

Photoreceptor vitality in organotypic cultures of mature vertebrate retinas validated by light-dependent molecular movements [☆]

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Abstract

Vertebrate photoreceptor cells are polarized neurons highly specialized for light absorption and visual signal transduction. Photoreceptor cells consist of the light sensitive outer segment and the biosynthetic active inner segment linked by a slender connecting cilium. The function of mature photoreceptor cells is strictly dependent on this compartmentalization which is maintained in the specialized retinal environment. To keep this fragile morphologic and functional composition for further cell biological studies and treatments we established organotypic retina cultures of mature mice and *Xenopus laevis*. The organotypic retina cultures of both model organisms are created as co-cultures of the retina and the pigment epithelium, still attached to outer segments of the photoreceptor cells. To demonstrate the suitability of the culture system for physiological analyses we performed apoptotic cell death analyses and verified photoreceptor viability. Furthermore, light-dependent bidirectional movements of arrestin and transducin in photoreceptors *in vivo* and in the retinal cultures were indistinguishable indicating normal photoreceptor cell-biologic function in organotypic cultures. Our established culture systems allow the analysis of mature photoreceptor cells and their accessibility to treatments, characteristic for common cell culture. Furthermore, this culturing technique also provides an appropriate system for gene delivery to retinal cells and will serve to simulate gene therapeutic approaches prior to difficult and time-consuming *in vivo* experiments.

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1. Introduction

In the vertebrate eye, the retina is responsible for light perception and also for the first processing steps in vision. The retina consists of different neuronal cell types which are organized in different layers (Fig. 1G and H). The photoreceptor cells are localized within the innermost layer of neuronal retina. Rod and cone photoreceptor cells are highly polarised sensory neurons which consist of morphologically and functionally specialized compartments. The light-

sensitive photoreceptor outer segment is linked with the inner segment and the cell body by a slender non-motile cilium, the so-called connecting cilium (Fig. 1I). The outer segment is arranged as hundreds of stacked membrane disks and contains all the components of the visual transduction cascade. The inner segment compartment mainly houses all organelles for biosynthesis of proteins and energy production, necessary for maintenance of the cell function (Fig. 1I).

Photoexcitation of the visual pigment rhodopsin activates the visual heterotrimeric G-protein transducin, leading to cGMP hydrolysis in the cytoplasm and closing of cGMP-gated channels in the plasma membrane of the outer segment (Molday & Kaupp, 2000). Arrestin binding to activated phosphorylated rhodopsin (R*P) terminates the visual transduction by preventing further binding of transducin to R*P. A fast light adaptation of photoreceptor cells relies on

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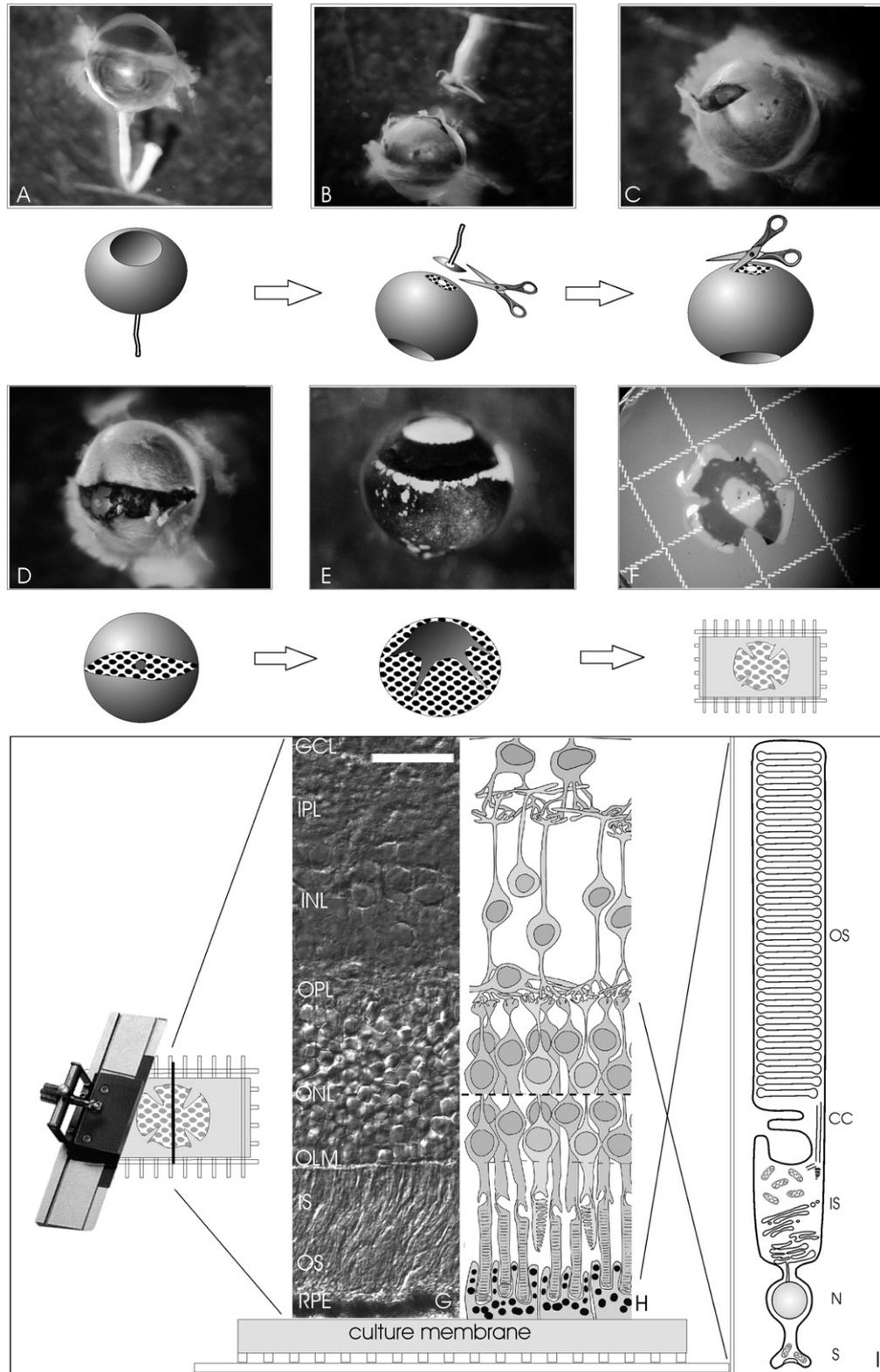


Fig. 1. Dissection and preparation of organotypic retina culture. (A) Eyeball with optic nerve prepared from a sacrificed mouse. (B) Cutting off the optic nerve under slight tension with forceps. (C) Incision of sclera by gently inserting scissor between the retinal pigmented epithelium and the sclera. (D) Complete incision of sclera around eyeball to each side reaching the cornea. (E) Incisions in the retinal cup, previously removed from sclera, cornea, lens, vitreous, iris, and hyaloid vessel. (F) Flattening of spread retina on culture membrane attached to nylon spacer. (G) Differential interference contrast picture of a retinal cryosection indicating the orientation of retinal explant with pigmented epithelium attached to the culture membrane. (H) Schematic representation of retina and cell organization. (I) Schematic representation of vertebrate photoreceptor cell. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 13.2 μ m.

feedback mechanisms based on changes in the intracellular free Ca^{2+} -concentration which affect several steps in the visual transduction cascade (Palczewski, Polans, Baehr, & Ames, 2000). However, massive bidirectional translocation of transduction proteins, in particular of arrestin and of transducin, between the functional compartments of photoreceptor cells contribute to a much slower adaptation of rod photoreceptor cells (Sokolov et al., 2002).

Light-induced exchanges of signal cascade components were first noted about two decades ago (Broekhuysen, Tolhuizen, Janssen, & Winkens, 1985; Brann & Cohen, 1987; Philp, Chang, & Long, 1987; Whelan & Mc Ginnis, 1988) and are currently of prominent interest in the field (e.g. (Hardie, 2002; Gießl, Trojan, Pulvermüller, & Wolfrum, 2004; Burns & Arshavsky, 2005)): upon illumination, 80% of transducin moves in minutes from the outer segment to the inner segment and the cell body of rod photoreceptor cells. The G-protein subunits return to the outer segments in the dark in a longer time course of hours. In contrast, arrestin translocates under these light conditions in a reciprocal direction. The intersegmental translocations of arrestin and transducin are thought to contribute to long term adaptation of rod photoreceptor cells (Sokolov et al., 2002; Strissel, Sokolov, Trieu, & Arshavsky, 2006). Nevertheless, the molecular and cellular mechanisms underlying these adaptive movements of arrestin and transducin still remain elusive (Chen, 2005; Strissel et al., 2006).

In cell biology, molecular intracellular movements and their association with the cytoskeleton are studied mainly in well-defined and accessible cultured systems. The accessibility of cultured cells allows the application of chemical reagents and well-defined external stimulation. However, so far no applicable method exists to cultivate differentiated photoreceptor cells without losing their compartmentalization. After dissociation from their retinal environment, photoreceptor cells lose their functional integrity in primary cell culture (Fintz et al., 2003; Leveillard et al., 2004). Cultivation of photoreceptor cells in their native environment the retina may solve this problem. However, in most attempts, organotypic cultures of mature vertebrate retinas which contain differentiated compartmentalized photoreceptor cells survived only for very short times, e.g. several hours (Ogilvie, 2001). Here, we show that a co-culture of the neuronal retina and retinal pigment epithelium not only survives for several days, but also keeps the physiological conditions for molecular movements between the outer and inner segment of the fragile photoreceptor cells. After introducing this culturing system in rodents we also adapted the organotypic culture to the retina of *Xenopus laevis*, a well-accepted model organism in retinal cell biology.

2. Methods

2.1. Animals

C57BL/6J mice were maintained on a cycle of 12 h of light (200 lux) and 12 h of darkness, with food and water *ad libitum*. In light adaptation

experiments mice were illuminated (200 lux), or kept in the dark before dissection of eyes and fixed with 4% formaldehyde in soerensen's phosphate buffer.

Wild-type *Xenopus* frogs were obtained from *Xenopus* Express (Plant City, FL). Frogs were either light-adapted for 60 min (800 lux), or kept in the dark and subsequently fixed with 3.7% formaldehyde in 73% methanol.

All animals used in these experiments were cared for and handled according to the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Vision and Ophthalmic Research and according to institutional animal care and use guidelines.

2.2. Retina culture

The retina culture system was established according to the experimental procedures previously published (Caffe, Visser, Jansen, & Sanyal, 1989) and altered as described in the following. In brief, intact eyes of postnatal day 12–14 C57/BL6J mice were immediately removed from sacrificed animals and incubated with 1.2 mg/ml Proteinase K (Sigma–Aldrich, Germany) for 15 min at 37 °C. Proteinase K activity was stopped by transferring eyes to culture medium containing 10% fetal calf serum (10 ml) for 5 min. After rinsing the eyes three times in serum-free culture medium (5 ml), retinas were dissected in basal culture medium by removing sclera, ocular tissue, and the hyaloid vessel under preservation of the pigmented epithelium (Fig. 1). Retinas were spread with the retinal pigmented epithelial cells facing down (Fig. 1F and G) on ME 25/31 culture membranes (Schleicher and Schuell, Germany), and cultured in Dulbecco's Modified Eagle's Medium with F12 supplement (DMEM-F12) and 10% fetal calf serum, L-glutamine, penicillin and streptomycin (Sigma–Aldrich, Germany) and maintained at 37 °C with 5% CO_2 . Specimens of postnatal day (pn) 12 to 14 were cultured for at least 1 day (12 h light-emitting diodes (LED) 200 lux/12 h dark) before start of light conditioning. Tissues from litter-matched specimens of pn 14 were used as controls.

In dark to light studies, cultured retinas and control mice were dark-adapted for 4 h and then exposed to 200 lux of light for 30 min by light-emitting diodes (LED). In light to dark studies, cultured retinas as control mice were exposed to light of 400 lux for 60 min before darkening. Since the optic apparatus is removed, retina cultures were illuminated with less light intensity than control animals.

2.3. Dissection and preparation of *Xenopus* tissue

Retinas from *Xenopus* eyes were dissected and prepared as described for mouse eyes till the stage when lens is removed (Fig. 1E). Retinas of *Xenopus* were cultured with the retina surrounding the lens. Eyes of control animals were fixed with 3.7% formaldehyde in 73% methanol, were rehydrated through a graded series of methanol in phosphate-buffered saline, equilibrated overnight in 30% sucrose, and then embedded in OCT medium (Sakura FineTek, Torrance, CA). Cryosections (12 μm) were processed for immunocytochemistry as previously described (Peterson et al., 2003).

2.4. TUNEL staining

For TUNEL staining, the "In Situ Cell Death Detection Kit" (Boehringer Mannheim, Germany) was used per instructions. The incubation with the "TUNEL-mixture" for 1 h at 37 °C in a humid chamber was terminated by multiple washes in PBS. Dried sections were mounted in Mowiol (HOECHST 4.88, Hoechst, Germany).

2.5. Antibodies and fluorescent dyes used on mouse tissue

Affinity-purified polyclonal rabbit antibodies against the α -subunit of the G protein were obtained from Biomol Research Laboratories, Inc. (PA) and used on mouse retina slices. Mouse antibodies directed to arrestin (clone 3D1.2) were applied on mouse retina slices as previously characterized (Nork, Mangini, & Millecchia, 1993). *Xenopus* retina slices were

immunocytochemical incubated using an anti-arrestin monoclonal antibody (1:50 xAr1-6). Labeling was detected with an anti-mouse-FITC conjugate (1:100). Slides were imaged with a fluorescence microscope (Zeiss, AxioVision Release 4.4).

2.6. Fluorescence microscopy

Cultured retinas, as eyes of control mice were cryofixed in melting isopentane, cryosectioned and treated as described (Reiners et al., 2003; Wolfrum, 1991). Secondary antibodies conjugated to Alexa 488 or Alexa 568 (Molecular Probes), sections were mounted in Mowiol 4.88 (Farwerke Hoechst, Frankfurt, Germany), containing 2% *n*-propyl-gallate. There was no reaction observed in control sections. Mounted retinal sections were examined with a Leica DMRP microscope. Images were obtained with a Hamamatsu ORCA ER charge-coupled device camera (Hamamatsu, Germany) and processed with Adobe Photoshop (Adobe Systems, USA).

3. Results and discussion

Previous studies indicated that photoreceptor cells lose their compartmentalization and functional integrity in primary cell culture (Fintz et al., 2003; Leveillard et al., 2004). In the present work, we cultivated photoreceptor cells in their native environment in organotypic retina cultures. Cultivation of neonatal retinas is a standard method for the analysis of retina differentiation and for pharmacologic and electrophysiologic studies on the developing neuronal retina network (Jablonski, 2003; Caffè, Soderpalm, Holmqvist, & van Veen, 2001; Perez-Leon et al., 2003). However, in neonatal retinas, the photoreceptor differentiation is still in progress and the compartmentalization of photoreceptor cells is not complete. So far, most attempts to culture mature vertebrate retinas have failed and the differentiated photoreceptor cells survived only for very short times, e.g. several hours (Ogilvie, 2001). To extend the life span of the

retinal cells in the organotypic culture of mature retinas, we customized culturing conditions. Our investigations revealed that cyclic light during culturing contributes to visible maintenance of the retina morphology. The stability of the fragile photoreceptor outer segments was enhanced by co-culturing with attached pigment epithelium cells immobilized on the nitrocellulose cultivation membrane (Fig. 1G and H). Mouse ages of post natal days (PN) 12–14 have been estimated to be a good compromise between photoreceptor integrity and outer segment length. This improvement in culturing of mature retinas results in enhanced viability and retain physiological activity *ex vivo*.

Viability of the organotypic retina culture was assessed by TUNEL staining after different culture time periods (Fig. 2). Fig. 2A shows a (positive) control slice treated with DNase, where all nuclei of retinal cells are stained by the TUNEL method. In retinas cultured for 1–3 days there are only a few TUNEL positive nuclei stained in the outer nuclear layer (Fig. 2B–D). After 7 days of culturing, numerous nuclei in almost every layer of cultured retinas are stained indicating a high amount of apoptotic cells (Fig. 2E). This evaluation of the viability state of the cultured retinal explants from mice by TUNEL staining, confirmed a low rate of apoptotic retinal cells during the first three days of culturing. Present results indicate that at least for the first three days *ex vivo*, the organotypic retina culture is a viable system suitable for physiological experimental approaches.

3.1. Light-dependent translocation of arrestin and transducin also occurs in organotypic cultures of the mouse retina

Light-driven translocation of arrestin and transducin between the inner and outer segment compartment had

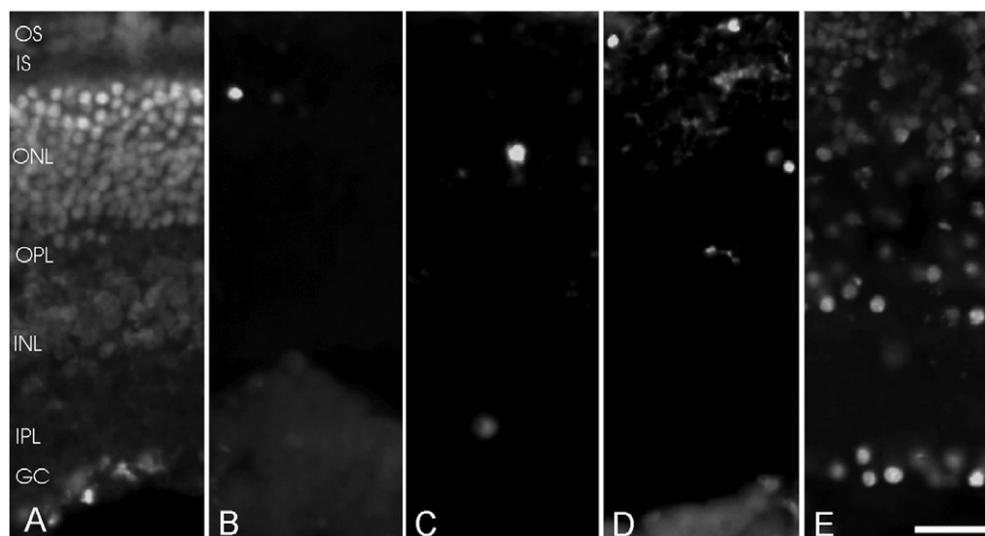


Fig. 2. Viability assessment of retinas cultured for different time periods. (A) TUNEL staining of nuclei in a longitudinal cryosection of DNase-treated mouse retina. (B) TUNEL staining of nuclei in a longitudinal cryosection of cultured retina after one day *ex vivo*. (C) TUNEL staining of nuclei in a longitudinal cryosection of cultured retina after two days *ex vivo*. (D) TUNEL staining of nuclei in a longitudinal cryosection of cultured retina after 3 days *ex vivo*. (E) TUNEL staining of nuclei in a longitudinal cryosection of cultured retina after seven days *ex vivo*. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 13.2 μ m.

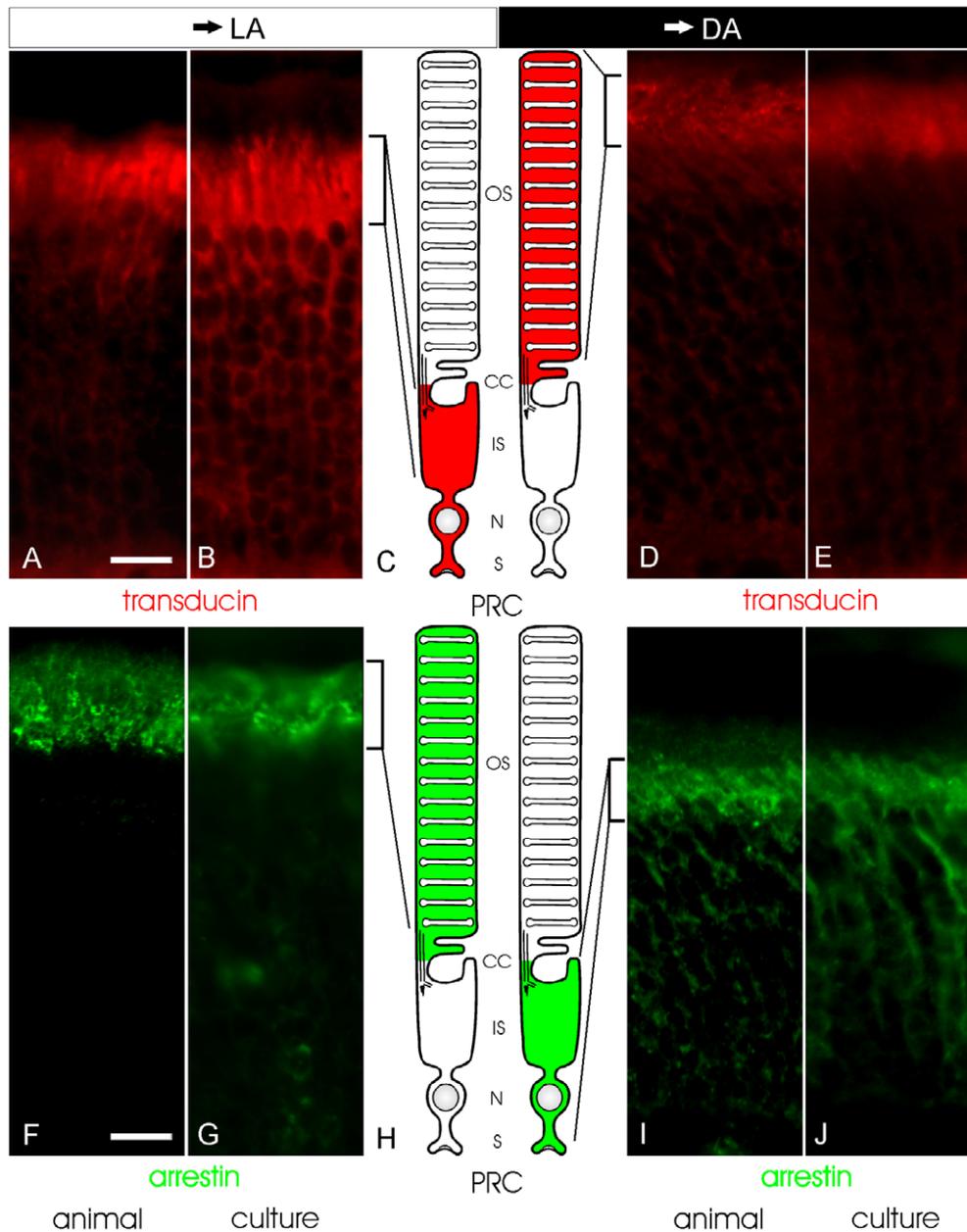


Fig. 3. Light-dependent translocation of transducin and arrestin in mammalian rod photoreceptor cells. (A) Immunocytochemical localisation of transducin in a longitudinal cryosection through a light-adapted mouse retina. (B) Immunocytochemical localisation of transducin in a longitudinal cryosection through a cultured light-adapted mouse retina. Transducin can be localized in inner segments of photoreceptor cells in retinas from light-adapted animals and in inner segments of light-adapted cultured retinas. (C) Schematic rod photoreceptor cells (PRC) with visualized transducin localisations in red. OS, outer segment; CC, connecting cilium; IS, inner segment; N, the nucleus; S, synapse. (D) Immunocytochemical localisation of transducin in a longitudinal cryosection through a dark-adapted mouse retina. Transducin can be localized in outer segments of photoreceptor cells in retinas from dark-adapted animals as in outer segments of dark-adapted cultured retinas. (E) Immunocytochemical localisation of transducin in a longitudinal cryosection through a cultured dark-adapted mouse retina. (F) Immunocytochemical localisation of arrestin in a longitudinal cryosection through a light-adapted mouse retina. (G) Immunocytochemical localisation of arrestin in a longitudinal cryosection through a cultured light-adapted mouse retina. Arrestin can be localized in outer segments of photoreceptor cells in retinas from light-adapted animals as in outer segments of light-adapted cultured retinas. (H) Schematic rod photoreceptor cells (PRC) with visualized arrestin localisations in green. (I) Immunocytochemical localisation of arrestin in a longitudinal cryosection through a dark-adapted mouse retina. Arrestin can be localized in inner segments of photoreceptor cells in retinas from dark-adapted animals as in inner segments of dark-adapted cultured retinas. (J) Immunocytochemical localisation of arrestin in a longitudinal cryosection through a cultured dark-adapted mouse retina. Arrestin can be localized in inner segments of photoreceptor cells in retinas from dark-adapted animals as in inner segments of dark-adapted cultured retinas. Scale bars, 7.5 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

been described two decades ago (Philp et al., 1987; Whelan & Mc Ginnis, 1988). Although the phenomenon was intensively investigated during the last years (Pulvermüller et al.,

2002; Sokolov et al., 2002; Wolfrum, Giessl, & Pulvermüller, 2002; Peterson et al., 2003; Mendez, Lem, Simon, & Chen, 2003; Nair et al., 2004), the molecular processes

underlying these movements still remained elusive. In the present study, we investigated the suitability of the organotypic retina culture for the analysis of physiological processes *ex vivo*, in particular light-dependent protein translocations.

To validate whether retinas remain in the physiological condition for the analysis of light-driven protein movements during cultivation, in a first set of experiments, we compared the movement of transducin in retinas of mice and explanted cultured mouse retinas (Fig. 3). After fully light (>1/2 h) and dark adaptation (>3 h), the endpoints of light-induced molecular translocations (Elias, Sezate, Cao, & McGinnis, 2004), we found no differences between the *in vivo* and the *ex vivo* condition. Immunofluorescence cytochemistry revealed that during light adaptation, transducin translocated to the inner segment of photoreceptors in the cultured retina as in the control animal (Fig. 3A and B). Likewise, after dark adaptation, transducin was found in the outer segment compartment in both the cultured retina and control animal (Fig. 3D and E).

The localization of visual arrestin has been described to be reciprocal to that of transducin in light and dark adaptation, respectively. Consequently, we also verified the movement of arrestin in light-adapted and dark-adapted retina cultures. Under light-adapted conditions, arrestin is localized to the outer segment of photoreceptors in retinas of intact animals as of cultured retinas (Fig. 3F–H). In contrast, arrestin localized to the inner segment during dark adaptation for the retina from the control animal as well as the compared explant (Fig. 3I and J). These normal movements of both arrestin and transducin provide evidence for the continuing physiological activity of the retina cultures.

3.2. Light-dependent translocation of arrestin in organotypic cultures of the *Xenopus* retina

In a next set of experiments, we asked the question whether the successful culturing technique developed for mouse retinas is adaptable to other vertebrate species. To expand the possibilities of analyses we transferred the culturing technique to the amphibian *X. laevis*, a well-accepted animal model in retinal cell biology. In contrast to the mouse culture, we kept the lens during the dissection in the retinal sphere to remain stability of the relatively thin *Xenopus* retina.

Cultured mature retinas of *X. laevis* under dark or light conditions as retinas of light treated mature frogs were analyzed for the localization of arrestin (Fig. 4). Arrestin localizations in fully light or dark-adapted photoreceptor cells were indistinguishable between control mature *Xenopus* and previously studied *Xenopus* tadpoles (Peterson et al., 2003; Peterson et al., 2005) and the *ex vivo* cultures. Photoreceptor outer segments of light-adapted cultured retinas and of control animals show a prominent localization of arrestin in outer segments of photoreceptors (Fig. 4A and B). In contrast, arrestin is mostly localized to inner segments of photoreceptors after dark adaptation of frogs or cultured retinas, respectively (Fig. 4D and E).

We were able to show translocation of visual arrestin also in photoreceptor cells of cultured frog retinas as in light-adapted intact frogs. The amphibian photoreceptor cell has slightly different morphology and dimensions compared to the mammalian photoreceptor like bigger outer segments, which makes it a suitable organism to visualize protein distributions in outer segments for example.

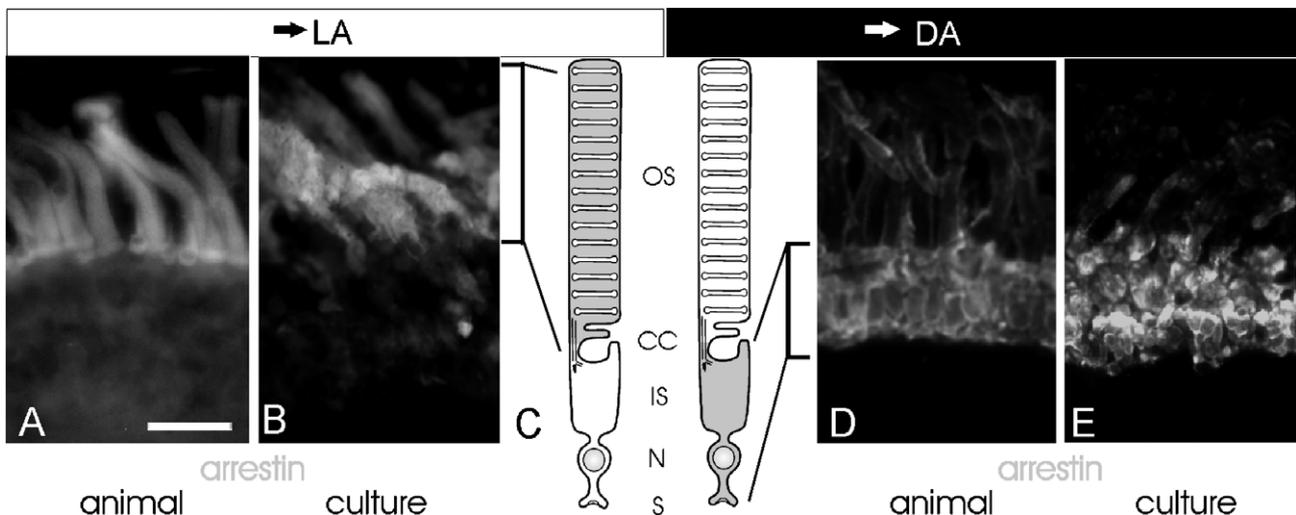


Fig. 4. Light-dependent translocation of arrestin in amphibian rod photoreceptor cells. (A) Immunocytochemical localisation of arrestin in a longitudinal cryosection through a light-adapted *Xenopus* retina. (B) Immunocytochemical localisation of arrestin in a longitudinal cryosection through a cultured light-adapted *Xenopus* retina. Arrestin can be localized in outer segments of photoreceptor cells in retinas from light-adapted animals as in outer segments of light-adapted cultured retinas. (C) Schematic rod photoreceptor cells (PRC) with visualized arrestin localisations in green. OS, outer segment; CC, connecting cilium; IS, inner segment; N, nucleus; S, synapse. (D) Immunocytochemical localisation of arrestin in a longitudinal cryosection through a dark-adapted *Xenopus* retina. (E) Immunocytochemical localization of arrestin in a longitudinal cryosection through a cultured dark-adapted *Xenopus* retina. Arrestin can be localized in inner segments of photoreceptor cells in retinas from dark-adapted animals as in inner segments of dark-adapted cultured retinas. Scale bar, 22.5 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

To study the molecular mechanisms during adaptation in consideration of different molecular and morphological features between the two photoreceptor types (mouse and *Xenopus*), could give us new insights in photoreceptor function in general.

4. Conclusions and perspectives

Isolated photoreceptor cells in primary cultures lose their compartmentalization (Fintz et al., 2003; Leveillard et al., 2004). Also in cultured embryonic retinas photoreceptors do not develop complete compartmentalization, especially with regard to differentiate no distinct outer segments (Stiemke & Hollyfield, 1994). To analyze protein localization, like the light-dependent redistribution of arrestin and transducin in photoreceptor cell compartments, we established an organotypic retina culture of mature mice. In these cultures it is possible to localize components in photoreceptors in differentiated compartments of the inner and outer segment.

Our present data show the retina culture system to be a viable environment for photoreceptor cells and suitable for the analysis of light-dependent protein movements between photoreceptor cell compartments. This is principally supported by the ability of the cultured photoreceptors to still react to different light conditions by moving proteins of the visual signal transduction in and out of cellular compartments. As only fully adapted stages were analyzed, we cannot exclude differences in kinetics or extents of protein trafficking altered by the cultured conditions in comparison to the *in vivo*-state.

One of the major advantages in using organotypic retina cultures is the accessibility of this *ex vivo* system. Our culturing technique provides the opportunity to add and test substances or apply gene transfer systems directly into the culture medium. This includes for example pharmacological treatments. In contrast to other culture systems, such as eyecup cultures, treatments can directly reach the photoreceptors in the organotypic retina culture. In eyecup cultures, added substances have to penetrate or pass many layers of the retina from the vitreal side until they can reach the photoreceptor cells. In our preparation, the retina is exposed to added reagents on both the vitreal surface and the RPE surface. The culturing of eyecups and retinas could be compared with intraocular injections in either the vitreous (to target ganglion cells) or the sub retinal space to especially target photoreceptor and/or pigment epithelium cells. Our organotypic retina culture gives us a model system, which can be used to simulate sub retinal injections. This kind of “*in vivo*-like” environment can also be utilized for the simulation of gene therapeutic approaches. In preliminary experiments, vector systems and their gene (repair) constructs can be validated on the organotypic retina culture prior to injection into living animals. These proceedings can reduce the amount of animal experiments to a minimum and also decrease material consumption. For example the extensive production of viruses for gene

delivery can be shortened, by expressing the gene constructs with easier to handle transfection systems on retinal tissue explants first.

Until now, developed and differentiated photoreceptor cells with distinct outer segments could only be studied *in vivo* or *ex vivo* eyecup cultures, e.g. (Nair et al., 2005). In contrast to eyecup cultures, our culturing system of mature retinas provides more accessibility to photoreceptor cells. Furthermore, the maintained attachment of the pigment epithelium cells provided extended stability and vitality of photoreceptor cells of mature retina culture. In the present study, we introduced a technique of culturing mature retinas and evaluated their physiological activity by visualizing light-dependent protein translocations in fully compartmentalized photoreceptor cells. This organotypic culture of retinas could provide a new powerful experimental tool to answer extended cell biological questions which previously could not be addressed experimentally.

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