Organotypic Cultures of Adult Mouse Retina: Morphologic Changes and Gene Expression

Brigitte Müller, Franziska Wagner, Birgit Lorenz, and Knut Stieger

Department of Ophthalmology, Justus-Liebig-University Giessen, Giessen, Germany

CORRESPONDENCE: Brigitte Müller, Department of Ophthalmology, Faculty of Medicine, Justus-Liebig-University Giessen, Friedrichstr. 18, Giessen 35392, Germany; Brigitte.Mueller@augen.med.uni-giessen.de.

Submitted: September 8, 2016
Accepted: February 28, 2017

Citation: Müller B, Wagner F, Lorenz B, Stieger K. Organotypic cultures of adult mouse retina: morphologic changes and gene expression. Invest Ophthalmol Vis Sci. 2017;58:1930-1940. DOI:10.1167/iovs.16-20718

PURPOSE. The purpose of this study was characterization of adult murine neuroretina in organ culture to investigate its suitability for use in preclinical therapeutic applications. In retinal disorders, neurodegeneration of mature retinal cells takes place. Therefore, neonatal retina cultures are not adequate for therapeutic applications, such as genome editing, as the retina is still developing with cells dividing and differentiating into highly specialized cell types such as photoreceptors.

METHODS. Retinal explants were prepared from 3-month-old wild-type C57Bl6 mice and evaluated after 1 to 10 days in culture by immunohistochemistry or quantitative (q)PCR. Histologic modifications of the neuroretina were evaluated with TUNEL assay and immunohistochemical markers for neurons, glia cells, and apoptosis.

RESULTS. During the first week, disruption and truncation of outer segments were detectable. Unspecific Müller cell reaction was detected from 4 days in culture. Sprouting of individual rod bipolar cell dendrites into the outer nuclear layer (ONL) was visible during all explant stages. During the second week in culture, cell death in the ONL became more prominent.

CONCLUSIONS. Adult organotypic retinal culture was successful for at least 10 days with characteristic alterations of the morphology during this period. This characterization forms the basis to establish retinal explants for gene therapeutic applications as an intermediate step between cell culture and experiments on adult animals, thus reducing the load of animal experimentation.

Keywords: retinal explant, animal models, photoreceptors, apoptosis

Retinal explant culture systems have the potential to mimic the functional dynamics of the organ beyond those of the dissociated cells, thus making this technique a very powerful intermediate model system between in vitro cell cultures and in vivo animal models. The different retinal layers made of highly specialized cell types remain intact, while glia cell reactions and/or intercellular interactions can be evaluated under well-defined conditions in the lab.

In the past, organotypic culture of the neonatal mouse retina has been very useful for improving the knowledge of both normal and retinal degeneration to study the role of various factors in photoreceptor degeneration, retinal cell fate determination, and development.1-4 It is now a widely used tool, with broad applications in the field of ophthalmology. Many alterations observed during in vitro retina culturing3,5-6 resemble some characteristics of experimental retinal detachment and diabetic retinopathy in vivo, respectively.7,8 Other researchers have studied the relationship between retinal development, maturation, degeneration, and gene transfer in culture.9,12 Furthermore, many studies evaluated the therapeutic effect and potential toxicity of substances.13,14

Several methods have been described for culturing retinal explants from different species.5,3,5,15-17 The method of Caffé and colleagues,5 in which the neonatal mouse retina is placed with the photoreceptor layer facing downward on rafts made of nitrocellulose filters and polyamide gauze grids, has been used in variations in several studies.9,18

Neonatal retinal organotypic cultures differ from adult ones since the immature retinal neurons go through phases of differentiation and pruning, under the control of various growth factors during the first 3 weeks postnatally.1,19-24 Additionally, neonatal organotypic retinal cultures have the fundamental problem that outer and inner segments of photoreceptors do not develop correctly.1 So far, only Moritoh and colleagues25 have kept adult rat and murine retinal explants in culture for up to 4 days after particle-mediated acute gene transfer in vitro. Mouse retinas showed less viability after 4-day culture.

In this study, we characterized the organ culture of adult murine retinas (>12 weeks) after up to 10 days in culture to establish survival, cellular changes, and early degeneration patterns of neuronal and glial cells.

METHODS
Animal Handling and Ethics Statement
Three-month-old wild-type C57Bl6 mice (Jackson stock no. 000664; Charles River, Sulzfeld, Germany) were used in this study. The mice were housed and bred in the animal facility of the University of Giessen under a cycle of 14-hour light (200 lux illumination in the cage) and 10-hour dark. All procedures concerning animal handling and euthanizing complied with the European legislation of Health Principles of Laboratory Animal Care in accordance with the ARVO Statement for the Use of.
**Preparation of Organotypic Retina Culture**

Animals were killed by cervical dislocation. The eyeballs were enucleated and the anterior part was excised. The eyeballs were then rinsed with Hanks' balanced saline solution (GIBCO HBSS; no. 14025076; Thermo Fisher Scientific, Dreieich, Germany) and incubated in basic culture medium lacking fetal bovine serum. Complete culture medium was a composite made of 50% Dulbecco’s modified Eagle’s medium (DMEM) (PAN Biotech, Aidenbach, Germany), 25% fetal bovine serum (FBS) (PAN Biotech), and 25% HBSS supplemented with 2 mM L-glutamine (PAN Biotech), 5.75 mg/mL glucose, and antibiotics (100 μg/mL streptomycin and 100 units/mL penicillin (PAN Biotech), according to the manufacturer’s instructions (Fluorescein no. 01684795910; Roche Diagnostics GmbH, Mannheim, Germany). It preferentially marks DNA strand breaks generated during apoptosis by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction. For controls, terminal deoxynucleotidyl transferase enzyme was either omitted from the labeling solution (negative control), or sections were pretreated for 10 minutes with RNAse-free DNAse I recombinant (Roche, no. 04536282001, 300 U/mL) in 50 mM Tris-HCl, pH 7.5, 1 mg/mL BSA to induce DNA strand breaks (positive control). While negative controls gave no staining, positive controls stained all nuclei in all layers of the retina.

**TUNEL Assay**

For the visualization of dying cells, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using an in situ cell death detection kit according to the manufacturer’s instructions (Fluorescein no. 11684795910; Roche Diagnostics GmbH, Mannheim, Germany). It preferentially marks DNA strand breaks generated during apoptosis by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction. For controls, terminal deoxynucleotidyl transferase enzyme was either omitted from the labeling solution (negative control), or sections were pretreated for 10 minutes with RNAse-free DNAse I recombinant (Roche, no. 04536282001, 300 U/mL) in 50 mM Tris-HCl, pH 7.5, 1 mg/mL BSA to induce DNA strand breaks (positive control). While negative controls gave no staining, positive controls stained all nuclei in all layers of the retina.

**Hematoxylin and Eosin Staining**

Hematoxylin staining was performed with hematoxylin solution according to Gill II (no. T864.1; ROTH, Karlsruhe, Germany) for 20 to 30 seconds. After a wash with running
water, eosin staining was performed with 0.5% eosin Y solution (no. X883.2, ROTH) for 10 to 15 seconds. The sections were washed and dehydrated with ethanol of serial dilution and enclosed with Roti-Histokitt II (no. T160.1, ROTH).

**Immunohistochemistry: Tissue Processing**

Frozen sections were preincubated in PBS containing 10% normal donkey serum (NDS), 1% bovine serum albumin, and 0.5% Triton X-100 for 1 hour. Antibodies were diluted in PBS containing 3% NDS and 0.5% Triton X-100. Sections were incubated overnight in primary antibodies (see Table 1), followed by three washes and a 90-minute incubation in secondary antibodies that were conjugated to Alexa 488 (no. 21202; Thermo Fisher Scientific) or Alexa 594 (no. 21207; Thermo Fisher Scientific). 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (no. D1306; Molecular Probes, Paisley, UK) was used at 1 μg/mL to counterstain the retinal nuclei during incubation with secondary antibodies. After a final wash, sections were coverslipped with Dako embedding fluorescence mounting medium (no. S3023; DakoCytomation, Glostrup, Denmark). For double immunolabeling, the respective primary and secondary antibodies were applied as a cocktail.

**Gene Expression Analysis by qPCR**

Three fresh retinas or retinal explants (10–15 mg) were pooled and homogenized with a Precellys homogenizer (Peqlab, Erlangen, Germany) using Precellys CK14S vials for 25 seconds at 2800 g. Total mRNA was purified from retinal explants by using the RNasey Micro Kit (no. 74004; QIAGEN, Hilden, Germany) according to protocol, and cDNA was synthesized from the mRNA by PrimeScript RT Master Mix (Cat. no. RR036Q; Takara, Saint-Germain-en-Laye, France). Sequences of primers are listed in Table 2. The real-time PCR system Mastercycler ep realplex (Eppendorf, Hamburg, Germany) was used. In order to reduce confounding variance, three or four independent biological samples from different litters were analyzed in technical triplicates. Technical replicates were averaged before fold change calculation.

The delta delta Ct method (ΔΔCt) was used to analyze the relative gene expression; that is, the fold change of mRNA up-or downregulation of different target genes was determined.39,40 Untreated retinas (0 days in culture) were used as control group and glyceraldehyde-3-phosphate-dehydrogenase (Gapdh) as housekeeping gene.

### Table 2. Primer Sequences

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primer Sequence</th>
<th>qPCR Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodopsin</td>
<td>5'-GAAGACCACACCCATGATAG-3'</td>
<td>208 bp</td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>5'-ATTCCACCCACCCCTTCA-3'</td>
<td>208 bp</td>
</tr>
<tr>
<td>Gfap</td>
<td>5'-GCTGGACCCAGCTACGGCCACAG-3'</td>
<td>169 bp</td>
</tr>
<tr>
<td>Gfap</td>
<td>5'-AGCCAGGGTGCTTCTG-3'</td>
<td>169 bp</td>
</tr>
<tr>
<td>AifI Iba1</td>
<td>5'-GATGATGAGGAGAAAACAAGACAC-3'</td>
<td>177 bp</td>
</tr>
<tr>
<td>Iba1AifI</td>
<td>5'-TTATTTAGCTGACGTGGCTCA-3'</td>
<td>177 bp</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5'-GCTCGGTGGAACGGAATTTG-3'</td>
<td>199 bp</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5'-CCGTGGATGAAGCTTCC-3'</td>
<td>199 bp</td>
</tr>
</tbody>
</table>

**FIGURE 1.** Vertical frozen sections through organotypic cultures of adult mouse retina during the first week stained with hematoxylin and eosin (A–C). Most obviously, the thickness of the entire retina changes over time. Up to 6 days in culture the ONL keeps at least eight rows of nuclei (C). Retinal layering is preserved through all time points. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 50 μm.
Microscopic Analysis

Hematoxylin- and eosin-stained specimens were viewed and analyzed with a Keyence Biozero8000 microscope equipped with epifluorescence and bright-field and CCD camera (Keyence International, Mechelen, Belgium). Confocal images were taken using an Olympus FV10i (Hamburg, Germany) confocal microscope equipped with argon and HeNe lasers. High-resolution scanning of image stacks was performed with an UPlanSApo x60/1.35 (Olympus) oil immersion objective at 1024 × 1024 pixels and a z-axis increment of 0.3 μm. For analysis of immunolabeled cells and their processes, a stack of 2 to 14 sections was taken (0.7-μm z-axis step size). Cell processes were reconstructed by collapsing the stacks into a single plane. Brightness and contrast of the final images were adjusted using Adobe Photoshop CS5 (San Jose, CA, USA).

FIGURE 2. Vertical frozen sections through organotypic cultures of adult mouse retina stained by rhodopsin (A–F) (green) and S-opsin antibodies (G–L) (yellow), markers for rod and short wavelength–sensitive cone photoreceptors, respectively. Retinal structure is given as overlay of bright field and immune staining at the left side of the micrographs. Rhodopsin is mainly located in the outer segments but detectable at lower concentrations in the cytoplasm around the nucleus in the outer nuclear layer (A–E). S-opsin is detectable in all compartments of the S-cones, most brightly in the outer segments at day 0 to 4 (G, H). After 4 days in culture outer segments get shorter and many of them are lost. At all stages of organotypic retina culture, the outer segments of rod and S-cone photoreceptors are immunopositive for their respective photopigment. Nuclei of S-opsin-immunoreactive cones are located in the outer third of the ONL (arrows). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 50 μm.
Quantification of TUNEL-Positive Nuclei and Number of Nuclear Rows in the Outer Nuclear Layer

TUNEL assay was performed on vertical frozen sections of retinal explants. An interval of 175 to 210 \( \mu \)m in length was defined in each image, and all TUNEL-positive and all DAPI-labeled nuclei of the outer nuclear layer (ONL) were counted. Only central retinal regions were analyzed. TUNEL-positive nuclei were given as percentage of all nuclei in the ONL in the respective field. At least three micrographs per retinal explant culture period were analyzed. Single images or image stacks 2 to 9 \( \mu \)m in depth were taken.

To quantify the change in number of nuclear rows in the ONL, vertical sections of central retina counterstained for DAPI were evaluated. At least six micrographs per retinal explant culture period were analyzed. Vertical sections came from two eyes of two individuals for each culture period. Single images or image stacks 2 to 4 \( \mu \)m in depth were taken.

Statistical Analysis

Statistical comparisons among different experimental groups were made using a 2-tailed Student’s \( t \)-test and SigmaPlot 12 software (Systat Software GmbH, Erkrath, Germany). Error bars indicate SD in all quantitative evaluations and SEM in all scatter plots of qPCR results.

RESULTS

Gross Morphologic Alterations Over Time

We successfully kept adult mouse retina in culture for up to 2 weeks. In hematoxylin- and eosin-stained vertical frozen sections we checked on the overall appearance of the plexiform and nuclear layers in retinal explant cultures in comparison to normal retina (Figs. 1A–C). During the whole culture period, all retinal layers were clearly detectable, that is, stayed neat and tight. The major difference between freshly isolated retina and retinal explants observed in vertical frozen sections was that the retina got thinner during culture due to condensation in the plexiform layers and photoreceptor death. During the first 4 days in culture, this was mainly related to truncation and degeneration of outer segments and morphologic change of inner segments to spherical structures since no regenerating pigment epithelium could be cocultured. On average, cells at both nuclear layers appeared densely packed and only a moderate reduction in the number of nuclei was obvious in the inner nuclear layer (INL). By the end of the second week the retinal thickness was significantly reduced, that is, \(<100 \mu m\) compared to \(\sim 220 \mu m\) before culturing.

Despite these morphologic changes during retinal explant culture, all photoreceptor types were detectable by immunostaining their respective photopigment at all time points investigated (Fig. 2). Rhodopsin immunoreactivity was mainly located in photoreceptor outer segments but detectable at lower concentrations in the cytoplasm around the nucleus in the ONL (Figs. 2A–E). With respect to cone photoreceptors, only S-opsin immunohistochemistry was shown in Figures 2G through 2L. The S-opsin antibody labeled all compartments of the cone, while the outer segment was labeled most brightly (Figs. 2G–I). M-opsin immunohistochemistry was detected in the outer segments of retinal explant culture at all time points investigated (Figs. 2J). Rhodopsin immunoreactivity was mainly located in photoreceptor outer segments but detectable at lower concentrations in the cytoplasm around the nucleus in the ONL (Figs. 2A–E). With respect to cone photoreceptors, only S-opsin immunohistochemistry was shown in Figures 2G through 2L. The S-opsin antibody labeled all compartments of the cone, while the outer segment was labeled most brightly (Figs. 2G–I). M-opsin immunohistochemistry was detected in the outer segments of retinal explant culture at all time points too (data not shown). After 8 days in culture, most photoreceptor outer segments were lost and inner segments were reduced to little spheres (Figs. 2F, 2L).

To assess the degeneration during retinal explant culture, the number of rows of photoreceptor nuclei in the ONL was quantified in central retinal regions. Vertical frozen sections of
retinal explants harvested after 0, 2, 4, 6, 8, and 10 days in culture were evaluated. A slow drop in number of rows was obvious during the first 4 days in culture (Fig. 3A). Numbers dropped from 13 rows in freshly isolated retina (0 days in culture) to 11 rows after 4 days in culture. Significant loss of nuclei rows occurred between 4 and 8 days in culture, and after 10 days, only approximately five rows of photoreceptor nuclei were left in central retina.

To assess the translational activity, total RNA was isolated from retinal explants harvested after 0, 1, 2, 3, 4, 6, and 8 days in culture. Even at early time points in culture, mean RNA yield per microgram retinal tissue was considerably low compared to freshly isolated retina (Fig. 3B). This was statistically highly significant ($P > 0.001$). Since 80% of all neurons in the mouse retina are photoreceptors, production of the photopigment rhodopsin very likely accounted for the vast majority of protein synthesis, including ribosomal RNA and tRNA. Adult retina culture was made without RPE cells and functional outer segments were decreasing with time in culture, very likely resulting in a drop of rhodopsin production and general protein synthesis in photoreceptors. To prove this hypothesis, we analyzed the fold change of rhodopsin gene expression by qPCR (Fig. 3C). It decreased more than 30-fold with increasing time in culture.

Immunohistochemical Characterization

Rod bipolar cells stained by protein kinase $\alpha$ (PKC$\alpha$) antibody appeared densely packed in the inner nuclear layer (INL) (Fig. 4). Bipolar cells kept their typical morphology during the entire length in culture. From day 1 up to day 10, individual dendrites showed sprouting into the ONL (arrows in Fig. 4). CtBP2-immunoreactive ribbon synapses were found at all stages of organotypic retina culture. Their appearance gets more dot- and less ribbon-like. After 4 days in culture, axonal sprouts are detectable in the GCL and NFL too (arrowheads). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 50 µm.

Figure 4. Vertical frozen sections through organotypic cultures of adult mouse retina stained by neuronal markers for photoreceptor synapses CtBP2 (red) and rod bipolar cells PKC$\alpha$ (green) (A–F). Nuclei are stained by DAPI (blue, only partially shown). During the entire length in culture bipolar cells keep their typical morphology. From day 2 up to day 10, individual dendrites show sprouting into the ONL (arrows). CtBP2-immunoreactive ribbon synapses can be found at all stages of organotypic retina culture. Their appearance gets more dot- and less rod-like. After 4 days in culture, axonal sprouts are detectable in the ONL and NFL too (arrowheads). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 50 µm.

Morphologic Changes in Adult Retinal Explant Culture

Retinal explants harvested after 0, 2, 4, 6, 8, and 10 days in culture were evaluated. A slow drop in number of rows was obvious during the first 4 days in culture (Fig. 3A). Numbers dropped from 13 rows in freshly isolated retina (0 days in culture) to 11 rows after 4 days in culture. Significant loss of nuclei rows occurred between 4 and 8 days in culture, and after 10 days, only approximately five rows of photoreceptor nuclei were left in central retina.

To assess the translational activity, total RNA was isolated from retinal explants harvested after 0, 1, 2, 3, 4, 6, and 8 days in culture. Even at early time points in culture, mean RNA yield per microgram retinal tissue was considerably low compared to freshly isolated retina (Fig. 3B). This was statistically highly significant ($P > 0.001$). Since 80% of all neurons in the mouse retina are photoreceptors, production of the photopigment rhodopsin very likely accounted for the vast majority of protein synthesis, including ribosomal RNA and tRNA. Adult retina culture was made without RPE cells and functional outer segments were decreasing with time in culture, very likely resulting in a drop of rhodopsin production and general protein synthesis in photoreceptors. To prove this hypothesis, we analyzed the fold change of rhodopsin gene expression by qPCR (Fig. 3C). It decreased more than 30-fold with increasing time in culture.

Immunohistochemical Characterization

Rod bipolar cells stained by protein kinase $\alpha$ (PKC$\alpha$) antibody appeared densely packed in the inner nuclear layer (INL) (Fig. 4). Bipolar cells kept their typical morphology during the entire length in culture. From day 1 up to day 10, individual dendrites showed sprouting into the ONL (arrows in Fig. 4). CtBP2-immunoreactive ribbon synapses were found at all stages of organotypic retina culture in the outer plexiform layer (OPL) and inner plexiform layer (IPL). Their appearance got more dot-like and less ribbon-like. After 4 days in culture, axonal sprouts were detectable in the ganglion cell layer (GCL) and in the nerve fiber layer (NFL) (arrowheads in Figs. 4C, 4D, 4F).

Müller glia cells were detectable by GFAP antiserum due to upregulated GFAP (glial fibrillary acid protein) in retinal explants having been cultured for 4 days and longer (Figs. 5A–F). Microglia cells were detected by Iba1 antiserum, which labels nonactivated and activated microglia. After 4 days in culture, an increase of activated microglia cell processes was obvious in the inner retina (Fig. 5K), and microglia-derived macrophages were frequently found in the ONL (Fig. 5L). Further evidence was gained by analysis of gene expression of GFAP and Iba1 in organotypic cultures of adult mouse retina via qPCR analysis. Glial fibrillary acid protein was upregulated from day 2 in culture and peaked at 6 and 8 days in culture (Fig. 6A). The comparison of the fold change in GFAP gene expression between 1 and 3 days showed statistical significance. The level of gene expression of Iba1 was raised 4-fold at 3 days in culture and peaked at 6 and 7 days in culture (Fig. 6B). Fold change in Iba1 gene expression showed no statistical significance. Nevertheless, the levels of fold change of GFAP and Iba1 gene expression complemented our immunohistochemical results (Fig. 5).

Apoptotic Processes

To check for apoptotic processes we used an antiserum against apoptosis-inducing factor (AIF). Apoptosis-inducing factor is localized to the mitochondrial intermembrane space and gets translocated to the nucleus due to pathologic permeabilization of the outer mitochondrial membrane. At all time points of retinal explant culture, inner segments of photoreceptors were labeled brightly by AIF immunohistochemistry due to the densely packed mitochondria (Figs. 7A–F). Both plexiform layers showed some AIF immunoreactivity, also probably due to...
mitochondria localized in synaptic processes. Only minor dislocation of AIF could be observed in perikarya of the INL during retinal explant culture detectable as brighter AIF immunofluorescence compared to day 0 retina. Since rod photoreceptors have very little cytoplasm around their nucleus in the ONL, AIF immunoreactivity was detectable only as isolated puncta. These were more numerous after 6 days in culture (Figs. 7D–F). At 8 days in culture, AIF was brightly visible in processes in the GCL also, possibly Müller cell end feet.

We performed TUNEL assays to determine the number of dying cells during retinal explant culture. Vertical sections of retinal explant culture showed a moderate number of TUNEL-positive nuclei in the ONL after 4 days in culture (Fig. 7I). Retinal explants harvested after 6 days in culture showed a considerable number of TUNEL-positive nuclei in the ONL (Figs. 7J–L). The temporal appearance of TUNEL-positive cells in the ONL of retinal explant culture older than 4 days showed no strong conformance with AIF dislocation. In the INL and GCL, only very few TUNEL-positive nuclei could be detected. TUNEL-positive nuclei in the ONL were quantified in vertical sections of retinal explant culture and displayed as percentage of all nuclei labeled by DAPI in the field analyzed (Fig. 8). A significant increase in percentage of TUNEL-positive nuclei occurred between 4 and 8 days in culture.

**DISCUSSION**

In our study we kept retinas of adult wild-type mice in culture for up to 2 weeks with the intention to use the retinal organ culture as a model to study genome editing as treatment approach for retinal dystrophies in future experiments. Characterization of the retinal organ culture of fully differentiated retina is essential to investigate its suitability for genome editing for use in preclinical therapeutic applications.
We found that all retinal layers in retinal organ culture were clearly detectable at all time points. The major difference between freshly isolated retina and retinal explants observed in vertical frozen sections was that the retina got thinner during culture due to gradual primary loss of outer and inner photoreceptor segments and subsequent secondary loss of photoreceptor nuclei. This was probably due to the fact that the RPE could not be kept during dissection of the retina and consequently not be cocultivated. Therefore, recycling of photoreceptor outer segments was not possible during retinal explant culture and subsequent loss of whole retina tissue is grown in culture. No migrating or proliferating Müller cells were observed, which is in contrast to observations in the diseased retina. The loss of systemic circulating factors may account for some of the changes seen when isolated retinal tissue is grown in culture.

In the inner retina, very few TUNEL-positive nuclei, only minor dislocation of AIF, and not much thinning of the INL and GCL were observed during retinal explant culture over time. Therefore, we conclude that almost no necrotic or apoptotic degeneration processes occur in the inner retina. This is interesting and in contrast to genetic pathologic conditions like retinitis pigmentosa or pathologic conditions like retinal detachment.

In contrast, in the outer retina, after 4 days in culture, photoreceptor nuclei began to die through an apoptotic process detected by TUNEL reaction only (Fig. 7). Photoreceptor nuclei showed no unequivocal nuclear localization of AIF (Figs. 7A–F). This is in agreement with results from Sanges and colleagues showing that nitrosomethyl-urea treatment causing necrosis made almost all photoreceptor nuclei TUNEL positive, without nuclear localization of AIF. In contrast, in rd1 mice, degenerating photoreceptors showed AIF translocation and TUNEL reaction, specific to the genetic pathologic condition. Therefore, between 4 and 10 days in culture, photoreceptor apoptosis resembled the more random DNA destruction in necrosis, probably due to the absence of RPE and subsequent loss of outer segments. Our quantification data of photoreceptor nuclear rows at the different culture time points delineated cell death during retinal explant culture significantly between 4 and 8 days.

Mean RNA yield per microgram of adult mouse retina dropped down immediately to one-fourth at early time points in retinal explant culture. This correlated with the downregulation of rhodopsin, the photopigment of rod photoreceptors, Fold change of rhodopsin gene expression in retinal explants was downregulated most dramatically after 3 days in culture. Very likely, the in vitro conditions in organotypic retina culture, including the absence of RPE and little or no renewal of photoreceptor outer segments, accounted for these dramatic changes. The turnover rate for one entire photoreceptor nuclei row in primates is 10 days. If this is applicable to mice photoreceptors in organotypic retina culture with no visual stimulation, they gradually wear out in culture. Incidentally, our histologic findings of this study are in line with that. Besides, rods account for 80% of all retinal neurons and rhodopsin constitutes approximately 85% of the total protein content in photoreceptors. Its retention in processing organelles at the cell body due to loss of outer morphology known from mammalian retina. Sprouting of rod bipolar cell dendrites into the ONL and of axon terminals in the GCL appeared to be a common feature during the retinal organ culture. Especially the sprouting into the ONL is known as a feature associated with the loss of the synaptic partner in the ONL during pathologic conditions, alongside loss of photoreceptors. Immuno labeling of synaptic ribbons in photoreceptor synaptic terminals was feasible at all time points, implying that even though there was a considerable loss of photoreceptors during retinal explant culture the remaining neurons remained viable. However, the ex vivo state of the organotypic retina culture appeared to stimulate reactive Müller cell gliosis, which became obvious through high levels of GFAP shown by immunohistochemistry and fold change of GFAP gene expression (Figs. 5, 6). This can be seen as an early and unspecific reaction to retinal damage with the aim to support the survival of retinal neurons. Müller cells contain their adenosine triphosphate (ATP) principally from glycolysis and have a low rate of oxygen consumption, which may spare oxygen for retinal neurons, particularly in the INL and GCL. No migrating or proliferating Müller cells were observed, which is in contrast to observations in the diseased retina. The loss of systemic circulating factors may account for some of the changes seen when isolated retinal tissue is grown in culture.

We found that all retinal layers in retinal organ culture were clearly detectable at all time points. The major difference between freshly isolated retina and retinal explants observed in vertical frozen sections was that the retina got thinner during culture due to gradual primary loss of outer and inner photoreceptor segments and subsequent secondary loss of photoreceptor nuclei. This was probably due to the fact that the RPE could not be kept during dissection of the retina and consequently not be cocultivated. Therefore, recycling of photoreceptor outer segments was not possible during retinal explant culture and subsequent loss of whole photoreceptors was unavoidable. During the whole length in culture, rod and cone photoreceptors were positive for antibodies against rhodopsin and cone opsins, respectively (Fig. 2). We conclude that all photoreceptor types were still producing their respective photopigment during retinal explant culture, even though the expression rate dropped considerably as our rhodopsin qPCR data confirmed (Fig. 3). Protein kinase α consistently stained rod bipolar cells in retinal explant cultures (Fig. 4). They kept their typical rhodopsin considerably as our explant culture, even though the expression rate dropped producing their respective photopigment during retinal explant culture. They kept their typical rhodopsin considerably as our explant culture, even though the expression rate dropped producing their respective photopigment during retinal explant culture.
segments instigates photoreceptor toxicity resulting in apoptosis,\textsuperscript{44} which was more prominent after 4 days in retinal explant culture.

The results indicate that our retinal explants were in a viable condition during the first week of in vitro culture. Protein kinase \( \alpha \) and CtBP2 immunohistochemistry revealed that neurons in organotypic retina cultures displayed much of their in vivo phenotype in terms of cytoarchitecture and stratification as well as preserved synaptic connections between rod photoreceptors and rod bipolar cells (Fig. 4). Early gliosis in Müller cells and microglia activation can be taken as other proof for viability and metabolic activity of organotypic retina cultures.

**Figure 7.** Immunostainings of apoptosis-inducing factor AIF (A–F) and TUNEL assay (G–L) on vertical frozen sections through organotypic cultures of adult mouse retina. Compared to day 0 retina, perikarya in the INL of retinal explant cultures show brighter AIF immunoreactivity. In the ONL, AIF immunoreactivity is detectable only as isolated puncta. After 8 days in culture, AIF is brightly visible in the GCL. TUNEL assay on vertical frozen sections through organotypic cultures of adult mouse retina harvested after 0 to 10 days in culture (G–L). Nuclear DAPI stain is shown in blue only on the right side of the micrograph. After 2 days in culture, no retinal cells are TUNEL positive (damaged DNA gets labeled by dUTP nucleotides that get labeled enzymatically by a green fluorescent chromophore). After 4 days in culture, a number of nuclei in the ONL are moderately TUNEL positive (arrows). After 6 days in culture, number of TUNEL-positive nuclei in the ONL had increased considerably, many of them showing high level of fluorescence (J). In the INL and GCL, TUNEL-positive nuclei are found very rarely. After 8 and 10 days in culture (K, L), the number of TUNEL-positive cells found in the ONL is still high, even though the ONL is considerably thinner compared to 4 and 6 days (I, J). IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 50 \( \mu \)m.
during the first week—more so since clear signs of diseased retina, like Müller cell proliferation and migration, as well as dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed. Overall, this represents a widening of the time dislocation of AIF into the nuclear layers, could not be observed.