PRO RETINA
RESEARCH-COLLOQUIUM

CONFERENCE REPORT

Retinal Degeneration

From Bench To Bedside

An Interdisciplinary Dialogue

April 8th/9th, 2005

Potsdam, Seehotel am Templiner See

PRO RETINA DEUTSCHLAND e. V.
in association with the EU-project **"European Retinal Research Training Network (RETNET)"**

PREFACE

„From Bench To Bedside: An Interdisciplinary Dialogue“ what a wonderful theme for an international conference bringing together young scientists to learn more about retinal degenerative diseases.

Dialogue is the key to advancing our common objective to find a cure for retinal degenerative diseases. Research has made considerable progress in the last 20 years and diversified into several areas generating numerous results. Bringing this puzzle together to complete the full picture is the challenge of today and tomorrow.

The benefits of learning and talking to colleagues in the field are obvious, but to talk and interact with researchers from other fields will spawn new ideas and assist in creating the whole picture. Retina International congratulates Pro Retina Germany e.V. for organizing this brilliant research colloquium in association with the EU-project "European Retinal Research Training Network (RETNET)".

The fact that over 130 researchers spanning more than 17 countries have accepted the invitation to come to Potsdam is proof that the organisers have successfully created an attractive programme and created the opportunity for interaction at precisely the right time.

May your dialogue be fruitful and may you find new friendships fueling enthusiasm to continue to bring research from the laboratory to the bedside, i.e. to the patient!

With best wishes

Christina Fasser
President Retina International

PROGRAMME

Friday, 8th

13:15 F. Badura, ProRetina Deutschland: Welcome remarks
13:25 Prof. E. Zrenner, Tübingen: The development of research in hereditary retinal degeneration

13:45 – 15:30 **Session 1: The Functional Retina**
Chairman: E. Zrenner
1. M. Griffith: The patient's view
2. K.-W. Koch, Oldenburg: Regulation of phototransduction
3. K. Rüther, Berlin: The role of protein kinase C-alpha in the On-bipolar function
4. A. Reichenbach, Leipzig: The role of the Müller (Glial) cell in retinal degeneration

15:30 – 16:00 Coffee break

16:00 – 18:00 **Session 2: Degeneration of photoreceptors and the retinal pigment epithelium – molecular mechanisms**
Chairman: S. Bhattacharya
1. D. Besch, Tübingen: Dysfunction of the retinal pigment epithelium in inherited multifocal RPE diseases
2. C. Driessen, Nijmegen: Ocular retinoid biochemistry and its role in human disease
3. B.Wissinger, Tübingen: Pathomechanisms of cone photoreceptor function loss
4. B. Laggerbauer, Würzburg: Genetic and biochemical analysis of RP-linked pre-mRNA splicing factors

18:00 Dinner

19:15 Guest-Lecture: S. Bhattacharya, London:
The European dimension: Strategic approaches in retinal research

19:45 – Open End: WIP (Work in Progress) - Session with ‚Smart and Easy Jazz'

PROGRAMME

Saturday, 9th

8:30 – 10:15 **Session 3: The retinal proteome: new proteins – new views**
Chairman: O. Strauss
1. M. Ueffing, München: Analysis of protein expression and signaling in the Mueller cell
2. J. Förster, Würzburg: The MAGUK scaffolding proteins in the mammalian retina
3. J. H. Brandstätter, Erlangen: The proteome of the photoreceptor synapse
4. U. Wolfrum, Mainz: Molecular analyses of Usher 1 and 2 proteins in retinal photoreceptor cells

10:15 Coffee break

10:45 – 12:30 **Session 4: Animal models**
Chairman: B.H.F. Weber
1. E. Knust, Düsseldorf: Drosophila crumbs – from epithelial cell polarity to retinal degeneration
2. U. Luhmann, Zürich: Abnormal intraretinal vascular development and regression of hyaloid blood vessels in a knockout mouse model for Norrie disease/exudative vitreoretinopathy
3. M. W. Seeliger, Tübingen: In vivo confocal imaging of the retina in animal models using scanning laser ophthalmoscopy
4. K. K. Singh, Hannover: Genetics of retinal drusen formation in rhesus macaques

12:30 Lunch

13:45 – 15:30 **Session 5: Therapeutic strategies in hereditary retinal degeneration**
Chairman: A. Laties
1. A. Wenzel, Zürich: Apoptosis in retinal degeneration-Neuroprotection of photoreceptors
2. B. Weber, Regensburg: Rescue of photoreceptor degeneration in a genetic mouse model for X-linked juvenile retinoschisis
3. U. Bartsch, Hamburg: Stem cell based therapeutic approaches
4. P. G. Layer, Darmstadt: Reaggregates and stem cells for retinal tissue engineering

15:30 Coffee break
16:00 – 17:00 Round Table Discussion
17:00 Farewell Remarks

SPEAKER ABSTRACTS

The Development of Research in Hereditary Retinal Degeneration

E. Zrenner, University Eye Hospital, Tübingen

Since the first descriptions of the various forms of retinitis pigmentosa and allied diseases, numerous attempts have been made to improve understanding the origin of these diseases, their pathophysiological processes and strategies of therapeutical intervention.

In the beginning research was concerned with the proper description of the various diseases, their inheritance, the course of progression and especially the different phenotypes. The correlation of phenotypes with genotypes is an important process still going on today with great intensity, employing new methods of non-invasive assessment of function as well as morphology.
Another very important stream was the discovery of mutations and deletions in genes that lead to hereditary retinal degenerations. So far 158 gene loci for retinal degenerations have been described with 110 genes being cloned (www.sph.uth.tmc.edu/retnet). Lately causal proteins are increasingly investigated with their expression in the retina and the consequences of their dysfunction on a cellular level.
Furthermore the availability of different animal models offers the possibility to study very thoroughly the consequences of the mutations with invasive and non-invasive methods on a molecular and cellular as well as systemic level in these animals.
This interdisciplinary research succeeded in elucidating many pathophysiological mechanisms, which is a prerequisite for the development of successful therapies. Consequently based on experimental results new therapeutic approaches could be initiated giving further information on targeted pharmacological intervention including new drug delivery systems; application of cell transplants including stem cells and especially the application of gene therapy with various viral and non-viral vectors. For those patients who have lost treatable cells, the development of electronic retinal prosthesis has achieved major breakthroughs.

All these achievements justify the hope that successful therapeutic strategies can be developed for the various forms and stages of hereditary retinal degeneration in the (foreseeable) future.

Regulation of Phototransduction

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The light sensitivity of a vertebrate photoreceptor cell (light adaptation) depends critically on the precise operation of several Ca^{2+}-dependent control steps. Ca^{2+}-sensor proteins detect changes in the cytoplasmic Ca^{2+}-concentration and regulate their targets. Guanylate cyclase-activating proteins (GCAPs) are among these Ca^{2+}-sensors, they activate retina specific membrane bound guanylate cyclases in a calcium-dependent fashion and thereby adjust light sensitivity and adaptation in rod and cone cells. Prominent isoforms in the mammalian retina are GCAP1 and GCAP2. Both calcium- sensor proteins show remarkable differences with respect to calcium-sensitivity, catalytic efficiency and target recognition. These differences allow fine tuning of the target and probably account for an optimized adjustment of the photoresponse. Some inherited retinal dystropies correlate with mutations in the gene coding for photoreceptor specific guanylate cyclase type 1 (ROS-GC1) for example type 1 Leber's congenital amaurosis (LCA1) and cone-rod dystrophy type 6 (CORD6). The LCA1-linked mutations are distributed over almost the entire ROS-GC1 coding sequence but the CORD6-linked mutations are restricted to three positions only, E786, R787 and T788, located within the putative ROS-GC1 dimerization domain. The biochemical explanation of the LCA1 phenotype was attempted for the mutation F514S. It causes complete loss of GCAP1-sensitivity. Biochemical analyses of the consequences of the CORD6-causing mutations showed a complex pattern of GCAP regulation, e.g. a decrease of basal cyclase activity in concert with an increase in GCAP-dependent maximal velocity. These results showed the importance of the region E786-T788 for dimerization of ROS-GC1 and proved a molecular basis for understanding defective calcium-signalling in CORD6.

Hwang, J.-Y., Lange, C., Helten, A., Höppner-Heitmann, D., Duda, T., Sharma, R.K. and Koch, K.-W. (2003) Regulatory modes of rod outer segment membrane guanylate cyclase differ in catalytic efficiency and Ca^{2+}-sensitivity. *Eur. J. Biochem.* 270, 3814-3821
Duda, T. and Koch, K.-W. (2002) Retinal Diseases Linked with Photoreceptor Guanylate Cyclase. *Mol. Cell. Biochem.* 230, 129-138

The Role of Protein Kinase C-alpha in the On-bipolar Function

Klaus Ruether [1], Michael Leitges [2], Ji-Jie Pang [3], Mark E. Pennesi [3], Samuel M. Wu [3], Wolfgang Baehr [4,5,6], Olaf Strauss [7]

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Purpose: Protein kinase Cα (PKCα) is abundant in retinal bipolar cells, but its role in transduction of the light signal has not been determined. To gain insight into its function, we examined PKCα knockout (PKCα-/-) mice using electroretinography (ERG) and single cell recordings.

Methods: Immunocytochemical staining with polyclonal anti-PKCα antibody was used to detect PKCα in rod bipolar cells. For the elucidation of retinal function, a-, b- and c-wave Ganzfeld-ERG-recordings were performed in knockout and control animals. On the cellular level, light-evoked current responses at various holding potentials were recorded under voltage clamp from rod bipolar cells.

Results: PKCα-/- mice showed no signs of retinal degeneration up to six months of age, but ERG measurements indicated a delayed return of the scotopic (rod-sensitive) b-wave to baseline. Direct recordings of the light-evoked bipolar response demonstrated a prolonged b-wave profoundly altering the waveform. These results indicate that PKCα is an important modulator affecting bipolar cell signal termination. In addition the implicit time of the ERG c-wave, originating from the retinal pigment epithelium (RPE), and recovery of photoreceptors from bleaching conditions were substantially faster in knockout animals than in wild type controls.

Conclusions: These results suggest that PKCα also plays a vital role in the photoreceptor – retinal pigment epithelium interaction.

The Role of the Müller (Glial) Cell in Retinal Degeneration

Bernd Biedermann[1], Andreas Bringmann[2], Mike Francke[1], Ivona Goczalik[1], Ianors Iandiev[1,2], Thomas Pannicke[1], Maik Raap[1], Ortrud Uckermann[1,2], Sebastian Wolf[2], Peter Wiedemann[2] and Andreas Reichenbach[1]

[1]Paul Flechsig Institute of Brain Research and [2]Department of Ophthalmology, Leipzig University.

Purpose: In the healthy retina, Müller cells are performing a wealth of glia-neuron interactions, crucial for signal processing and for survival of retinal neurons; many of these functions depend on the expression of inwardly rectifying K^+ (Kir4.1) channels. We studied whether and how Müller cells may also contribute to retinal degeneration.

Methods: We used two animal models (retinal detachment / proliferative vitreoretinopathy (PVR) in rabbits, and transient ischemia in rats), as well as retinal tissue excised from human patients in the course of therapeutic interventions. Immunocytochemistry, patch-clamp electrophysiology, and vital dyes / imaging techniques were applied to wholemount preparations/isolated Müller cells.

Results: In various types of retinal injury or disease, the expression of functional Kir4.1 channels by Müller cells is down-regulated. This results in a depolarization of the membrane potential of the cells, and in a loss of crucial glial functions such as K^+ buffering, neurotransmitter uptake, and water homeostasis. As an example, after transient ischemia Kir4.1 immunoreactivity disappears from Müller cell endfoot membranes whereas the immunocytochemical localization of aquaporin-4 (AQP4) water channels remains unchanged. When retinal wholemount preparations from such animals are exposed to hypotonic solutions, the Müller cells swell. Such osmotic swelling does not occur in normal Müller cells but can be induced in these cells by blockage of the K^+ channels. This indicates that Müller cell-mediated K^+- and water clearance are coupled, and require the co-expression of Kir4.1 and AQP4. In the cases of detachment and PVR, the down-regulation of K^+ currents in Müller cells is accompanied by an up-regulation of functional purinergic (P2Y) receptors. This combination of features is similar to the situation in immature Müller cells / progenitor cells, and may contribute to the uncontrolled cellular proliferation characteristic for PVR.

Conclusions: Müller cells respond to retinal injury by a de-differentiation in respect to the expression of ion channels and ligand receptors. In particular, the down-regulation of functional Kir channels seems to constitute a key event, resulting in functional deficiencies of retinal glia, and in an aggravation of the pathomechanisms of retinal degeneration including edema, excitotoxicity, and retinal destruction / detachment due to pathological cell proliferation.

Dysfunction of the Retinal Pigment Epithelium in Inherited Multifocal RPE Diseases

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Alterations of retinoid cycle genes are known to cause retinal diseases that can be marked by focal white dot fundus lesions. Fundus appearances reveal tiny local RPE-changes, although generalized metabolic defects and global functional abnormalities are present. As a possible explanation, topographic inhomogeneities of the human photoreceptor mosaic and the role of a cone specific visual cycle will be discussed.

Recently, novel catalytic activities in membrane fractions of cone-dominant chicken and ground-squirrel retinas have been identified and it has been proposed that these activities represent catalytic steps in a particular visual cycle that serves cones. This cone-specific visual cycle does not involve the RPE but probably Müller cells. From a number of other studies we know that the mosaic of rods and L-, M, and S-cones in the human retina is topographically very heterogeneous. This implies that single RPE cells may subserve a quite different composition of photoreceptor types and subtypes. Due to these particular characteristics of photoreceptor subtypes as well as different pathways for photopigment regeneration the metabolic demand of individual RPE cells might differ. In "flecked retina diseases" heterogeneity of metabolic demand in individual RPE cells could therefore be responsible for their multifocal appearance.

Ocular Retinoid Biochemistry and its Role in Human Disease

Carola Driessen[1], Huub Winkens[1], Niyazi Acar[2], Wolfgang Baehr[3], Klaus Peter Hofmann[4], Mathias Seeliger[5], Krzysztof Palczewski[6], Jacques Janssen[1]

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Purpose: Retinoids carry out essential functions in vertebrate development and vision. Many of the retinoid processing enzymes remain to be identified at the molecular level. One of which RDH5, was cloned by us. Mutations in this gene have been reported to cause a human retinal disease designated as fundus albipuntatus. This is a more or less non-progressive disease of which the main features are extremely prolonged dark adaptation and white flecks in the fundus of affected patients. RDH5 knock out mice are were found being still capable of regenerating their rod visual pigment in the dark. Hence, in addition to RDH5, other additional enzymes capable of oxidizing 11-cis-retinol will need to be present. Three candidates, RDH11, RDH12 and RDH15, were clonedsubjected to intensive analysis of their presence in the retina, cofactor usage, and substrate-specificity. To expand the knowledge of retinoid biochemistry in vertebrates. ,we We will work to generate mice in which the genes encoding these three retinol dehydrogenases are no longer functional.

Methods: RDH5 knock out mice were further analysed using fluorescein and ICG angiography and electronmicroscopy. Targeting vectors were constructed for the genes encoding RDH15 and RDH12. Embryonic stem cells were electroporated and cell lines with appropriate targeting were identified. These cell lines were used for blastocyst injection. Chimeric offspring were used in a breeding program.

Results: RDH5 mice showed a flecked retina, resembling the retina of fundus albipunctatus patients. Electronmicroscopy showed an accumulation of lipid compared to wildtype animals. RDH11 and RDH12 are both able to convert retinoids. RDH11 is present in the retinal pigment epithelium and co-purifies with RDH5. RDH12 mRNA is present in the outer nuclear layer and outer limiting membrane. Though RDH15 is highly homologous to RDH5, RDH15 is most likely involved in steroid metabolism. RDH15 chimaeric mice show massive cell expansions in different tissues.

Conclusions: The RDH5 mouse is a good animal for fundus albipunctatus. Three candidate enzymes are analysed for their role in the retinal retinoid metabolism. Of these enzymes,

RDH12 is the most important candidate for playing a in role formation of 11-cis-retinal. Recently mutations in this enzyme were found to be associated with childhood-onset severe retinal dystrophy.

Pathomechanisms of Cone Photoreceptor Function Loss

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Purpose: To study the genetic basis of cone dystrophies and investigate the molecular mechanisms of disease

Methods: Linkage analysis/positional cloning and/or candidate gene screening were employed to identify disease genes. Mutation screening was done by RFLP, SSCP, dHPLC and DNA sequencing. Potential splicing mutations were assayed by expression of minigene constructs in COS cells. Heterologously expressed CNGA3 mutations were analyzed by Western blotting, Ca^{2+} imaging and patch clamp recordings.

Results: In recent years we could show that mutations in CNGA3, CNGB3 or GNAT2 can cause autosomal recessive achromatopsia. Screening of these genes in a large cohort of patients enabled us to establish a comprehensive mutation spectrum and to extract epidemiological data for the central European population. In addition we could show that mutations in these genes may cause a variety of phenotypes including incomplete achromatopsia, oligo cone trichromacy, progressive cone dystrophy and macular dystrophy.
Functional analysis of heterologously expressed CNGA3 mutants convincingly correlates basal channel activity of mutant polypeptides with residual cone function in patients. Yet some CNGA3 mutants require co-expression of the CNGB3 subunit for channel activity.
More recently we could show that achromatopsia can also be caused by mutations in PDE6C. Mutations in PDE6C include several nonsense, missense and splicing mutations as demonstrated by minigene expression experiments. A mutation in pde6c was also found in a spontaneous mouse mutant that lacks cone function and shows early loss of cone photoreceptors in the retina. The mutant pde6c allele is characterized by a 1.6 kb insertion in intron 4 that causes the activation of a cryptic exon upon mRNA splicing.

Conclusions: Genes that encode cone photoreceptor specific isoforms of crucial components of the phototransduction cascade are a rich resource for the identification of disease genes that cause hereditary retinal disorders characterized by the loss or reduction of cone function. Functional studies of mutant proteins and the availability of animal models will help to improve our still limited knowledge about cone photoreceptor biochemistry and physiology.

Genetic and Biochemical Analysis of RP-linked pre-mRNA Splicing Factors

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Aim of this study: In recent years, several mutations in general pre-mRNA splicing factors have been linked to autosomal dominant forms of Retinitis pigmentosa (RP). In line with this, a novel RP-mutation in the processing factor hPrp4 was identified (group of H. Boltz). The finding that mutations in splicing factors that are required in every tissue can cause a tissue specific phenotype is surprising. Our groups have therefore dedicated efforts to a biochemical analysis of this phenomenon and to further screening for RP loci.

Methods: We have initially analysed the effect of a RP-causing mutation in hPrp4 on its interactions with other known splicing factors. For this purpose, we have employed an assay that monitors direct protein-protein interactions in vitro. In addition, we test the integration of the mutated protein into functional splicing units (U snRNPs) via co-immunoprecipitation. We are now in the process of combining gene silencing in cultivated cells or zebrafish embryos with complementation studies. These approaches should help to investigate whether mutated splicing factors linked to RP lead to inefficient or aberrant pre-mRNA splicing.

Results: Through linkage analysis in a collective of RP patients, a novel mutation in hPrp4 was identified. This missense mutation substitutes an arginine in the central region of the protein by a histidine. We have observed that this mutation dramatically reduces binding of hPrp4 to its known interacting partner hPrp3. The binding defect is confined to this interaction, since association with Cyp-20 (its second known interactor) remains unaffected. As a consequence, the mutated hPrp4 can no longer be integrated into the spliceosome. In addition, we have obtained preliminary data suggesting that the mutated hPrp4 does not confer a trans-dominant negative effect on pre-mRNA splicing in vitro.

Conclusions: The inability of a RP-linked hPrp4 mutant to bind hPrp3 and UsnRNPs indicates that this mutation elicits a loss of function rather than an aberrant function. Thinking along these lines, this biochemical defect induces a cellular situation similar to haploinsufficiency. Together, the data support the idea that inefficient splicing can be a cause for Retinitis pigmentosa.

Analysis of Protein Expression and Signaling in the Mueller Cell

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Purpose: The retinal Mueller glia (RMG) plays a pivotal role in supporting structure and function of the retina. The molecular mechanisms of how RMG support retinal integrity especially with respect to photoreceptor survival remains unclear so far. With the purpose to systematically analyse RMG function towards photoreceptor support, we have employed proteomic techniques to analyse protein expression patterns as well as single biologically relevant proteins.

Methods: The analytics is based on: 1) isolation of primary porcine RMG by panning, 2) high-resolution protein separation at high resolution by chromatography and two- dimensional gel electrophoresis (2D-GE), and, 3) protein identification by MALDI-TOF mass spectrometry.

Results:
Proteome mapping: By comparative 2D-GE mapping of isolated primary RMG cells in culture over time we have found that protein expression remains largely stable during initial culturing, but loss of Müller glia cell-specific proteins, such as glutamate synthetase can be seen over time. The changes in expression reflect the trans-differentiation from a multifunctional, highly differentiated phenotype to a de-differentiated monolayer of cells in culture.
Secreted factors: Müller glia cells secrete factors that show a pronounced effect on photoreceptor survival by yet unknown means. By combining very sensitive methods forto searching for secreted proteins from conditioned media using an *in vivo* 35-trans-S-label in combination with column chromatography and gel-electrophoresis we have detected proteins secreted by Müller glia cells that support photoreceptor survival. We have investigated the effect of glia cell derived neurotrophic factor (GDNF), a potent neurotrophic factor for retinal neurons and found that it targets RMG rather than photoreceptors. RMG in turn secrete distinct other neurotrophic factors that help photoreceptors to survive. From these results we propose, that RMG are likely to be the endogenous source of neurotrophic agents to support photoreceptor survival and function.

Conclusion: In vitro analysis of differentiated RMG function requires the use of first passage primary cells. We propose, that primary RMG are a major endogenous source of neurotrophic agents to support photoreceptor survival and function.

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The MAGUK scaffolding proteins in the mammalian retina

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Purpose: The subfamily of p55-like membrane-associated guanylate kinases (MAGUKs) is a group of cytoplasmic adapters characterized by a number of conserved protein interaction domains mediating the organization of biologically active molecules at sites of cell-cell contacts. Notably, MPP4 and MPP5 have been implicated in retinal function; however, their specific roles in the cellular mechanisms underlying vision are largely unknown. To further elucidate the *in vivo* function(s) of MPP4 and MPP5, we aim at the precise localization of their cellular site of action and the identification and characterization of the components of multiprotein complexes recruited by these molecules in the retina.

Methods: Specific antibodies against MPP4 and MPP5 were generated and used for immunolabelling of the different cell types of the retina. Association to candidate binding partners was studied by colocalization and direct interaction tests. Protein complexes from native retinal tissue were isolated by GST-pulldown assays and immunoaffinity chromatography. Bound proteins were separated by 2D-gel-electrophoresis and visualized by silver staining.

Results: MPP4 and MPP5 localize to distinct subcellular domains of the retina; while MPP4 is an abundant component of the photoreceptor synapses and associates with Veli3, MPP5 is exclusively present at the outer limiting membrane junctions where it colocalizes with Veli3 and CRB1. A proteomic approach was established to purify the scaffolding proteins and interacting molecules from bovine retina providing the basis to decipher large protein networks.

Conclusions: The characteristic subcellular expression of MPP4 and MPP5 and their binding partners suggest specific functions of these molecules in defined domains of the retina and imply a crucial role in cell adhesion, polarity and communication. Dysfunction in the interplay of molecules within and between the MAGUK-associated protein complexes may be an important mechanism underlying human retinal disease.

The Proteome of the Photoreceptor Synapse

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Purpose: Bassoon, a cytomatrix protein at the active zone (CAZ), plays a prominent functional role at chemical synapses in the CNS. In a mutant mouse retina lacking a functional Bassoon protein, the photoreceptor ribbons, unique presynaptic structures in form of vesicle-covered plates, are not anchored to the presynaptic membrane and photoreceptor synaptic transmission is greatly impaired (Dick et al., 2003, *Neuron* 37: 775-786). We exploited this synaptic phenotype to dissect the molecular structure of the photoreceptor ribbon complex.

Methods: With immunocytochemistry and biochemical and molecular methods, we looked for candidate molecular partners of Bassoon at the photoreceptor ribbon synapse, comparing wild-type and mutant retinae.

Results: RIBEYE is a protein postulated to build the ribbon. In the wild-type retina, RIBEYE and Bassoon are present in the same subcellular complex at the presynaptic site as shown by immunocytochemistry and co-immunprecipitation. Yeast-two-hybrid experiments demonstrate a direct interaction between the RIBEYE B-domain and the region between the coiled coil domains 1 and 2 of Bassoon. In the mutant mouse this middle part of Bassoon is absent, and, indeed, with immunocytochemistry we find RIBEYE in the cytoplasm of the photoreceptor terminals, away from the presynaptic membrane. Most importantly, we found that CAZ proteins segregate into two molecular compartments at the photoreceptor synapse: Proteins that are associated with the ribbon, and proteins that are associated with the arciform density / presynaptic membrane.

Conclusions: Our results demonstrate two molecular compartments at the photoreceptor synapse, and a direct interaction between Bassoon and RIBEYE. This interaction links the two compartments and is responsible for the physical integrity of the photoreceptor ribbon synaptic complex. As this link is lost in the Bassoon mutant mouse, it explains the morphological phenotype of free floating ribbons, and the functional phenotype of impaired photoreceptor synaptic signaling, in the mutant photoreceptors.

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Molecular Analysis of Usher 1 an Usher 2 Proteins in Retinal Photoreceptor Cells

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Cell and Matrix Biology, Institute of Zoology, Johannes Gutenberg-University of Mainz, Germany.

Purpose: Human Usher syndrome (USH) is the most common form of deaf-blindness. USH is genetically heterogeneous with at least eleven chromosomal loci assigned to the three clinical USH types (USH1A-G, USH2A-C, USH3A). Among the USH-forms, USH1 is the most severe form which is characterized by profound congenital deafness, constant vestibular dysfunction and prepubertal-onset retinitis pigmentosa. The products (proteins) of the identified USH-genes belong to different protein families. The aim of our project is to elucidate the molecular and cellular function of these diverse proteins related to USH and the identification of potential molecular linkages between these heterogeneous molecules in the vertebrate retina. The knowledge of the cellular function of the USH-proteins is a necessary pre-request in the future evaluation of founded therapy strategies of USH.

Methods: In vitro and in vivo binding assays (e.g. GST-pull downs, co-transfection, yeast-two-hybrid system) were applied. Subcellular fractions of photoreceptor cells were generated. Isoform-specific antibodies to USH-proteins were raised and used in Western blot analysis and for immunocytochemistry.

Results: Our studies reveal the scaffold-protein harmonin (USH1C) as a key integrator within a network of USH-proteins. Protein-protein interaction assays reveal harmonin specifically interacts with all known USH1- and USH2-protein predominantly via the three PDZ-(postsynaptic density 95, discs large, zonula occludens-1)-domains. Our investigations further demonstrate that harmonin isoforms are molecularly linked, either directly to F-actin or indirectly through actin binding proteins to the actin cytoskeleton. All identified network proteins are expressed in the neuronal retina as well as in the inner ear. Immunocytochemistry and biochemical analyses reveal that the identified components of the supramolecular USH-network co-localize at the ribbon synapse of retinal photoreceptor cells.

Conclusions: At synapses, the USH-network may contribute to the cortical cytoskeletal matrices of the pre- and postsynaptic regions which are thought to play a fundamental role in the structural and functional organization of the synaptic junction. Defects in any of the USH-network partners may result in the dysfunction of the entire network and in turn affect synaptic function causing retinitis pigmentosa, the clinical phenotype in the retina of USH-patients.

Supports: FAUN-Stiftung; Forschung contra Blindheit - Initative Usher Syndrom; DFG (Wo548/4), GRK 1044; ProRetin

Drosophila Crumbs – from Epithelial Cell Polarity to Retinal Degeneration

Elisabeth Knust, Sandra Berger, Mélisande Richard, Ferdi Grawe, Natalia Bulgakova, André Bachmann, Özlem Kempkens

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Purpose: The aim of our research is to understand the genetic, cellular and molecular basis that prevents light-dependent retinal degeneration. We are using the *Drosophila* eye as model and study the function of the Crumbs-protein complex, which plays also a role in the control of epithelial polarity in the embryo.

Methods: We are using mutations in the genes encoding members of the Crumbs-complex and analyse their phenotype in homozygous mutant embryos and in flies carrying mutant eyes by immunohistochemistry and electron microscopy. In addition, we are studying the composition and function of the Crumbs-complex by biochemical and molecular methods.

Results: The *Drosophila* MAGUK (membrane-associated guanylate kinase) protein Stardust (Sdt) organises a protein scaffold in the apical cytocortex of embryonic epithelial cells by recruiting the transmembrane protein Crumbs (Crb) and the PDZ-domain proteins DPATJ and DLin-7. This complex, in turn, stabilises the zonula adherens, a belt like structure encircling the apex of cells, which mediates adhesive interactions and thus guarantees the integritiy of the epithelial tissue. *crumbs* and *stardust* are required later in the photoreceptor cells. These cells, which develop from epithelial cells, express a similar protein complex, composed of Crumbs, Stardust, DPATJ and DLin-7 at the stalk membrane, which topologically corresponds to the subapical region of epithelia cells. Loss of *crumbs* results in defective morphogenesis and in light dependent retinal degeneration. Degeneration can be prevented by reduction in rhodopsin levels. *stardust* encodes several isofoms, some of which are exclusively required in the embryo. In the eye, some alleles only affect morphogenesis, while others lead to light-induced retinal degeneration. It has been shown that in human, mutations in CRB1 are associated with Retinitis pigmentosa 12 (RP12) and Lebers's congenital amaurosis (LCA), the two most severe forms of retinal dystrophy. Similarly, mice lacking Crb1 exhibit light-dependent degeneration of photoreceptors. CRB1 co-localises with Pals1/Mpp5, the mammalian homologue of Stardust, at the outer limiting membrane of mouse photoreceptor cells.

Conclusion: The striking similarity of the structure and function of crumbs with the mammalian homologue makes *Drosophila* an ideal model to study the genetic, cellular and molecular basis of RP12- and LCA-related retinal degeneration.

Abnormal Intraretinal Vascular Development and Regression of Hyaloid Blood Vessels in a Knockout Mouse Model for Norrie Disease/Exudative Vitreoretinopathy

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Purpose: To characterize early postnatal development of hyaloid and retinal vasculature and to identify morphologic and underlying molecular changes in a mouse model for Norrie disease/ exudative vitreoretinopathy.

Methods: We used Scanning laser ophthalmoscopy-angiography and electroretinography (ERG) *in vivo* as well as histological stains, quantitative RT-PCR (Taqman), ELISA- and Western Blot-analyses.

Results: We observed a delayed development of the superficial retinal vasculature and a block of the vessel-outgrowth into the deep retinal layers due to a defect in angiogenic sprouting before postnatal day p10 leading to the developmental lack of the deep retinal vasculature. Subsequently, increasing levels of hypoxia inducible factor-1 alpha (HIF1a) and *Vegfa*/VEGFA-mRNA and proteins as well as a characteristic ERG-pattern indicated hypoxic conditions in the inner retina of the *Ndph* knockout mice. The development of microaneurysm-like lesions, which contributed significantly to the leakiness of the retinal vasculature, a late reaction of glial cells as well as drastically delayed regression of the hyaloid vasculature of *Ndph* knockout mice might be consequences of hypoxia. In addition, transcriptional alterations of genes encoding angiogenic signaling molecules, including the Norrin-receptor FZD4, were found.

Conclusion: These data suggest a defect in sprouting angiogenesis as the underlying cause of Norrie disease and indicated an early and a late phase of Norrie disease defined by the development of retinal hypoxia. They might also explain similar symptoms in allelic retinal diseases like familial exudative vitreoretinopathy, retinopathy of prematurity and coats disease as secondary consequences of pathological hypoxia.

In Vivo Confocal Imaging of the Retina in Animal Models Using Scanning Laser Ophthalmoscopy

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Scanning-laser ophthalmoscopy (SLO) is a technique for confocal imaging of the eye. The use of lasers of different wavelengths allows to obtain information about specific tissues and layers due to their reflection and transmission characteristics. In addition, fluorescent dyes excitable in the blue and infrared range offer a unique access to the vascular structures associated with each layer.

The adaptation of SLO imaging for use in animal models allows to obtain valuable *in vivo* information about a number of ocular tissues like lens, iris, vitreous, retina, retinal pigment epithelium (RPE), and choroid, and the respective vascular elements. A further enhancement in specificity can be obtained by GFP expression under control of tissue-specific promotors in transgenic animals.

In a number of models of inherited retinal degenerations and other disorders with retinal involvement, the changes associated with the disease were studied. The correlation of functional (ERG) and morphological data allowed to investigate the pathophysiology in detail and led often also to a better understanding of retinal function in general.

Another important field of application is the follow-up of therapeutic intervention. We have focussed so far on the investigation of acute and long-term success of gene therapy. The non-invasive detection of expression markers like GFP and of the functional and structural improvements helped to assess the therapeutic efficacy.

Keywords:
scanning-laser ophthalmoscopy, animal models, retinal degenerations, angiography

Genetics of Retinal Drusen Formation in Rhesus Macaques

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Objective: Macular drusen formation is a hallmark of ARMD (Age Related Macular Degeneration), but the mechanism underlying their formation is not well characterized. It is known that rhesus macaques (*Macaca mulatta*) present a natural animal model for retinal drusen, and in both, man and monkey, genetic factors are implicated. We tried to identify the drusen associated genes in rhesus macaques.

Methods: A search using 42 microsatellite markers, linked to 7 different autosomal loci implicated in retinal pathology in humans, was performed in a single rhesus matriline, followed by a positional candidate gene approach. Several genes implicated in the etiology of ARMD with and without drusen formation have been mapped to the 6q region in humans. Two of the candidate rhesus orthologs, *ELOVL4* (Elongation Of very Long Chain Fatty Acids 4) and *IMPG1* (Interphotoreceptor Matrix Proteoglycan-1) were searched for mutation using PCR, SSCP (Single Stranded Conformational Polymorphism) and sequencing.

Results: An association between two alleles at heptallelic marker D6S1036 and the severity of drusen formation was found (P combined: 0.012). For *ELOVL4*, no sequence changes were found in the exons, but a polymorphism was found for the 3' UTR region which was not associated with the drusen pathogenesis and could play a protective role. In *IMPG1*, 6 SNPs were identified. Haplotype frequencies of these six SNPs were found to differ significantly between affected and non-affected animals (chisquare=10.102, 3 d.f., p=0.018). This difference was mainly due to a haplotype which was found on 17% of chromosomes in animals with drusen, but not in controls.

Conclusion: Our data demonstrate that one or several genes on the rhesus homologue of human 6q are likely to play a role in retinal drusen formation.

Apoptosis in Retinal Degeneration – Neuroprotection of Photoreceptors

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Purpose: Photoreceptor loss due to retinal degeneration in most cases occurs by apoptosis. Although different pathogenic stimuli may trigger the apoptotic program via different routes, there may be a certain level at which these private pathways converge. We aim at understanding apoptosis at the molecular level and try to interfere with it beyond the level of convergence in order to obtain a potential therapy applicable for many different forms of retinal degeneration.

Methods: We use mouse models of inherited and induced retinal degeneration to study the sequence of events during photoreceptor apoptosis. Modulation of the visual cycle, interference with phototransduction, inhibition of AP-1, modulation of PrP expression and deletion of caspase-1 have all been shown to be beneficial in our model of light-induced photoreceptor apoptosis. Here we test the effect of these treatments on retinal degeneration induced by mutations in the rhodopsin gene (VPP).

Results: Retinal degeneration in the VPP mouse could be positively influenced by: Modulation of the visual cycle (RPE65), inhibition of phototransduction, and inhibition of caspase-1. Inhibition of c-Fos and application of PrP was without effect.

Conclusion: Degeneration in the VPP mouse may follow the degenerative pathway induced by low-level, long-term light exposure. Strategies for neuroprotection may be tested using this model of induced degeneration and could involve pharmacological slowing of the visual cycle (accutane) and inhibition of caspase-1 by locally applied inhibitors.

Rescue of Photoreceptor Degeneration in a Genetic Mouse Model for X-linked Juvenile Retinoschisis

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Objective: Deleterious mutations in the RS1 gene on Xp21 are associated with X-linked juvenile retinoschisis (RS), a common form of macular degeneration in males. We have generated a knock-out mouse deficient in Rs1h, the murine ortholog of RS1. The knockout mouse exhibits many characteristic features of human RS including the presence of small cyst-like structures in the inner retina, and disorganization and displacement of cells within the retinal layers. Accordingly, the Rs1h-deficient mouse serves as a valuable model system to develop possible therapeutic interventions for human RS.

Methods: To explore the latter goal, we have generated a construct containing the human RS1 cDNA under the control of the mouse opsin promoter in the replication-deficient adeno-associated virus (AAV) serotype 5. The RS1-AAV construct was injected into the subretinal space of one of the eyes of 15 day old Rs1h knockout mice. The uninjected eye served as a control.

Results: The injected and uninjected eyes were evaluated at various time points by ERG and microscopic as well as immunolabeling techniques to assess the effect of gene transfer on photoresponse, protein expression and morphology of the retina. Our data show that retinoschisin is expressed and secreted from photoreceptors of the RS1-AAV injected eyes. Like normal mice, the expressed protein localizes to rod and cone photoreceptors of the outer retina and bipolar cells of the inner retina at essentially normal levels. Importantly, the injected eyes showed a significant improvement in the ERGs, retinal tissue morphology and preservation of photoreceptors.

Conclusion: Our study demonstrates that gene therapy can restore visual function in a Rs1h-deficient mouse to nearly normal levels. This visual improvement is accompanied by a corresponding recovery of retinal morphology at both the inner and outer retinal layers. Implications for clinical intervention in human RS are apparent.

Stem Cell-based Therapeutic Approaches

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Purpose: Stem cells are multipotent cells with the capacity to self-renew, and are thus considered as candidate cells to develop cell-based therapies for various disorders. We will review experiments with embryonic stem cells and various tissue-specific stem cells aimed to establish cell replacement strategies or ex vivo gene therapies for retinal disorders. We will also summarize transplantation experiments with neural stem cells (NSCs) that were performed to study neuron-glia interactions and molecular aspects of glial cell differentiation in vivo.

Methods: Cell culture experiments were carried out to study the ability of embryonic, mesenchymal, neural and retinal stem cells to differentiate into retinal cell types. Transplantation experiments were performed to investigate the integration and differentiation of these cells in pathologically altered retinas. A non-viral transfection technique was developed to genetically modify NSCs and mesenchymal stem cells. NSCs from wild-type and knock-out mice were grafted into developing retinas to study neuron-glia interactions and to elucidate the role of distinct proteins for terminal differentiation of glial cells, respectively.

Results: Differentiation of the various stem cell types into retinal cell types was observed in vitro and after transplantation in vivo. The extent of integration into the host tissue was highly variable and dependent on the grafted stem cell type, and the age and the phenotype of recipient retinas. Intraretinally grafted NSCs integrated into dystrophic retinas of adult ß2/ß1 knock-in mice and differentiated into glial cells. Extensive integration and differentiation into glial cells was also observed for NSCs transfected with EGFP or the cell recognition molecule L1. Intraretinally grafted wild-type and ErbB3-deficient NSCs differentiated into oligodendrocytes that myelinated the retinal nerve fibre layer, whereas Sox10-deficient cells failed to terminally differentiate into oligodendrocytes.

Conclusions: The current interest in cell replacement strategies for retinal disorders is based on the availability of expandable stem cells that are capable to differentiate into cells with a retinal phenotype. Strategies are currently being developed to increase the integration of grafted cells into adult retinas and to direct differentiation of multipotent cells into distinct cell types. Genetically engineered stem cells might be used to target therapeutic gene products to diseased retinas. Intraretinal transplantations of NSCs provide a tool to study neuron-glia interactions and differentiation of glial cells in vivo.

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Reaggregates and Stem Cells for Retinal Tissue Engineering

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Purpose: A major advantage of the reaggregation approach is to reveal prospective potencies of cells and of whole tissues (3-dimensionality). From reaggregates, we can learn about the genetic and environmental constraints of formation, stability and regenerative capacities of cells and tissues. Moreover, reaggregates using stem cells could become central at making progress into tissue engineering and regenerative medicine.

Methods: Cells from dissociated embryonic avian retinae are reaggregated in rotation culture to form cellular spheres reconstituting a complete arrangement of all retinal layers. This phenomenon is based upon in vitro proliferation of multipotent precursor stem cells and spatial organisation of their differentiating descendants. Moreover, the addition of soluble factors from cultured retinal pigmented epithelial (RPE) or radial glial cells is essential to revert inside-out spheres into correctly laminated spheres, a process which seemingly involves the Wnt-cascade (Nakagawa et al., 2003). Such complete restoration of a brain tissue by cell reaggregation has been achieved only for the embryonic avian retina, but not to the same extent for the mammalian retina, nor for other brain parts.

Results: Therefore, as a model for future tissue engineering of a human retina, we attempted to reconstruct a retina from the postnatal gerbil (Meriones unguiculatus). If gerbil retinal cells alone are reaggregated, regions homologous to the inner half of the retina are discernible. Similar to avians, a major improvement is achieved through addition of mouse RPE-supernatants, leading to highly organised retinal spheres, consisting of a GCL, an IPL and an INL, but without a photoreceptor layer. The in vitro-formation of an IPL can be studied particularly well in this model.

Conclusion: These studies represent a biomedically relevant step towards tissue engineering of a mammalian retina, as soon as stem cell biology will open ways to produce large amounts of human retinal precursors.

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POSTER ABSTRACTS

Identification of Mutations in the 51 Novel Exons of Usherin (USH2A)

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PURPOSE: The aim of this work is the screening for mutations in the 51 novel exons of USH2A gene in Spanish patients with Usher syndrome type II, atypical Usher and non-syndromic retinal degeneration, in which only one pathogenic mutation was detected after screening exons 1-21, in order to detect the second mutation responsible for the disease.

METHODS: DNA from 31 unrelated patients were extracted from peripheral blood samples as described elsewhere. From these, one was diagnosed as maculopathy, three presented with ARRP, other three suffered from atypical Usher and the last 24 had USH2A.
The 51 novel USH2A exons and their intron/exon boundaries were amplified by PCR. PCR products were analysed by SSCP. The fragments displaying an abnormal electrophoretic pattern were subsequently sequenced.

RESULTS: The mutation screening in our patient sample revealed 5 frameshift mutations (two of them were deletions and the other three were insertions) and one in-frame insertion of three nucleotides. All these changes were private. We also found 10 different amino acid changes. Five of these were private, other 4 were found in two families and the last one was present in four families.

CONCLUSIONS: A high number of changes have been found in the 51 novel exons of USH2A gene in this study. However, the majority of them are amino acid changes which need further studies to elucidate its pathogenic implications.
Our results confirm that mutations in the 51 novel exons of Usherin can cause USH and ARRP in combination with a mutation in exons 1-21. Further studies are needed to know whether two mutations in these novel 51 exons are disease-causing or not.

Degeneration of Different Classes of Photoreceptors in Drosophila rdgC Mutant Studied by ERG

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PURPOSE: *Drosophila* RdgC is an eye specific phosphatase required for dephosphorylation of rhodopsin. Genetical elimination of RdgC leads to an apoptotic light dependent degeneration of photoreceptors, caused by massive internalization of complexes of phosphorylated rhodopsin and arrestin. In the dominant photoreceptor class (peripheral, R1-6), the degeneration is completed within a few days, while in the central photoreceptors (R7 and R8) it is slower. However, the residual ERG in *rdgC* originating from the central receptors is not equal to the ERG in *ninaE*, a fly strain where only R7 and R8 cells are functional. A subset of R8 cells contains a long wavelength sensitive Rh6 rhodopsin with a thermolabile and hypsochromic metarhodopsin conformation and undetectable level of phosphorylation. We hypothesized that this photoreceptor class could exhibit a different degeneration time course due to a putatively different rhodopsin inactivation and phosphorylation pathway.

METHOD: Utilizing mass electrical recording from flies' eyes, electroretinography, we examined the ERG, PDA (prolonged depolarizing afterpotentials, caused by excess metarhodopsin over arrestin) and the spectral sensitivity of *rdgC* and *ninaE* flies after different periods of exposure to 12h/12h day/night cycle and different carotenoid content in the food.

RESULTS: We found that after 10 days, PDA in *rdgC* cannot be elicited neither with UV nor blue light. The complex spectral sensitivity of *rdgC* with degenerated R1-6 cells is the same as in *ninaE*, a sum of absorbances of four classes of rhodopsin (Rh3-UV, Rh4-UV, Rh5-blue, Rh6-green). After a few days, the peak in the blue is decreased, and the peak in the UV is shifted towards shorter wavelenghts. Fitting the nomograms to the complex sensitivity spectra didn't yield satisfying results due to lack of information on the b-bands of rhodopsin absorbance spectra. Carotenoid deprivation prevented the degeneration in *rdgC*, thus resulting in a spectral sensitivity equal as in the wild type, but it did not alter the spectral sensitivity in

Autosomal Dominant Retinitis Pigmentosa: Linkage Analysis in a Large German Pedigree Excludes Known Loci

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Retinitis pigmentosa (RP) is the most common form of inherited retinal dystrophies, with a prevalence of about 1/5.000. It is characterized by early night blindness, loss of the peripheral visual field, and can lead to blindness in late stages. RP is phenotypically and genetically heterogeneous. In 20% of cases, family history indicates autosomal dominant inheritance (ADRP). Twelve ADRP loci have been mapped to date, with genes identified in all cases. However, mutations in these genes only account for half of all ADRP cases, suggesting the existence of additional disease genes. We studied a large German pedigree with ADRP. Both onset of symptoms and severity of the disease show intrafamilial variation: Some of the nine affected family members noticed visual impairment in the early 3rd decade, others not before early 4th decade. Progression of visual impairment ranges from rapid to moderate. Linkage studies using microsatellite markers for all known ADRP loci excluded *NRL* (*RP27*), *CRX, RP1, PIM1K (RP9), IMPDH1 (RP10), CA4 (RP17)*, and *FSCN2* as causative genes in this family. Where markers were not informative (loci for *PRPF31, RDS,* and *RHO,* respectively), the entire coding regions of the corresponding genes were sequenced. In case of *PRPF8* and *HPRP3*, mutations have only been described in restricted parts. We found no mutations in either gene by sequencing these regions. As there is no male-to-male transmission in this family, and since mutations in *RPGR* (underlying X-linked RP3) have been described in families with apparent ADRP, markers flanking the RP3 locus where also typed and showed no linkage. Genome-wide linkage analysis will be performed in order to identify the ADRP locus in this family.

Functional and Morphological Characterization of a Mouse Model for X-linked Retinitis Pigmentosa (RP)

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Purpose: To understand the molecular mechanisms that lead to photoreceptor degeneration in a mouse model for X-linked recessive RP with a mutation (deletion of exon 4, DEx4) in the gene encoding the retinitis pigmentosa GTPase regulator (*Rpgr*).

Methods: Breedings were performed for eight generations to the C57BL/6 background and subsequently to Balb/c for three generations. Functional and histological examinations of mutant male mice and wild type control littermates in both genetic backgrounds were accomplished by electroretinography (ERG) and light microscopy of retinal sections. ERGs were recorded under standard conditions for scotopic measurements at different flash light intensities (10 mcds/m^2 to 3000 mcds/m^2). Paraffin sections of mouse eyecups were analysed by measuring length of the outer nuclear layer (ONL) and inner and outer segments (IS+OS) in different regions of the retina. Additionally, the number of photoreceptor rows in the ONL was determined.

Results: Rpgr mutant mice show a progressive retinal degeneration upon ERG recordings and histological examinations. In the BL/6 background, hemizygous mutant mice revealed a significant decrease of the rod a-wave amplitude (19% at the age of 6 to 7 months up to 34 % at 13 to 14 months). In the Balb/c backcross line, the a-wave amplitude was reduced in-line with the increase of Balb/c background. While F1 and F2 offspring of the BL/6$^{\Delta Ex4}$ X Balb/c crosses exhibit a similar but more pronounced decrease in retinal function compared to BL/6$^{\Delta Ex4}$, F3 mutant mice displayed a significantly reduced a-wave as early as 3 month of age. Histological examinations of eye sections from F1 mice revealed a significant reduction of the ONL in thickness and the number of photoreceptor nuclei, predominantly in the peripheral retina.

Conclusions: Hemizygous male mice with an exon 4 deletion in the Rpgr gene provide a potential model to study the mechanisms of photoreceptor degeneration in X-linked recessive RP. Similar to the human disease course rod photoreceptors start to degenerate in the retinal periphery in our Rpgr mutant mice. Furthermore, our results show that the genetic background has an impact on the progression of photoreceptor degeneration.

Approach to Molecular Diagnosis of Retinitis Pigmentosa in Uncharacterised Families: a Proposed Model

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Introduction: Retinitis Pigmentosa (RP) is a group of inherited and progressive retinal dystrophies that lead to blindness. There are more than 150 loci responsible for these dystrophies. Their genetic characterisation becomes even more challenging, because of their additional allelic and clinical heterogeneity. Genetic diagnosis was facilitated with the introduction of three genotyping microarrays, but these only account for 17 genes related to RP.

Purpose: To design an approach that not only aims to shorten the time needed for a diagnosis, but could also help in the diagnosis of certain sporadic cases, which account for 40% of all non-syndromic forms of RP in Spain.

Patients and methods:

Patients:
> 200 uncharacterised RP Spanish families (20 ADRP families: 139 individuals; 84 ARRP families: 361 individuals; and 105 SRP families: 134 individuals).

Molecular analyses:
– 55 known genes: small sets of STR markers (3 to 7), closely linked to each of the 55 most prevalent genes known to cause RP in Spanish population (17 ADRP genes, 31 ARRP genes and 7 ADRP/ARRP genes).
– Genome-wide scan: 400 additional STR markers (Linkage Mapping Set MD10, Applied Biosystems).

Software and Linkage analyses:
All data, including the vast quantities of genotyping data produced by this project, will be managed by a proprietary computer system, in order to minimize user input error and automate many tasks, such as checking the data for genotyping errors, re-calculation of allele frequencies, calculation and visualization of LOD scores, etc.

Results: Haplotype analysis will be performed on samples from dominant, recessive and sporadic cases of RP. This will help to:

– ARRP: rule out genes and select candidate genes due to identity by descent (IBD).
– ADRP: rule out genes.
– SRP: select candidate genes due to identity by descent (IBD) or uniparental isodisomy.

Direct genetic studies will follow, such as traditional mutation screening techniques, automatic DNA sequencing, or genotyping microarrays.

If the genetic cause of the disease in each family is not found, a genome-wide scan will be performed, followed by linkage analysis, in informative families.

Functional Implications of the Sorsby Fundus Dystrophy Mutation Timp3(Ser156Cys) in the Inhibition of Matrix Metalloproteinases

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Purpose: Sorsby fundus dystrophy (SFD) is a rare autosomal dominant disorder of the central retina. It is characterized by subretinal neovascularization, atrophy of the retinal pigment epithelium and the choriocapillaris. Several independent point mutations in the tissue inhibitor of metalloproteinases-3 (TIMP3) gene have been associated with SFD, all localized at the C-terminal region of the mature protein and all resulting in a single unpaired cysteine residue in the mature protein. The aim of this study is to assess the implications of Timp3 mutation Ser156Cys and Timp3-deficiency with regard to inhibition of selected matrix metalloproteinases such as Adamts4, Adamts5 and Adam17.

Methods: Chondrocyte cell lines were established from Timp3 knock-out (+/-, -/-), Ser156Cys knock-in (+/-, -/-) and wt (+/+) mice. This cell type expresses most of the known metalloproteinases and their substrates (e.g. aggrecan, collagen type II) and thus represents an excellent in vitro system to studying Timp3 function. Immunocytochemistry was used to assess cleavage intensity of aggrecan. ELISA was employed to quantify the cleavage rate of aggrecan and membrane bound TNFa.

Results: Timp3 has the capacity to specifically inhibit Adamts4 and Adamts5, two enzymes that are involved in degradation of the extracellular proteoglycan aggrecan. Adam17 is the protease responsible for the shedding of membrane bound TNFa to its soluble form and it is efficiently regulated by Timp3. Elevated levels of aggrecan protein and a higher cleavage rate of aggrecan was found in the Timp3-deficient chondrocytes compared to wild type (wt). Shedding of cell surface TNFa is not inhibited in $Timp3^{-/-}$ chondrocytes leading to a significantly reduced level of membrane-bound TNFa on the surface of Timp3 (-/-) chondrocytes. Conclusion: Timp3-deficiency results in an imbalance in the aggrecan catabolism. In addition, the shedding of cell surface TNFa is impaired in the absence of Timp3. Taken together, these results suggest that Timp3 is actively involved in the inhibition of several matrix proteinases in particular Adamts4, Adamts5 and Adam17. The consequence of the Timp3 mutation Ser156Cys for these processes is under investigation.

Assessing the "Common-Disease, Common Variant" Hypothesis in Age-related Macular Degeneration (AMD)

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Purpose: Age-related macular-degeneration (AMD), a late onset disorder of complex etiology, is the leading cause of blindness in industrialized countries. To date, only two genes including apolipoprotein E (APOE) and complement factor H (CFH) have repeatedly been associated with the disease. Here, we test the hypothesis whether linkage disequilibrium (LD)-based single nucleotide polymorphism (SNP) analysis in candidate genes may be a useful method to identify additional, possibly minor, genetic factors contributing to the disease.

Methods: In the Franconian AMD Study we have collected 800 unrelated AMD patients and 600 age-, gender- and ethnically-matched controls. Past and current smokers were excluded from the case group to eliminate the known impact of this risk factor. Common SNPs with minor allele frequencies >0.2 were selected from two gene loci including APOE and the G protein-coupled receptor-75 (GPR75). The latter gene was previously extensively analyzed in AMD but no disease-associated mutations were identified thus making this gene an attractive "negative" control for our study. SNP typing was done using the MALDI-TOF Sequenom Technology. Haplotypes, single locus and multi-marker haplotype association tests were calculated using the program HAPLOVIEW.

Results: Four common SNPs were identified at the APOE locus and two common SNPs at the GPR75 locus. The frequencies of the minor alleles ranged between 0.27 and 0.45. No association with AMD was found based on a single SNP analysis. Upon defining regions of solid LD (D'>0.8), two blocks were detected, one spanning three SNPs of APOE, the other containing both SNPs of GPR75. Two APOE haplotypes, namely 111 and 211, were significantly associated with AMD reaching p-values of 0.0038 and 0.0103, respectively. Further analysis showed that haplotype 111 carries the epsilon2 allele while haplotype 211 carries the epsilon4 allele of APOE.

Conclusions: Our data reinforce the validity of the "common-disease, common variant" hypothesis and show that this is an appropriate approach to identify genetic risk factors even those which account for only a minor proportion of AMD.

Mutational Screening of the RP2 Gene in Spanish XLRP Families

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INTRODUCTION: Retinitis pigmentosa (RP) is a degenerative disease of the retina, characterized by night blindness and visual field constriction. X-linked form of RP (XLRP; MIM # 268000) is one of the most severe type of RP due to its early onset and its rapid progression. Five XLRP loci have been mapped: RP_{23}, RP_6, RP_3, RP_2, and RP_{24}. So far, only two XLRP genes have been identified RPGR (for P3 locus) and RP_2, which account for 60-90% and 10-20% of XLRP respectively.

PATIENTS AND METHODS: In our study, we have analyzed the RP_2 gene in 24 unrelated XLRP Spanish families which were excluded from having RPGR mutations in a previous screening. PCR-SSCP analysis of all 5 exons of the RP_2 gene was performed using pairs of primers flanking each exon. Any fragment displaying an abnormal pattern in SSCP was automatically sequenced and analysed by the Sequence Analyser program in the ABI Prism 310 Genetic Analyser (Applied Biosystems) in order to identify the mutation. To detect the possibility of an alternative splicing we analysed the RP_2 at mRNA level by 2 specific RT-PCRs.

RESULTS AND CONCLUSIONS: We have identified 2 novel nonsense mutations (Glu_{18}Stop and Glu_{134}Stop) and 1 novel missense mutation (Ser_{140}Phe). In addition, in other previously reported Spanish family the disease was found to be associated with a frameshift mutation (303 InsT). Three of the mutations identified truncate the protein and the remaining mutation is localized in the cofactor-C homologous domain of the protein and presumably affects its function. Our results show that mutations in RP_2 are responsible of 11,8% of XLRP in the Spanish population.

MPP4 – a Scaffolding Protein in the Connecting Cilia of Photoreceptors

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Purpose: MPP4 (membrane protein, palmitoylated 4) is a retina-specific, putative scaffolding protein belonging to the family of membrane-associated guanylate kinases. The MPP4 protein is mainly localized at the synapses of photoreceptors but has also been found at the connecting cilium of rods and cones. Connecting cilia are modified non-motile primary cilia linking the outer and inner segments of photoreceptor cells. The current study aims at the further elucidation of MPP4 presence in the unique ciliary structure of photoreceptors.

Methods: Antibodies raised against various epitopes of the MPP4 protein together with antibodies against molecular markers for connecting cilia were used for immunofluorescence labelling of fixed and unfixed frozen retinal sections, of isolated individual photoreceptor inner and outer segments and of intact photoreceptor microtubule-based axonemes. Cytoskeletal fractions from isolated bovine rod inner and outer segments (RIS-ROS) were prepared by sucrose-density-centrifugation.

Results: Comprehensive immunolabelling studies on intact and dissociated retinal cells from cow and mouse show that MPP4 is localized to the connecting cilia. In addition, Western blot analyses of detergent-extracted photoreceptor cytoskeleton indicate an association of MPP4 with the ciliary axonemes.

Conclusions: Our data obtained from a combination of different approaches provides further evidence that MPP4 is a component of the ciliary structure of photoreceptor cells. A growing number of ciliary proteins have been implicated in retinal disease (e. g. RPGR, RPGRIP, RP1). The elucidation of MPP4 function is expected to contribute to the understanding of the mechanisms underlying cilia-associated and other retinopathies.

MPP1 and MPP4 are Novel Members of the Intracellular CRB1 Protein Scaffold in the Retina

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Purpose: The human Crumbs homologue 1 (CRB1) protein is a 154 kDa transmembrane protein that is preferentially present in photoreceptors. CRB1 is thought to organize an intracellular protein scaffold in the human retina that is involved in photoreceptor polarity. Mutations in the *CRB1* gene have been found in patients with different severe inherited degenerations of the retina. Our project aims at the identification of members of the CRB protein complex in the human retina in order to i) obtain functional clues for the retinal pathway(s) in which CRB1 is involved, and ii) obtain novel candidate genes for inherited retina disorders.

Methods: The membrane-associated guanylate kinase protein (MAGUK) protein MPP5 (also known as PALS1) binds to the 37-amino acid intracellular domain of CRB1. We have used yeast two-hybrid (Y2H) screens of retina cDNA libraries to search for proteins that interact with MPP5. Domains for which no interactors are known were used as a bait. Novel interactions were confirmed using GST pull-downs, co-IPs, and liquid β-galactosidase assays. Their localization in the retina is determined by immunohistochemistry.

Results: Using MPP5$^{SH3+HOOK}$ as a bait in our Y2H screens, we have identified two family members, MPP4 and MPP1, as part of the CRB1 protein scaffold. Both MPP1 and MPP4 are expressed in the retina and MPP4 co-localizes with MPP5 and CRB1 at the outer limiting membrane. Using 3D homology modeling, we predict that MPP5 and MPP1/MPP4 form homo-, as well as heterodimers by a domain swapping mechanism that involves the GUK domain. This corroborated the Y2H interaction results. Our current hypothesis suggests the involvement of an adaptor protein that triggers the unfolding of the MAGUK module in order to enable heterodimerization of MPP family members.

Conclusion: MPP4 as well as MPP1 are part of the CRB1 protein scaffold in the retina via binding to MPP5. These data implicate a function for MPP proteins in photoreceptor polarity and, by association with CRB1, pinpoint the cognate genes as functional candidate gene

GDNF-induced Signalling in Retinal Mueller Glia

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Purpose: Apoptotic cell death of photoreceptors is the final event leading to blindness in the heterogeneous group of inherited retinal degenerations. GDNF was found to very effectively rescue function and survival of photoreceptors in an animal model of retinal degeneration (Frasson et al., 1999), but the cellular mechanism of GDNF-action has been unresolved.

Methods: GDNF-receptor localization was investigated by immunohistochemistry on porcine retinal sections and by RT-PCR on isolated retinal cells. Isolated primary RMG were stimulated with GDNF, neurturin (NRTN) and artemin (ARTN) and phosphorylation of RET transmembrane receptor, as well as downstream kinases was monitored with phospho-specific antibodies. Expression of GDNF-target gene was monitored by northern blot and ELISA.

Results: We found, that GDNF receptors GFRa-1 and RET are expressed on retinal Mueller glia (RMG) and not on photoreceptors in porcine retina. Additionally, RMG express the receptors for Artemin and Neurturin (GFRa-2 and GFRa-3). We further investigated GDNF, ARTN and NRTN-induced signalling in isolated primary RMG and demonstrate, that three intracellular cascades are activated *in vitro*: MEK/ERK, SAPK and PKB/AKT pathways. We correlate the findings to intact porcine retina, where GDNF induces phosphorylation of ERK in nuclei located in inner nuclear layer. GDNF was further found to induce transcription of bFGF, and bFGF was found to support photoreceptor-survival in an *in vitro* assay.

Conclusion: We provide here a detailed model for GDNF-induced signalling in RMG and propose that the rescue effect on mutated photoreceptors induced by GDNF is an indirect effect and is mediated by RMG.

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Dynamics of cGMP Synthesis in Photoreceptor Cells – Application and Use of Mutated and Chemically Modified GCAPs

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Purpose: Particulate guanylate cyclases (GCs) are key enzymes in phototransduction of vertebrate photoreceptors. GCs regulate, in interplay with a phosphodiesterase, the concentration of the intracellular messenger cyclic GMP (cGMP). After photoreceptor illumination the intracellular concentrations of cGMP and Ca2+ decrease. At low Ca2+ concentrations guanylate cyclase activating proteins (GCAPs) activate GCs. These steps are part of a negative feedback loop to adjust the light sensitivity of the cell. The GC/GCAP-system is also affected in several inherited retinal dystrophies, e.g. Leber's congenital amaurosis or cone-rod dystrophy. To understand the GC/GCAP system at the molecular level is a prerequisite to unravel the mechanisms of these retinal diseases.

Methods: Cysteines in GCAP1 and GCAP2 offer the unique opportunity to allow or restrict accessibilities of certain labels (dyes, fluorescent markers). Several cysteine-mutants of GCAP2 were cloned, heterologously expressed and purified. Ca2+-dependent GC-activation by the mutants was tested. The Ca2+-dependent accessibility of cysteines for the thiolreactive substance DTNB was tested as a monitor for Ca2+-dependent conformational changes. In addition, mutants were labelled by fluorescent dyes to monitor conformational changes by fluorescence spectroscopy.

Results: All mutants exhibited IC50-and EC50-values comparable to wildtype GCAP2. However, mutants with no cysteine in the first EF-hand motif exhibited a weaker GC-activation, indicating an important function of this cysteine in GC-activation. The cysteine in the first EF-hand motif was accessible at low Ca2+-concentrations but not at high Ca2+-concentrations. This result showed that a Ca2+-dependent conformational change in the first EF-hand motif must have occurred, which was unexpected since the first EF-hand is considered as a non-functional EF-hand. Although the fluorescence studies revealed Ca2+-induced changes in fluorescence we could relate these effects to the interaction of GCAP2 with membranes.

Conclusions: Cysteine mutants of GCAP2 are useful tools to investigate Ca2+-sensitive processes in guanylate cyclase regulation. They allow monitoring the Ca2+-dependent conformational changes in GCAP2, which are critical for the control of cGMP synthesis.

SPECTRUM OF MUTATIONS IN GENES CAUSING AUTOSOMAL DOMINANT RETINITIS PIGMENTOSA IN A SPANISH POPULATION: GENOTYPE/PHENOTYPES ASSOCIATION

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PURPOSE: To identify mutations in genes causing autosomal dominant retinitis pigmentosa (adRP) in a Spanish population.

METHODS: 170 index patients of adRP families together with 100 simplex cases of RP, and 80 controls were screened for mutations in the coding sequence and intronic splice sites of the RHO, RDS, CRX, NRL, RP1 (exon 4), FSCN2, PRPF31, PRPF8 (exon 42) and PRPF3 (exon 11) and IMPDH genes by denaturing gradient gel electrophoresis (DGGE) or single-strand conformation polymorphism (SSCP) and direct genomic sequencing. Ophthalmic and electrophysiological examination of the patients was performed according to previously established protocols. Phenotype/genotype correlation in adRP families is discussed.

RESULTS: The prevalence of disease-causing mutations in each of the analyzed genes showed that 27 of 170 in the adRP families were caused by rhodopsin mutations, 5 (2.5 %) were detected in a 1000 bp segment of exon 4 of the RP1 gene, 3 mutations (1.7 %) in the peripherin/RDS and 2 in the NRL gene. One additional mutation in NRL was found in a simplex RP case. The NRL mutations were expressed in vitro showing an over transactivation of the RHO promoter respect to the wild type NRL gene. We also detect 9 mutations (5.2 %) in pre-mRNA splicing factor genes causing adRP and two independent families with the Arg224Pro mutation in the IMPDH1. Although we have detected 16 mutations in FSCN2, an adRP associated gene, none of these mutations co-segregates with the disease. Several mutations reported are novel or only found in the Spanish population

FUNCTION OF PDE6 GAMMA IN RETINAL DEVELOPMENT AND DEGENERATION

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The photoactivable phosphodiesterase 6 (PDE6) is the primary effector of phototransduction in vertebrate photoreceptors. This enzyme is composed of two catalytic subunits Pα (88 kDa) and Pβ (84 kDa) and two inhibitory subunits Pγ (11 kDa). Previous studies have shown that Pγ also has the ability to interact with SH3 containing proteins of the endocytosis-MAP kinase signaling network (Wan et al, 2001 ; Morin et al, 2003).

Objectives:
To characterize Pγ expression in the developing rat retina
To identify SH3-containing proteins partners of Pγ in photoreceptors

Results:
In rat retina, Pγ mRNA was detected as early as P0 whereas catalytic subunits mRNA were not detectable before P4. Pγ mRNA is expressed in photoreceptors progenitors as shown by in situ hybridization.
Pγ protein was detected by immunohistochemistry at P0. At this stage, the immunoreactivity is surprisingly located is the Inner Plexiform Layer (IPL). At P5, Pγ immunoreactivity is present in the IPL, the OPL and the outer segment.
Pγ interacts and colocalizes with pacsin in photoreceptors inner segments and synapses. Pacsin is a synaptic protein involved in the endocytosis machinery and a substrate of the protein kinase C.

Together, these data point to a novel role of Pγ in photoreceptor synapses. A developmental study is underway to examine photoreceptors in Pγ knock-out mice where a precocious retinal degeneration occurs.

Eleven novel Myosin VIIA mutations in Spanish patients with Usher syndrome type I

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PURPOSE: The screening of *MYO7A* gene in Spanish patients with Usher syndrome type I in order to detect the mutations responsible for the disease in each patient.

MATERIALS AND METHODS: Seventy-five patients belonging to 48 families were studied. Genomic DNA was extracted from peripheral blood samples as described elsewhere. Individual exons and exon/intron boundaries were amplified and SSCP analysis was performed. In those cases where an abnormal electrophoretic pattern was observed, DNA fragments were directly sequenced.

RESULTS: In this study eleven novel mutations were found. Five of them were amino acid changes and five cause a premature stop codon: two nonsense and three frameshift mutations (two deletions and one insertion). A silent change was found and considered as mutation. All changes found were private with the exception of the silent mutation which has been found in two families.

CONCLUSIONS: Usher syndrome type Ib is caused by a high diversity of mutations in *MYO7A* gene. No prevalent mutation has been found, being most of them private.

Mutations in RDH12, encoding a photoreceptor cell retinol dehydrogenase, in autosomal recessive retinal dystrophy

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Purpose: To describe the mutation spectrum of the retinol dehydrogenase 12 (*RDH12)* gene in autosomal recessive and sporadic retinal dystrophies.

Methods: Samples from 1080 patients with retinal dystrophies were screened for mutations in the coding region of *RDH12* by dHPLC and sequencing. Missense mutations were transiently expressed in COS-7 cells and the conversion of all-*trans* retinal to all-trans retinol was determined by HPLC.

Results: In addition to our 5 previously identified most likely pathogenic changes (T49M, R62X, Q189X, 806delCCCTG, Y226C; Janecke et al. 2004), we identified 20 additional *RDH12* mutations in patients with arRD: M1?, A47T, N34fsX62, T55M, R65X, L99I, N125K, G127X, [c.429C>G;c.430C>G;c.432delG], G145E, H151D, T155I, Y195X, A206D, A206V, R234H, R239W, L274P, C285Y, and R295X. Haplotype analysis identified three founder mutations. Each of the missense variants tested exhibited decreased or aberrant activity relative to wild-type when assayed for their ability to catalyze the interconversion all-*trans* retinol and all-*trans* retinal. In all patients with *RDH12* mutations, the disease affected both rods and cones with onset of symptoms in early childhood (2-4 y) and progression to legal blindness in early adulthood (18-25 y).

Conclusions: Our studies suggest that *RDH12* mutations account for about 5% of cases of severe, childhood-onset arRD, and for 1.5% of arRD cases in total. Our studies identify a cohort of patients whose disease may be amenable to therapies effective for visual cycle defects currently under development by the vision research community.

Abnormalities in vessel formation in a mouse model of Timp3 deficiency

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Purpose: Sorsby fundus dystrophy (SFD) is an autosomal dominant disease of the macula caused by mutations in the tissue inhibitor of metalloproteinases-3 (TIMP3). Choroidal neovascularization is a hallmark of this condition which closely resembles the exudative form of age-related macular degeneration (AMD), a highly prevalent blinding disorder of multifactorial etiology. TIMP3 is a multifunctional protein and known to be a potent inhibitor of angiogenesis. It competitively blocks the binding of VEGF to its receptor VEGFR2, thus regulating VEGFR2-associated intracellular signalling. Here we investigate VEGF-mediated angiogenesis in the Timp3-deficient mouse.

Methods: We have generated a mouse model of TIMP3 deficiency by disrupting the orthologous murine Timp3 gene via homologous recombination in EC cells. This model was analysed by indocyanine green angiography (ICG), light microscopy, transmission- and laser scannig electron microscopy. Angiogenic response was analysed by the mouse aortic ring assay. Protein analysis was done by Western blotting.

Results: Light- and transmission electron microscopy of wild-type (wt) and knock-out (ko) mice show an intact structure of the different retinal layers, the complex of Bruchs membrane/RPE and the choroid. ICG angiography and scanning laser electron microscopic observations reveal a striking phenotype in the ko mice, indicating numerous expanded and dilated vessels in the choroid. Vascular response, analysed by the aortic ring showed strongly enhanced vessel outgrowth under spontaneous conditions. Addition of recombinant Timp3 and VEGFR2 Inhibitor reduced this response to a level comparable to wt. Furthermore VEGFR2 downstream molecules (e.g. ERK1/2 and p38 tyrosinkinases) were activated in ko mice, indicating a crucial role for this pathway.

Conclusion: In wt mice VEGF binding to VEGFR2 is competitively be inhibited by Timp3. In the absence of Timp3, VEGF/VEGFR2 binding is unopposed leading to enhanced intracellular signalling. This may finally lead to abnormal vessel formation.

Functional Characterization of Bestrophin and Potential Role in Best Disease

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Purpose: Bestrophin gene mutations cause Best's vitelliforme macular dystrophy. Reduction of the light-peak in the EOG was formerly regarded as a leading symptom, but gene analysis revealed patients with macular degeneration and unchanged light-peaks.

Results: In the rat DC-ERG, L-type channel block reduced the light-peak amplitude. Transfection with bestrophin accelerated the activation time constant of the light peak with unchanged amplitude. So far, bestrophin was described as Ca^{2+}-dependent weakly outwardly rectifying Cl^- channel. To study bestrophin function, RPE-J cells (no endogenous bestrophin expression) were transiently transfected with bestrophins. Membrane currents were measured using the patch-clamp technique.
Cells showing bestrophin in the cell membrane revealed no changes in membrane conductance, but modulations of Ba^{2+} currents through L-type Ca^{2+} channels. Voltage-dependent activation shifted towards more negative values and time-to-peak accelerated in transfected cells. Mutant bestrophins showed same effects on voltage-dependence, but different kinetic modulations. W93C bestrophin led to slower activation kinetics and inactivation. R218C bestrophin led to faster inactivation kinetics.
Bestrophin appeared as regulator of L-type channel activity which might also explain light-peak alterations in patients EOG. To further clarify the relation between Cl^- channels and Ca^{2+} channels we started to compare fresh RPE cells from wild-type versus bestrophin-knockout mice in whole-cell patch-clamp recordings. In preliminary experiments the latter do still show outwardly rectifying currents under control conditions. But further analysis and a pharmacological scrutiny will be obligatory.

Conclusion: Thus, bestrophin likely might not only function as Cl^- channel. More detailed studies on either bestrophin deficient mice or transgenic mice will show that either one of the hypothesis is right or, may be, both will be proofed to be true; bestrophin as Cl^- channel and Ca^{2+} channel regulator.

Evaluation of recent techniques for the diagnosis of inherited retinal dystrophies

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Background: To evaluate the clinical relevance of new diagnostic techniques to examine inherited retinal dystrophies.

Methods: Between 1986 and 2005 2050 Patients with RD underwent clinical and electrophysiological examinations in the Centre of Ophthalmology in Essen (1986-1990), Dept. of Ophthalmology Charité Campus Benjamin Franklin (1990-2003) and the Eye Centre Siegburg (2004-5). In last decade the multifocal electroretinogram (mfERG; n=622), the measurement of the autofluorescence of the retinal pigment epithelium (AF; n=531) and the molecular genetic proof of disease causing mutations (MG; n=175) could be added as new methods for differential diagnosis. These methods were retrospectively analysed regarding their diagnostic relevance.

Results: The major relevance of the mfERG is the possibility of early detection of macular dysfunction prior to ophthalmoscopically visible lesions. However, the potential for differential diagnosis is limited. Follow-up examinations with the mfERG may be advisable in disorders with late onset macular involvement, e.g. retinitis pigmentosa. The AF can be used to detect RPE alterations prior to ophthalmoscopically visible lesions and to follow them with non-invasively. The differential diagnostic potential of the AF is higher than that of the mfERG. The molecular genetic proof of disease causing mutations has a high diagnostic relevance, however, it is of limited value for the individual prognosis. The combination of all three methods reveals a high clinical variability between patients with mutations in the same gene.

Conlusions: Ophthalmoscopy and full-field ERG recording remain the most important methods for the diagnosis of RD. The addition of mfERG and AF allows an earlier diagnosis in several patients. AF and MG are important for differential diagnosis. AF is an easy non-invasive technique for follow-up. MG alone is not sufficient for counselling of the patient.

Characterisation of retinoschisin binding properties to plasma membrane and phospholipids

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Purpose: X-linked juvenile retinoschisis (RS) is caused by mutations in the RS1 gene encoding a 24 kDa protein, termed retinoschisin (RS1). Retinoschsin is secreted from photoreceptor and bipolar cells as a disulfide-linked oligomeric complex. Although loss of retinoschisin function has been established as the underlying disease mechanism, little is known about the molecular events leading to the degeneration of the neural retina. In this study, we focus on the binding mechanism of retinoschisin to the outer membrane surface.

Methods: Retinal membranes from murine eye cup were isolated and treated with various solutions (hypo- and hypertonic buffer, ß-mercaptoethanol, detergents and buffers with different pH-values) to dissociate membrane associated proteins. Soluble and membrane bound fractions were assessed by SDS-PAGE separation and Western blot analysis. The binding of heterologous RS1 to artificial phospholipid membranes (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, phosphatidylglycerol) was analysed by ELISA.

Results: Native retinoschisin is released quantitatively from the membrane under alkaline conditions. In contrast, in the presence of ß-mercaptoethanol and under hypertonic conditions native RS1 only partially dissociates from the membrane. Other conditions have no effect on native RS1 binding. Furthermore, recombinant retinoschisin shows no binding to artificial phospholipid membranes.

Conclusion: Our data suggest that native RS1 membrane binding is mediated by hydrogen bonds and ionic interactions. Only part of the 8 subunits constituting the functional oligomeric RS complex may be involved in the binding. This binding appears independent of the major phospholipids of the plasma membrane.

The scaffold protein harmonin (USH1C) also integrates Usher syndrome 2 proteins into synaptic Usher protein complexes in retinal photoreceptor cells

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Purpose: The Usher syndrome (USH) is the most common form of combined deaf-blindness. USH is divided in three clinical distinct types USH1 to USH3. USH1, the most severe form, is characterized by profound congenital deafness, constant vestibular dysfunction and prepubertal onset of retinitis pigmentosa. USH2 is a mild form with moderate to severe sensorineural hearing impairment at birth, normal vestibular responses and progressive retinitis pigmentosa. USH1C encodes for the scaffold protein harmonin, which contains PDZ-motifs known to organize protein complexes via protein-protein interaction. Recently, harmonin was found to bind all known USH1-proteins via its three PDZ-domains. Their co-localization in the photoreceptor synapses suggests that these interactions occur in the synaptic compartment of the specialized neurons, where they may constitute the basis for a supramolecular USH1-complex. Here, we addressed the extent of the known USH1-protein/ network to interactions with USH2-molecules.

Methods: Recombinant USH-proteins/ fragments were used for protein-protein interaction analysis by GST-pull downs and by the yeast two-hybrid system. Antibodies against the USH-proteins were generated and applied in western blot analysis as well as in immunocytochemical studies on the subcellular localization of the USH-molecules.

Results: Sequence analysis of usherin (USH2A), the 7-transmembrane very large G-protein coupled receptor 1 (VLGR1, USH2C), and the putative USH2B-protein NBC3 (Na^{2+}-bicarbonate co-transporter) identified PDZ-binding motives at their C-terminal tail. Our *in vitro* and *in vivo* protein interaction assays revealed that all three USH2-proteins bind to the PDZ1 domain of harmonin. Immunocytochemical analysis of cryosections of rat and mouse retinas further demonstrates additional co-localization of USH2-proteins with USH1-proteins in photoreceptor synapses.

Conclusions: Our findings strongly suggest that ribbon synapses of photoreceptors bear supramolecular protein network composed of USH1- and USH2-proteins. Mutations of one of the components may cause dysfunction of the entire network which probably leads to synaptic defects and in turn to retinal degeneration (retinitis pigmentosa), the phenotype observed in USH-patients.

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RNAi-based therapeutic suppression of rhodopsin and rds/peripherin in retinal explants

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Purpose: A cumulative number of some 150 different mutations in rhodopsin (rho) and rds/peripherin (rds) account for up to 30% of dominant retinitis pigmentosa cases. Clearly, extensive mutational heterogeneity presents a significant barrier to development of therapeutic intervention. Here, a mutation-independent suppression strategy targeting rho and rds and exploiting RNA interference (RNAi) has been assessed in a murine system.

Methods: Wild type and replacement rho and rds cDNAs plus short hairpin (sh)RNA vectors targeting rho and rds were co-transfected into COS-7 cells. Transcript levels were determined by real-time RT-PCR. One day-old retinas were in vitro electroporated with bicistronic EGFP-expressing shRNA vectors and evaluated by immunofluorescence microscopy and RT-PCR 14 days post-electroporation. To extract RNA from targeted cells transduced retinas were dissociated and EGFP-positive cells isolated by FACS sorting.

Results: In COS-7 cells, shRNAs specific for either rho or rds decreased expression by up to 77% while mRNA levels were maintained by replacement cDNA expression. In retinal explants, similar suppression of target mRNAs was achieved; some other photoreceptor-specific genes were also down-regulated, while housekeeping genes were unaffected. Significant reduction of rho protein was also evident in transduced photoreceptor cells.

Conclusions: RNAi can significantly silence mouse rho and rds expression in COS-7 cells and organotypic retinal cultures. Replacement mRNAs providing wild type proteins but refractory to RNAi silencing can be engineered using codon degeneracy. A given RNAi molecule is mutation-independent, i.e. can target all mutant alleles (except those carrying mutations in the RNAi target sequence) in addition to the wild type allele. This study provides a proof of principle of mutation-independent suppression and replacement, which could potentially be applied in the development of therapies for the wide range of dominant disorders with mutational heterogeneity.

Analysis of subunit assembly in bestrophin, the protein mutated in Best vitelliform macular dystrophy

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Purpose: The VMD2 gene is mutated in Best vitelliform macular dystrophy (BMD), an early-onset autosomal dominant disorder characterized by deposits of lipofuscin-like material in the subretinal space. Functionally, VMD2 has been suggested to encode a putative Ca^{2+}-dependent chloride channel, termed bestrophin. The number of subunits forming the Cl-channel is estimated at 4 to 5. With the first N-terminal half of Bestrophin, there is a high sequence identity to conserved family members VMD2L1, VMD2L2 and VMD2L3 suggesting similar functional properties for this recently identified novel protein family. Little is known about the molecular mechanisms underlying the more than 90 distinct mutations associated with BMD. With this study, we aim to identify the structural properties of bestrophin required for oligomerization and functional channel formation in wild type (wt) and mutated protein.

Methods: VMD2 constructs with C-terminal and N-terminal truncations, internal deletions and wt sequence were generated. All constructs include a tagged epitope sequence at the C-terminus. The various constructs were cotransfected together with wt VMD2 in EBNA cells. Solubilized protein was co-immunoprecipitated with a polyclonal VMD2 antibody. Labbeling was done with an antibody directed against the tagged epitope.

Results: The oligomerization complexes obtained by co-immunoprecipitation were analyzed for their ability to bind the respective C-terminal, N-terminal truncated, internal deletions, mutant and wt subunits of bestrophin. All C-terminal truncated peptides were able to bind wt VMD2. The immediate N-terminal 55 amino acids (aa) have not been tested so far. N-terminal truncations showed interaction with wt VMD2, so did the constructs containing internal deletions. Amino acids 75-87 contain a putative oligomerisation domain. The construct with the aa exchange S79C, a variant which is thought to disrupt the oligomerisation signal still shows strong interaction with wt VMD2.

Conclusions: The VMD2 protein sequence was analysed for its oligomerisation potential with truncations from the C-terminus, the N-terminus as well as internal deletions and a point mutation, likely to directly interfere with a potential oligomerisation signal. Thus far, the potential oligomerisation signal encoded by aa 75-87, does not appear to act in vivo as a site for VMD2 dimerisation. The possibility that two domains interactively contribute to VMD2 subunit assembly is being investigated.

Light-induced translocation of the signal transduction proteins transducin and arrestin analyzed in photoreceptor cells of organotypic retina culture.

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Purpose: The visual G-protein transducin and arrestin - responsible for the "turn off" of activated rhodopsin - migrate between the inner and outer segment of the photoreceptor cell in a light dependent way. Upon illumination transducin moves from the outer to the inner segment of the photoreceptor cell and arrestin in the opposite direction. The transport mechanisms and regulation factors underlying this intracellular translocation of transducin and arrestin still remain elusive. In the present study, we evaluated whether or not the molecular translocations are mediated by the actin cytoskeleton. Therefore we studied the movement of these signaling proteins in the organotypic retina culture. The aim was to establish a reliable organotypic retina culture to analyze molecular mechanisms.

Methods: For better accessibility and simple application of cytoskeletal drugs we established an organotypic retina culture. Apoptotic cell death was analyzed via the "TUNEL" assay. The light dependent movement of the signaling molecules and the actin cytoskeleton was analyzed by immunocytochemistry.

Results: There are only few "TUNEL"-positive, apoptotic cells in the organotypic retina culture during the first five days of culturing. The signal transduction proteins arrestin and transducin also translocate under illumination in the retina culture system, as they do in the retinas of light adapting animals. There is a reduced or incomplete translocation of the two proteins by disruption/stabilization of actin filaments in the retina after the treatment with cytoskeletal drugs during dark- but not during light-adaptation.

Conclusions: The organotypic retina culture proofs as vital system by the evidence of light-induced translocation of signal transduction proteins ex vivo and by keeping a non-apoptotic state for at least 5 days. Addressing possible transport mechanisms for the translocation of transducin and arrestin our data suggest that the actin cytoskeleton is necessary for this shift during dark-adaptation. In contrast the movement in the opposite direction during light-adaptation seems to be independent of actin filaments and could probably be mediated by diffusion, which would also explain the higher velocity of the light-adaptation process.

Supports: ProRetina Deutschland

RPE-Autofluorescence and OCT in Vitelliform Macular Dystrophy

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Purpose: To evaluate the relevance of autofluorescence (AF) imaging of the retinal pigment epithelium (RPE) and optical coherence tomography (OCT) and the clinical assessment of vitelliform macular dystrophy, i.e. adult vitelliform macular dystrophy (AVMD) and Best macular dystrophy (BMD).

Methods: Consecutive AVMD and BMD patients have undergone complete eye examination, including AF, since March 2001, and OCT, since July 2004.

Results: To date, 28 AVMD patients and 9 VMD2 gene mutation associated BMD patients have undergone AF, and 4/28 AVMD and 1/9 BMD patients OCT. At the time of the first AF/OCT, the mean age of the AVMD patients was 59.9 years (35.1-78.1) and median visual acuity was 0.6. The fundus findings (n=54 eyes) included a single vitelliform lesion smaller than 1 disc diameter (n=38), RPE defects (n=9), subtle RPE changes (n=4), and a normal macular (n=3). AF was abnormal in 44 of 51 eyes with funduscopic changes: AF increased in 33/38 eyes with vitelliform lesions, in 3/4 eyes with RPE changes and in 2/9 eyes with RPE defects, but reduced in 6/9 eyes with RPE defects. Follow-ups (0.6-2 years) detected subtle changes in lesion size or no further changes with AF. OCT was abnormal in all 8 eyes showing a highly reflective thickened RPE/choriocapillaris band foveal, RPE detachment in 1 eye, and in 2 eyes a cystoid space at the RPE level. The mean age of the BMD patients was 48.7 years (22.6-63.7) and median visual acuity was 0.32; they (n=18 eyes) presented with vitelliform (n=1), pseudohypopyon (n=1), vitelliruptive (n=7), atrophic (n=7) lesions, and subtle RPE changes (n=2). Increased or decreased AF was seen in all eyes - correlating to clinically visible yellow material or atrophic areas, respectively. In 2 eyes reduced AF showed pattern configuration. Development from vitelliruptive to an atrophic stage in one patient within 1.5 years could be monitored using AF. OCT of this patient showed thinned retina in the atrophic lesion and RPE detachment of the vitelliform lesion in the fellow eye.

Conclusions: AF changes were seen in 86% of AVMD and in 100% of BMD. OCT was always abnormal; AF can be normal in very small AVMD lesions. AF and OCT enables fast and non-invasive detailed morphologic information to be obtained. Retinal thickness can be measured and both RPE detachment or cystoid spaces detected using OCT. AF is a valuable tool for progression monitoring and is more accurate than funduscopy.

Stargardt disease and Autosomal recessive cone–rod dystrophy caused by the homozygous 2888 del G mutation in the ABCA4 gene.

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INTRODUCTION: The photoreceptor cell-specific ATP-binding cassette transporter gene (ABCA4) is mutated in most patients with Stargardt disease (STGD) or fundus flavimaculatus (FFM). In addition, a few cases with AR retinitis pigmentosa (RP) and AR cone-rod dystrophy (CRD) have been found to have ABCA4 mutations. The mutant alleles are classified as mild, moderate or severe and the combinations of these alleles are correlated with the different phenotypes.

PATIENTS AND METHODS: A total of 85 STGD families were studied. DNA from every patient and relatives was analysed for variants in all 50 exons of the ABCA4 gene by screening on the ABCR400 microarray; the results were confirmed by direct sequencing. Haplotype analyses were also performed.

RESULTS: In two families, previously diagnosed with STGD and CRD respectively, we found that both were segregating the homozygous 2888delG mutation in the ABCA4 gene. They were also homozygous for the intragenic polymorphisms (N1868I, L1894L).

CONCLUSIONS: The present study suggests that mutations in the ABCA4 gene cause different phenotypes, even when it is due to a homozygous change (and not a compound heterozygote).
In these families a modulating effect due to intragenic polymorphism (SNPs) is not probable because they are homozygous for them.
However it is possible that other cis or trans extragenic factors may modulate ABCA4 activity; this could explain the existence of different phenotypes with the same genotype.

Analysis of Multicomponent Protein Networks in the Retina

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Purpose: A large group of genetically inherited blinding diseases is associated with mutations in genes expressed in photoreceptors. At the biochemical level, numerous proteins interact forming complexes to perform cellular tasks. Thus, mutation of one of the interaction partners can lead to disruption of functional network and consequently to severe physiological disorders. We have combined bioanalytical and molecular methods to describe functional relationships within protein networks in mammalian photoreceptors.

Methods: Mammalian photoreceptor outer segments (OS) were isolated from porcine retinae. The membranous OS fraction was solubilized using non-ionic detergents and native protein complexes were separated using density gradient centrifugation. Individual proteins were separated by SDS-PAGE and identified by MALDI-TOF mass spectrometry. Assembly and dynamics of specific protein complexes were further analyzed using affinity methods.

Results: Optimization of native separation methods, protein solubilization and separation conditions allowed us to isolate multiprotein complexes from mammalian photoreceptor outer segments. Using a proteomic approach (Zischka *et al.*, 2004), we have identified several candidates as novel components of the phototransduction pathway, including small GTPases from Rho and Rab families. RhoA and Rac1 are relevant for neuronal development and are able to rescue photoreceptor degeneration in a Rhodopsin-null mutant of *Drosophila* (Chang and Ready, 2000). We have established a preliminary protein interaction map for mammalian photoreceptors.

Conclusions: We have detected protein interactions linked to the physiology of vision in mammalian photoreceptors. Protein interaction maps and analysis of their dynamics can be correlated to physiological states and will be used for a better understanding of vision processes and their pathology in diseases causing blindness.

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Leber Congenital Amaurosis detected using a genotyping microarray: suspected gene modifier effect

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INTRODUCTION: Leber Congenital Amaurosis (LCA) is the most severe inherited retinopathy with the earliest age of onset. Individuals affected with LCA are diagnosed at birth or in the first few months of life with severely impaired vision or blindness, nystagmus and abnormal or extinguished electroretinogram (ERG). Non syndromic LCA has been associated with mutations in eight genes: AIPL1 (17p13.1), CRB1 (1q31-q32.2), CRX (19q13.3), GUCY2D (17p13.3), MERTK (2q14.1), LRAT (4q31), RPE65 (1p31) and RPGRIP1 (14q11). These genes are involved in different physiologic pathways in the retina.

MATERIAL AND METHODS: We report a mutational analysis of all eight genes in 127 unrelated families. Mutational analysis was performed in 30 families diagnosed with LCA, 60 with early onset Retinitis Pigmentosa and 37 not-early onset Retinitis Pigmentosa. Samples were studied with a microarray (Asperbio) followed by a family study and direct sequencing in the laboratory.

RESULTS: The respective frequencies of mutant alleles are: 23% (14/60) for LCA with 9 mutated families (one carried an homozygous mutation, three are compound heterozygotes, four are single heterozygotes, and one is digenic or possible triallelism), 16% (19/120) for early-onset ARRP with 15 mutated families (one carried an homozygous mutation, eleven are single heterozygotes and three are digenic or possible triallelism) and 7% (5/74) with 5 mutated families with ARRP not-early onset (all five are single heterozygotes). CRB1 is the most mutated gene in Spanish affected families.

CONCLUSION: The combination of microarray and laboratory analysis is an optimal option for finding new disease alleles and it allows detecting cases of possible triallelism.
There is a gradient in mutation frequencies with respect to onset and severity of retinal disease, so correct classification of families is fundamental.
The relative percentage of mutations found with the microarray suggests that more LCA-associated genes remain to be discovered.

Monitoring of the respiratory chain activity in vivo using dynamic optical reflection spectroscopy

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Mitochondria are among the key players in various aspects of the processes of tissue degeneration. A non-invasive method for monitoring their activity is therefore a key to assessing the metabolic state of the tissue as well as its general condition. We previously developed a method for monitoring redox states of respiratory pigments using the PCA-based spectral deconvolution of difference absorption spectra (Zupancic 2003, Pfluegers Arch 447: 109-119). In present work we applied this approach to dynamically monitor redox states of cytochromes a, a3, b and c as well as flavoproteins (mainly NADH dehydrogenase) in the eyes of blowflies (Calliphora vicina) and fruitflies (Drosophila melanogaster). With flies' eyes we used a regime of step changes in PO2 from normoxic to anoxic (pure N2) conditions and back by a rapid stop-flow system with which we could completely exchange the atmosphere in the recording chamber in under 2 s. All the respiratory pigments were reduced in anoxic conditions but the extent of change as well as the latency, and the time courses of reduction and oxidation differed significantly. In blowflies the latencies between the onset of the anoxic conditions and the start of reduction were approx. 3 s for flavoproteins, cytochromes a and c and 6-7 s for cytochromes a3 and b. The rates of respiratory pigment oxidation were approx. 3 times higher than the rates of reduction. The maximal amplitude of the reduced form concentration change was for flavoproteins 0.9±0.08 μMcm (mean±sem; units including the light path), cytochromes c 1.03±0.08, a 0.77±0.06, a3 0.46±0.03 and b 0.26±0.02. Assuming equimolar stoichiometry and complete reduction in anoxia these values should be proportional to the pre-anoxic oxidation states of cytochromes. Data from fruitflies were similar in shape but different in extent, consistent with morphological and physiological differences between the two species. The results show that the method and the preparations used allow a unique insight into the functioning of mitochondria inside the intact living tissue.

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