to react to the explant preparation. It is these phenomena that must be monitored during further development of the retinal explant culture system.

## 5. Conclusions

This report has conveyed the message that it is possible to culture the intact mouse retina, neonatal and late postnatal, in a serum-free medium for a considerable period of time. Since serum contains components of unknown nature and effects, this serum-free system avoids a serious drawback associated with the currently used serum-supplemented media for mammalian retinal explant. This technique will be applied to gain in-depth insight into the conditions for effective drug treatment of degenerative photoreceptor disorders. In addition, this system provides better prospects for successful analysis of changes at the mRNA and protein levels. This report has also demonstrated the usefulness of the explant culture system for the investigation of basic parameters of retinal development. Isolating the retinal explant at different developmental ages shows that green cone visual pigment differentiation will not proceed if the tissue is taken before the moment this process is initiated in vivo. Therefore, the green identity of cones is linked to signals present outside the cone cell body and perhaps even from molecules entering the retina from outside. This phenomenon might be relevant to pathological features encountered in the clinical setting as well (Verriest et al., 1980; Haider et al., 2000).

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