PRO RETINA
RESEARCH-COLLOQUIUM
CONFERENCE REPORT

Retinal Degeneration

Illuminating Molecular Complexities of the Retina

An Interdisciplinary Dialogue

April 7th/8th, 2006

Potsdam, Seehotel am Templiner See

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PRO RETINA DEUTSCHLAND e. V.

in cooperation with the **European IP "EVI-GenoRet" (LSHG-CT-2005-512036)**

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PREFACE

The First Pro Retina Research Colloquium in 2005 in Potsdam was one of the most outstanding scientific meetings ever in the field of retinal degeneration. An excellent program, the enthusiasm and energy of the highly motivated researchers who exhibited posters, the ideal format for communication and last but not least the perfect meeting place all combined to make a truly exciting and productive conference. Thus, it was not surprising that the participants voted unanimously to convene again in the following year.

Very timely topics have been chosen again this year by the organizers and the speakers, revolving around the molecular complexities of the retina. Given the rapid developments in the field of genomics and proteomics, the increasing insight into pathophysiological mechanisms on the cellular level and breakthroughs in our understanding of the triggering mechanisms for retinal degeneration and age-related macula degeneration have opened up unexpected areas of research. Moreover, the implementation of the European Integrated Program EVI-Genoret, successfully lanced by the European Vision Institute (EVI) under the scientific coordination of Prof. José Sahel and myself, has already passed its first year report with very positive feedback from the international reviewers. The Pro Retina Research Colloquium is an excellent platform for top-notch research presentation and discussion. It is therefore a logical next step to establish close cooperation between this conference and the European Integrated Project EVI-Genoret, joining all scientists in this field to provide a lively Europe-wide platform for research into retinal degeneration. This is a very important prerequisite for Europe taking the lead in this research area, for besides well established ophthalmic research institutes the network created in this way will also encompass the newly founded "Institut de la Vision" in Paris (Head Prof. José Sahel) and the new "Institute for Ophthalmic Research" in Tübingen (Head Prof. Eberhart Zrenner).

A group of more than 200 scientists is expected and it is very reassuring that many young biologists, ophthalmologists, molecular geneticists, bio-physicists and physiologists have selected retinal degeneration as their particular research topic. The multitude of national and supra-national funding activities, including those of the patient organizations, has ensured that there will be a sufficient number of positions available in this field in the future. With the ever increasing number of patients suffering from various forms of retinal degeneration, this research has great importance of this research for our society in general. Good vision is a very precious asset and nobody knows this better than the patient organizations. The close cooperation between researchers and patient organizations not only increases the awareness of scientists about the importance of their task and provides an enormous boost in motivation, but also allows them to communicate new findings quickly to those who need them most. Many new therapeutic approaches have been pursued quite successfully in the last few years in age-related macula degeneration as well as in hereditary retinal dystrophies, including

restoration of vision in the blind. To foster and to accelerate this process is a task of utmost importance. Very clearly, the organizers (Franz Badura, Klaus Rüther, Olaf Strauss and Bernhard Weber), deserve our highest respect and our deepest gratitude - and with them the many unnamed members of the organization committees, the speakers, the presenters, the participants and especially the patients, who helped to trigger and maintain this process with their donations and activities.

It is truly laudable that Pro Retina Deutschland e.V. is joining forces with Retina International, which is so well fostered by its President Christina Fasser, and with national organizations of patients and scientists. The conference host, Pro Retina Deutschland e.V., is represented by Kurt Schorn and Franz Badura, who is really the motor of this meeting. It is my pleasure as the Head of the Scientific Advisory Board of Pro Retina Deutschland e.V. and co-chair of the Scientific and Medical Advisory Board of Retina International to welcome you all to this scientific meeting and to wish you joy, high spirits, new friendships and especially maximum benefit for your scientific work.

Tübingen, March 27th, 2005

Prof. Dr. med. Eberhart Zrenner
Head of the Scientific Advisory Board of Pro Retina Deutschland e.V.
Co-chair of the Scientific and Medical Advisory Board of Retina International

PROGRAMME

Friday, 7th

13:15	Eberhart Zrenner/Tübingen: Opening Remarks
13:25	Christina Fasser/Retina International: From the Patient's View
13:40	Frank G. Holz/Bonn: Visualization and Diagnosis of Retinal Disease - Advances in Imaging Technology
14:00-15:30	**Session 1: The Connecting Cilium and Neurodegeneration** *Chairman: Frank G. Holz*
	1. Uwe Wolfrum/Mainz: Photoreceptor Cilium and Retinal Degeneration
	2. Ronald Roepman/Nijmegen: Getting a GRIP on RPGR – Dissection of the Protein Complex unveils its Importance for Retina Function
	3. Christine Petit/Paris: Hereditary Deafness: From Genes to Pathophysiology
15:30-16:00	Coffee break and scientific chitchat
16:00-17:30	**Session 2: Cone-rod-interaction** *Chairman: Bernhard H. F. Weber*
	1. Susanne Kohl/Tübingen: Genetic Complexity of Cone and Cone-Rod Dystrophies
	2. Thierry Leveillard/Paris: Rod-derived Cone Viability Factor
	3. Mathias Seeliger/Tübingen: Functional Rod Pathways in Mice dissected with Electroretinography
18:00	Joint Ceremonial Dinner
19:00- Open End	Postersession

PROGRAMME

Saturday 8th:

08:30-10:30 **Session 3: Signalling Processes in the Retina**
Chairman: Olaf Strauß

1. Ulrike Janssen-Bienhold/Oldenburg: Diversity of the Connexins in Retinal Network Signalling

2. Katja Rillich/Leipzig: Neuro-Glial Crosstalk in the Retina

3. Silke Haverkamp/Frankfurt: Bipolar Cells of the Mouse Retina: Types and Plasticity

4. Katharina Wycisk/Zürich: Structural and Functional Abnormalities of Retinal Ribbon Synapses due to Cacna2d4 Mutation

5. Christina Zeitz/Zürich: Night Blindness-associated Mutations in the Metabotropic Glutamate Receptor 6 show a Distinctive Scotopic 15-Hz Flicker ERG and abolish Protein Trafficking

10:30 Coffee break and scientific chitchat

11:00-13:00 **Session 4: The Biology of Neovascularization**
Chairman: Klaus Rüther

1. Ernst Tamm/Regensburg: Dissecting the Function of the Norrie Protein

2. Hans-Peter Hammes/Mannheim-Heidelberg: Neovascularization of Retinal Vessels

3. Olaf Strauß/Hamburg: The Role of the RPE Cells in Neovascularization of Choroidal Vessels

4. Andreas Bringmann/Leipzig: Osmotic Swelling of Glial Cells in the Diabetic Rat Retina: Implications for the Development of Retinal Edema

13:00 Lunch

14:15-15:45 **Session 5: Novel Aspects of AMD Etiology**
Chairman: Gareth McKay

1. Claudia Keilhauer/Würzburg: AMD Phenotypes and Aspects of Recruitment of Cases/Controls for Association Studies

2. Lars Fritsche/Regensburg: Susceptibility Factors in AMD

3. Peter Zipfel/Jena: The Role of the Complement System in AMD

4. Akshay Anand/Chandigarh: The Animal Models of AMD – a Critical Appraisal

15:45 Farewell Coffee

SPEAKER ABSTRACTS

Visualization of retinal disease – advances and perspectives in imaging technology

Frank G. Holz

Department of Ophthalmology, University of Bonn, Germany

Various innovations in imaging technology have considerably improved our understanding of the pathophysiology of retinal diseases. With the advent of confocal scanning laser ophthalmoscopy (cSLO) it is possible to record fundus autofluorescence (FAF) *in vivo*, and, therefore, lipofuscin accmulation in postmitotic RPE cells as a common pathogenetic pathway in various complex, degenerative diseases such as AMD as well as in monogenetic retinal diseases. Based on FAF-studies and basic science models the accumulation of the toxic lipofuscin compound A2-E will now be tackled therapeutically along with FAF monitoring. FAF imaging is also useful in identifying suitable areas of noncompromised RPE prior to autologous RPE-patch transplantation and demonstration of viable RPE following translocation. In hereditary retinal disorders including Stargardt disease, retinitis pigmentosa, LCA and cone dystrophies FAF imaging revealed further insights in underlying mechanisms of disease. Combining cSLO FAF imaging and microperimetry the impact of increased FAF levels on retinal sensitivity can now be determined. High-resolution FAF imaging even allows for delineation of individual RPE cells *in vivo*. Further improvements in resolution are emerging by applying two photon excited fluorescence imaging with a femtosecond Ti:sapphire laser in association with cSLO technology. Another cSLO method is applicable for determining topographic macular pigment optical densities. This not only allows for assessing the potential protective role of luteal pigment and its supplementation but also for refined phenotyping in diseases such as macular teleangiectasia. Finally, optical coherence tomography has revealed insights in dynamic changes in macular and vitreoretinal interface pathologies. These imaging tools can also be used in animal models of blinding retinal diseases and will help to monitor future therapeutic interventions.

Photoreceptor cilium and retinal degeneration

Uwe Wolfrum

[1]Cell and Matrix Biology, Institute of Zoology, Johannes Gutenberg-University of Mainz, Germany

Vertebrate photoreceptor cells are highly specialized, polarized neurons. The photoreceptor outer segment contains visual transduction cascade proteins arranged in hundreds of stacked membrane disks. It is connected to the inner segment and the cell body, the compartments of biosynthesis, via a narrow non-motile connecting cilium. This cilium corresponds in its basic molecular composition the transition zone at the base of a regular motile cilium. In photoreceptors all intracellular exchange between the inner and the outer segment compartment has to occur through the connecting cilium.
Photoreceptor cells are highly biosynthetic active and their light-sensitive outer segment turnover throughout lifetime. Every day 10% of the outer segment is shed at its tips and new disk membranes are assembledeplaced at its base. For photoreceptor maintenance, the vectorial transport of an enormous number of newly synthesized molecules, including rhodopsin, from the inner segment to the outer segment through the connecting cilium is necessary. Furthermore, in photoreceptors, bidirectional movements of molecules of the visual cascade also occur, which are light-dependent and participate in long-term adaptation. The bidirectional translocation of those molecules, e.g. transducin and arrestin, also occurs through the tiny connecting cilium. In this light, the connecting cilium is a cellular "bottle neck" for high-frequent molecular traffic. Therefore, it does not astonish that defects in ciliary components impair photoreceptor function leading to photoreceptor cell death and retinal degeneration.
Analyses of gene products related to several retina degenerative diseases revealed that these proteins participate in the ciliary function of photoreceptor cells. Good examples are, the RP1 protein (*retinitis pigmentosa* (RP) type 1), a microtubule-associated protein, specific for ciliary axonemes of photoreceptor cells and RPGR (RP G-protein regulator, X-linked *RP 3*) protein as well as its interacting partner RPGRIP-1 (*Leber congenital amaurosis*) which are suggested to participate in the function of the connecting cilium. Furthermore, defects in components of the ciliary apparatus can also cause syndromes with severe visual impairment. Proteins related to the *Bardet-Biedl syndrome* and to the *Usher syndrome* (USH) are found to be involved in the function of the ciliary apparatus of photoreceptor cells. Further analyses of the molecular composition of the ciliary apparatus of photoreceptor cells and their molecular interaction should provide important novel insights in photoreceptor function and will lead to a better understanding of mechanisms underlying the degeneration of photoreceptor cells which is a necessary prerequisite for the development of founded therapeutic strategies for RP.

Current Supports: DFG, FAUN, Forschung contra Blindheit Initiative Usher Syndrom, ProRetina Deutschland

Getting a GRIP on RPGR – dissection of the protein complex unveils its importance for retina function.

Ronald Roepman[1], Stef Letteboer[1], Heleen Arts[1], Sylvia van Beersum[1], Anneke Navis[1], Xinrong Lu[2], Elmar Krieger[3], Paulo Ferreira[2] & Frans Cremers[1]

[1]Dept. of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; [2]Departments of Ophthalmology, and Molecular Genetics and Microbiology, Duke University Medical Center, Durham, USA; [3]Center for Molecular and Biomolecular Informatics, Radboud University Nijmegen, Nijmegen, The Netherlands.

Purpose: RPGR interacting protein 1 (RPGRIP1) is a key component of cone and rod photoreceptor cells, where it interacts with RPGR (Retinitis Pigmentosa GTPase regulator). Mutations in *RPGRIP1* lead to autosomal recessive congenital blindness (Leber congenital amaurosis - LCA). Most LCA-associated missense mutations in *RPGRIP1* are located in an alternatively spliced segment that encodes two C2 domains. We have investigated the functional relevance of this domain by identifying novel interacting proteins and analyzing the effects of the mutations on the protein-protein interactions.

Methods: A homology model of the C-terminal C2 domain of RPGRIP1 was built using the programs WHAT IF, SCWRL and YASARA. Novel interacting proteins were identified by yeast two-hybrid screening of retinal cDNA libraries. Interactions were confirmed by GST pull-down analysis and coimmunoprecipitation assays. Colocalization was analyzed using GFP fused variants in mammalian cell lines or by immunohistochemistry of retinal cryosections.

Results: The C2-C homology model revealed a potential Ca^{2+}-binding site that was predicted to be disrupted by a missense mutation in *RPGRIP1*, which was previously identified in an LCA patient. Through yeast two-hybrid screening, we found this C2 domain to specifically bind to nephrocystin-4, encoded by *NPHP4.* Mutations in *NPHP4* are associated with nephronophthisis (NPHP), and a combination of NPHP and retinitis pigmentosa called Senior-Løken syndrome (SLSN). We show that RPGRIP1 and nephrocystin-4 interact strongly *in vitro* and *in vivo*, and that they colocalize in the retina, matching the panretinal localization pattern of specific RPGRIP1 isoforms. Their interaction is disrupted by either mutations in *RPGRIP1*, found in patients with LCA, or by mutations in *NPHP4*, found in patients with NPHP or SLSN.

Conclusions: We provide evidence for the involvement of a disrupted interaction with the C2 domain of RPGRIP1 in the retinal dystrophy of both SLSN as well as LCA patients. The identification of additional interactors builds evidence for an emerging protein complex with a pleiotropic and dynamic behaviour in the (patho-)physiology of the retina and other tissues.

Hereditary deafness : from genes to pathophysiology

Gaëlle Lefèvre, Elisabeth Verpy, Vincent Michel, Nicolas Michalski, Christine Petit

More than a hundred genes are predicted to underlie hereditary deafness in humans. To date, 42 of them have been identified. The pathogenic processes involved in each form of deafness are progressively deciphered. All but one are due to cochlear defects. We will discuss the gene defects underlying the cohesion of the hair bundle, the mechanoreceptive structure to sound stimulation. Our entry points in that respect are the Usher syndrome genes. Usher syndrome is the most frequent cause for a double sensorineural handicap combining severe sensorineural deafness and retinitis pigmentosa causing progressive blindness. Usher syndrome can be classified into three types depending on hearing loss severity, on the retinopathy onset date as well as on the possible presence of vestibular disorders. Usher type I syndrome is the most severe clinical form. We have managed to isolate three out of five of its known genes. Our research work has brought to light the crucial role, previously unnoticed, played by interstereociliary links in the initial cohesion of the hair bundle. Our investigations provided evidence that the five Usher I molecules directly interact with one another. One of them, harmonin (USH1C), a PDZ domain-containing protein can interact with the four other USH1 proteins, namely, myosin VIIa (USH1B), cadherin-23 (USH1D), protocadherin-15 (USH1F) and Sans (USH1G). In this cohesion process, cadherin-23 forms links that are early binding the hair bundle stereocilia both together, and to the kinocilium. Moreover, we have recently shown that usherin, a protein defective in Usher II type syndrome (USH2A), is likely to compose links interconnecting the base of the stereocilia. In addition, a new deafness gene product led us to detect a protein that caps and binds together the stereocilia of the outer hair cell hair bundle and anchors their longest stereocilia to the acellular gel (tectorial membrane) that conveys the sound stimulus to them. In its absence, none of the interstereocilia links of the mature hair bundle are able to withstand the sound mechanical stress.

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Genetic complexity of cone and cone-rod dystrophies

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Purpose: Cone and cone-rod dystrophies are a phenotypically and genetically heterogeneous group of disorders characterized by severely reduced visual acuity and color vision abnormalities. Only few genes (22 loci / 14 genes) and mutations have been identified in only a limited number of patients and families with these disorders. Here we want to provide an overview on the genes involved in cone and cone-rod dystrophy, and present results obtained in ongoing molecular genetic studies in a project of the Klinische Forschergruppe: Erbliche Netzhauterkrankungen: Klinik, Genetik und Tiermodelle.

Methods: Mutation screening was performed by PCR, SSCP, dHPLC, DNA sequencing and DNA Chip technology (Asper Biotech).

Results: Mutations in three genes (*CNGA3*, *CNGB3* and *GNAT2*) for autosomal recessive rod-monochromacy have been identified, and rearrangements and deletions as well as point mutations in the red-/green opsin gene cluster (*OPN1LW* / *OPN1MW*) have been shown to be the cause for X-chromosomal blue cone monochromacy. These disorders are described as congenital and non-progressive, yet certain mutations in *CNGA3*, *CNGB3* and *GNAT2* have been associated with incomplete achromatopsia or progressive cone dystrophy, and patients with blue cone monochromacy have been described with disease progression and macular dystrophy later in life. Mutations in only two genes in autosomal dominant (*GUCA1A* and *RDS/peripherin*) and in one gene (*RPGR*) in X-chromosomal progressive cone dystrophy have been described in few patients and families, and one locus for autosomal dominant and two loci for X-chromosomal cone dystrophy have been identified by linkage or deletion mapping. In autosomal dominant cone-rod dystrophy mutations in eight genes (*CRX*, *GUCY2D*, *RIMS1*, *RDS/peripherin*, *SEMA4A*, *GUCA1A*, *AIPL1* and *UNC119*) have been published and one locus is proposed by deletion mapping. In addition, one locus has been mapped to the X-chromosome. In autosomal recessive cone-rod dystrophy, three loci are known, one co-localizing with the *ABCA4* gene which has been originally identified in Stargardt's disease. Several independent reports and our own data show that mutations in *ABCA4* are responsible for 50-60% of all autosomal recessive cone-rod dystrophies.

Conclusions: The knowledge on the genetic basis of cone and cone-rod dystrophy is still small compared to the large number of genes and mutations identified for example in retinitis pigmentosa. Only one major locus for autosomal recessive cone-rod dystrophy has been identified with the *ABCA4* gene. Our aim is to describe a comprehensive mutation spectrum in already known genes associated with cone and cone-rod dystrophy, and to identify and analyse new candidate genes for these cone photoreceptor disorders.

Rod-derived cone viability factor, on the way for a therapy of retinitis pigmentosa

Thierry Léveillard[1], Marie Noëlle Delyfer[1], Anne Galy[1], Ying Yang[1], Angella Giangrande[2] Isabelle Audo[1], Olivier Poch[2], Saddek Mohand-Saïd[1], José Sahel[1]

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Retinitis Pigmentosa (RP) and other retinal degenerative diseases affecting 30,000 to 40,000 people in France and up to 1,500,000 people worldwide are part of a long list of orphan diseases for which no treatment is currently available. They constitute a heterogeneous group of disorders having common characteristics that lead to irreversible damage of the peripheral vision. In affected people, the retinal rod photoreceptors responsible for night vision and side vision slowly degenerate. The first clinical signs are night blindness and a narrowing of the peripheral field of vision which progressively worsens to become "tunnel-like". Frequently, the retinal cone photoreceptors are then secondarily affected. Retinal cones are responsible for both colour vision and high-contrast vision, visual acuity, detail perception and all visual functions in normal light. As the disorder progresses, the central vision is reduced until, in most cases, the person becomes blind. The first symptoms usually appear in teenagers and young adults and the disorder general develops over 20 to 30 years to its end-stage.

We have used several therapeutic strategies to prevent the secondary degeneration on cones. The indirect ones aim at preventing primary rod death using GDNF, a Glutamate receptor antagonist or the caspase inhibitor p35. To target cones directly, we have used a systematic strategy based on a functional assay using cone-enriched cultures to identify trophic factors expressed by rods and necessary for cones to survive. We constructed an expression library from wild-type mouse retina and used expression cloning methods to test all the genes for their potential to promote chicken cone survival. We screened 2,100 pools, corresponding to 210,000 individual clones. Pool number 939 contained twice as many living cells as the negative controls. Using limiting dilution, we isolated clone 939.09.08 and found that it contained a 502-bp insert with an open reading frame encoding a putative 109-amino acid polypeptide. We named this gene Rod-derived Cone Viability Factor. It represents a splice variant of the gene Txnl6 with homology with the thioredoxin family of genes. The identified factor has many characteristics of the postulated therapeutic gene. It is secreted by rods, has a protective activity on cones and its expression is lost after rods have degenerated. In RP, preventing cone cell death is a very promising therapeutic approach. Even when 95% of the cones have been lost in patients with a macular disease specifically affecting the cones, the vision remains substantial. This both gives greater hope of a therapeutic strategy aimed at preserving the remaining cones in RP patients and also opens a broad therapeutic window. Patients suffering from RP generally consult an ophthalmologist when rod loss has already occurred, meaning rod-targeted therapies have little use. Therefore, keeping the cones alive would prevent 1.5 million people worldwide becoming blind.

Functional rod pathways in mice dissected with electroretinography

M. W. Seeliger, C. Friedburg, E. Zrenner, M. Biel, N. Tanimoto

Purpose: Ganzfeld electroretinography (ERG) yields a sum response of retinal circuits to light stimulation. This response is complex due to the input from both rod and cone photoreceptors and the temporal overlap of the underlying components. Here, we focus on the functional role of specific elements in the signal pathway from rods to bipolar cells.

Methods: For this work, wild-type and functionally cone-deficient Cnga3$^{-/-}$ knockout mice were examined with Ganzfeld ERG. Recordings were performed under scotopic conditions following overnight dark adaptation. Rod photoreceptor responses were isolated with the paired flash method. Bipolar cell contribution was estimated from scotopic single flash data. The response dynamics were assessed from a series of flicker ERGs with flash frequencies ranging from 0.75 to 24 Hz, and intensities from 10^{-5} to 20 cd*s/m^2. Cnga3$^{-/-}$ mice were used to ensure rod-system specific responses at stimulus intensities above 10^{-2} cd*s/m^2 (the approximate threshold for cone signals).

Results: The rod outer segment responses corresponded well to the mathematical models available. At low flash intensities, it may be assumed that these signals arrive in practically unaltered shape at the rod synapse to its ON bipolar cell. The transition of the signal to the ON bipolar involves substantial compression, i.e. low intensity portions are overly enhanced and high intensity portions are attenuated. Modeling of this effect reveals this compression as the probable key factor in rod ERG waveform shape. The OFF system is structurally and functionally not well developed in mice and its contribution is negligible under the conditions used.
The dynamics of signal generation and transmission were assessed with flicker ERG. For each frequency, there was a monotonic rise in flicker amplitude with intensity up to a maximum value at about 10 mcd*s/m^2. The absolute value of that maximum decreased with frequency and vanished at around 15-20 Hz. A further increase in intensity led to a monotonic reduction of amplitude down to zero, which was at low frequencies preceded by a plateau at the maximum value. The intensity at the onset of the drop and at which zero amplitude was reached was inversely related to flicker frequency. The results were in good agreement with a newly developed model.

Conclusions: The role of specific functional elements in the signal pathway from rods to bipolar cells and the dynamics of the scotopic flicker ERG were described. At low light intensities, the absolute amplitude and the flicker fusion frequency was apparently limited by the timing properties of the rod outer segment. At high light intensities, the flash-to-flash interaction gained importance and could be well modeled by an equivalent background step increasing with intensity. These insights are important to better understand ERG changes in inherited diseases on a (sub)cellular level.

Diversity of connexins in retinal network signalling

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Purpose: In the vertebrate retina many types of neurons form networks via stereotyped patterns of gap junctional connections. Gap junctions (GJ) represent intercellular conduits consisting of two oligomeric hemichannels, the molecular composition of which determines the specificity of signalling within the neuronal networks of the retina. GJ are dynamically modulated by the ambient light conditions and neurotransmitters, and thereby make an important contribution to visual information processing. In order to get a better understanding of how the diversity of the GJ-forming proteins, the connexins, affects certain functions in retinal physiology, our group focussed on the identification and functional characterisation of connexins involved in the formation of coupled networks of retinal horizontal cells and the rod pathway in the mammalian retina.

Methods: We used different techniques to identify retinal connexins and investigate their expression patterns, as well as the morphology, molecular composition, regulation and function of the corresponding GJ. These include molecular methods (single-cell RT-PCR, RACE-PCR, molecular cloning, *in situ* hybridisation), immunocytochemistry using connexin-specific antibodies, dye injections (cell morphology, tracer coupling) and different imaging techniques (light, confocal laser scanning and electron microscopy). Transgenic mice (Cx45, Cx57 knock-out) were generated in the lab of Prof. K. Willecke (Institute for Genetics, University of Bonn).

Results: mmCx57 was identified as the connexin almost exclusively expressed in mouse horizontal cells. As shown by means of Neurobiotin injections into and intracellular recordings from horizontal cells of isolated wild type and Cx57-deficient mouse retinas, these retinal neurons lose the capability of tracer and electrical coupling, when Cx57 is deleted. In a parallel study, we cloned three orthologs (cpCx49.5, cpCx52.6, cpCx55.5) of mmCx57 from fish retinal cDNA, two of which (cpCx52.6 and cpCx55.5) are exclusively expressed in distinct horizontal cells of the fish retina as shown by *in situ* hybridisation. In the rod pathway of the mouse retina, we could identify mmCx36 and mmCx45 as the important connexins that play a pivotal role in this pathway, since the lack of either protein results in an impairment of visual transmission under scotopic conditions. Furthermore, we found immunocytochemical evidence for heterotypic Cx36/Cx45 gap junctions at the gap junctional connection between the processes of AII amacrine cells and the axon terminals of type 5 and 6 ON cone bipolar cells.

Conclusion: The diversity of connexins is the basis for the plasticity and flexibility of gap junctional coupling between retinal neurons and provides the retina with the potential to optimise parallel processing of visual information.

This work was supported by the Deutsche Forschungsgemeinschaft (JA 854/1-1 to U.J.-B. and SFB 517-A2 to R.W.)

Neuro-glial crosstalk in the Retina

Katja Rillich, Michael Weick, Andreas Reichenbach

Question: The retinal circuitry adapts during light- and dark-adaptation. Will Müller cells, the general glial element in the retina, also adapt to changed light conditions?

Approach: Müller cells control a variaty of homeostatic functions from neuronal energy supply and control of extracellular ion concentrations to neurotransmitter recycling. Calcium is one of the key modulators of cell functions. For instance, morphology, metabolism, and gene transcription are under control of calcium. We expect that changes in Müller cell calcium metabolism might indicate adaptation in Müller cell functional state, e.g. during light adaptation.

Method: Dark adapted Guinea pig retinal wholemounts and slices were loaded with X-Rhod-1 calcium indicator dye. Local and full field light stimulations were performed, and calcium changes of individual Müller cells were recorded.

Results: The cytosolic calcium concentration of Müller cells changes in a complex manner in due to light stimulation. Local, low light stimuli evoke increased calcium in local Müller cells. Full-field stimulation of retinal wholemounts with physiological intensities of light induced a slow but prominent calcium increase that run down after about 5 minutes. Superimposed on this slow calcium regulation, fast calcium rises could be observed. They reach maximum after a few seconds and may last up to one or two minutes. Pharmacological interception of neuronal signaling beyond photoreceptors by glutamatergic modulators, did not completely block the slow calcium increase found in Müller glial cells. Also other inhibitors of retinal receptors did barely affect the slow signal. Only Zaprinast®, an inhibitor of photoreceptor phosphordiesterase, which impairs phototransduction, did strongly reduce the slow and fast responses. Noteworthy, cylcopiazonic acid did not affect the slow signal but inhibited the fast calcium rises. Our current hypothesis is that the signal elicting the slow response originates from photoreceptors whereas the fast calcium rises are triggered via secondary or tertiary neurons.

Bipolar cells of the mouse retina: types and plasticity

HSilke Haverkamp

Department of Neuroanatomy, Max-Planck-Institute for Brain Research, Frankfurt/Main

With the ever growing number of transgenic mice being used in vision research, a precise knowledge of the cellular organization of the mouse retina is required. The bipolar cells can be subdivided into one rod bipolar and nine different cone bipolar cell types (Ghosh et al., 2004). The axons of the bipolar cells, which transfer the light signals of the photoreceptors to the ganglion cells, terminate at distinct levels within the IPL. Functionally, the cells can be subdivided into ON- and OFF-cells: On-bipolar cells show depolarizing light responses and terminate in the inner half of the IPL, OFF-bipolar cells hyperpolarize in response to light and terminate in the outer half of the IPL. Superimposed on this ON/OFF dichotomy are four types of OFF and five types of ON cone bipolar cells.

Immunocytochemical markers can be used to label different bipolar cell types: type 3, type 5 and rod bipolar cells express the calcium-binding protein CaB5, whereas type 1/2 bipolar cells express the neurokinin 3 receptor (NK3R) (Haverkamp et al, 2003). Type 7 and type 9 bipolar cells are labeled in transgenic mouse lines by the expression of green fluorescent protein (Huang et al., 2003; Haverkamp et al., 2005).

Cone bipolar cells contact all the cone pedicles (between 5-10) within their dendritic field - with one exception: the type 9 bipolar cell has a wide dendritic tree that selectively contacts S-opsin expressing cones. We were able to study these S-cone selective or blue cone bipolar cells in a transgenic mouse expressing Clomeleon, a chloride-sensitive fluorescent protein, under the *Thy1* promotor. The blue cone bipolar cells comprimise only 1-2% of the bipolar cell population, and in the ventral mouse retina, where most cones express both L- and S-opsin, blue cone bipolar cells contact only those cones, which express S-opsin only. They are the genuine blue cones of the mouse retina.

Previous studies have shown that rod bipolar cells form synaptic connections with cones when rods are absent. We now investigated whether cone bipolar cells also show synaptic plasticity in the absence of cone input. Using two knockout lines, one lacking cone function ($CNGA3^{-/-}$) and the other lacking both cone and rod function ($CNGA3^{-/-}Rho^{-/-}$), we found that cone bipolar cells form ectopic synapses with rods, but only when rods are functional.

Structural and functional abnormalities of retinal ribbon synapses due to Cacna2d4 mutation.

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Purpose: In a spontaneous mutant substrain of C57BL/10 mice, severely affected retinal ribbon-type synapses were previously described. The retinopathy was accompanied by a substantial loss in activities of the second-order neurons. Rod photoreceptor responses were maintained with reduced amplitude, whereas cone activities were absent. We focused on the identification of the genetic defect underlying this hitherto unknown autosomal recessive cone-rod dysfunction.

Methods: Genome-wide linkage analysis and screening of positional candidate genes were used to identify the causative mutation. Tissue-specific transcriptional activity of the defective gene was determined by northern blot analysis and RT-PCR approaches. Cone photoreceptor numbers were counted on immunohistochemically stained cryosections.

Results: We localized the mutation to a 275 kb region of chromosome 6. Within this candidate interval, we found a homozygous frameshift mutation (c.2547insC) in the Cacna2d4 gene of affected animals. This gene codes for an L-type calcium channel auxiliary subunit of the $\alpha_2\delta$ type. The mutation introduces a premature stop codon which truncates one third of the predicted Cacna2d4 protein. A severe reduction in Cacna2d4 transcript levels observed in mutant retinae probably results in lack of Cacna2d4 protein. The mutation leads to significant loss of rods, whereas cone cell number remains unaffected until 6 weeks of age.

Conclusions: The Cacna2d4 mutation underlies a novel channelopathy leading to cone-rod disease in the visual system of mice and provides a new candidate gene for human retinal disorders including night blindness, retinitis pigmentosa and cone-rod dystrophis.

Night blindness-associated mutations in the metabotropic glutamate receptor 6 show a distinctive scotopic 15-Hz flicker ERG and abolish protein trafficking.

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Purpose: To investigate the genetic basis defect of autosomal recessive congenital stationary night blindness (arCSNB), which is characterized by loss of night vision due to a defect in signal transmission from photoreceptor to the adjacent ON-bipolar cells.

Methods: Patients and unaffected relatives from 5arCSNB individuals of five families with presumably arCSNB were screened for mutations in *GRM6* and analyzed with standard and 15-Hz flicker electroretinography (ERG). These recordings were compared to those of patients with X-linked CSNB1. N-and C-terminal tagged wild-type (wt) and mutated *GRM6* constructs were transiently or stably transfected in mammalian cells. We discriminated between surface and intracellular protein with anti-V5 and anti-myc antibodies, respectively. Colocalization studies were performed with antibodies against endoplasmic reticulum (ER) and Golgi-markers. A possible effect of mutations on protein dimerization was investigated by Western blot analysis.

Results: Affected individuals in three of five families carried either compound heterozygous or homozygous mutations in *GRM6*. All displayed a distinctive abnormality of the rod pathway signals (scotopic 15-Hz flicker ERG). Also, WweWe demonstrate that mGluR6 localizes to the cell surface, to ER and Golgi compartments. In contrast, all disease-associated missense mutations lead to retention of the protein in the ER and Golgi apparatus, while dimerization seems not to be affected.

Conclusions: The novel ERG profile suggests the existence of more than two rod pathways. The distinctive ERG feature was not observed in patients with X-linked CSNB1 and additional affected individuals with unknown molecular defect. Our observations will help to discriminate autosomal recessive fromand X-linked recessive cases by ERG and at DNA level. CSNB-associated mutations in three different domains of mGluR6 abolish proper protein trafficking. We propose that the ligand-binding and the poorly characterized cysteine-rich domains in addition to the transmembrane domain have a pivotal role in correct trafficking of metabotropic glutamate receptors to the cell surface. Thus, the phenotype of arCSNB is due to a lack of mGluR6 on the cells surface.

Dissecting the Function of Norrin

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Purpose: To learn about the biochemical function(s) of norrin, the strategy of expressing ectopic norrin in the eye of transgenic mice by means of a strong and strictly lens-specific promoter was used. Norrin is a 133 aa secreted polypeptide with the predicted tertiary structure of a cysteine knot growth factor. Mutations in norrin cause Norrie's disease (ND) an X-linked retinal dysplasia that presents with congenital blindness, sensorineural deafness and mental retardation. Mutant mice with a targeted disruption of Ndp (*Ndp$^{y/-}$*) show complete absence of intraretinal capillaries and a progressive loss of vessels within the stria vascularis of the cochlea. The structural changes correlate with a marked loss of function, as mutant *Ndp$^{y/-}$* mice develop blindness and sensorineural deafness.

Methods: Four independent transgenic mouse lines were developed that overexpressed norrin in the eye under control of the βB1-crystallin promoter. Transgenic integration and copy number was characterized by Southern blot analysis. The phenotype of transgenic mice was analyzed from P2 to P28.

Results: Overexpression of norrin and its mRNA was confirmed by northern blot hybridization, and western blot analyses. Lenses of transgenic mice with ectopic expression of norrin showed significantly more capillaries in the hyaloid vasculature that surrounds the lens during development and overexpressed VGEG and PIGF. *In vitro*, lenses of transgenic mice in coculture with microvascular endothelial cells induced proliferation. Transgenic mice showed more BrdU-labeled retinal progenitor cells at embryonic day 14.5 and thicker retinas at postnatal life than wildtype littermates indicating a putative direct neurotrophic effect of norrin. The transgenic expression of ectopic norrin under control of a lens-specific promoter was completely sufficient to restore the formation of a normal retinal vascular network in *Ndp$^{y/-}$* mutant mice. The improvement in structure correlated with the presence of a normal ERG with a positive b-wave in Nor/*Ndp$^{y/-}$* mice indicating that the restoration in retinal vasculature correlated with that of retinal function.

Conclusions: Ectopic norrin, which is expressed in and secreted from the lens is completely sufficient to prevent the defects in retinal structure and function of *Ndp$^{y/-}$* mutant mice. Norrin restores the retinal vascular network by completely respecting the normal vascular archi-

tecture of the retina, while no abnormal vascular sprouting or signs of retinal neovascularization are observed. Our results provide direct evidence that pharmacologic modulation of norrin might be used to treat the vascular abnormalities in the retina of patients with Norrie disease. In addition, norrin might be used as therapeutic compound for treatment of other vascular disorders of the retina.

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Neovascularization of retinal vessels

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Retinal neovascularization depends on the induction of the angiogenic endothelial phenotype by the shifted balance between anti- and proangiogenic factors, and is also strictly controlled by cellular communications. Pericyte loss is a hallmark of early diabetic retinopathy, and was thought to be necessary to facilitate endothelial proliferation. However, recent data show that pericytes comigrate with the endothelial sprouts during physiological angiogenesis, and are present in early neovascular sprouts in the mouse model of retinopathy of prematurity. Pericytes were also considered to determine the window of plasticity of retinal capillaries. We have demonstrated that not pericyte recruitment, but the upregulation of enodthelial survival factors determine the resistance of the retinal vasculature towards regressive signals such as VEGF-depletion. Pro- and antiangiogenic factors are also provided to the endothelium by intravascular components such as platelets. During angiogenesis, platelets release proangiogenic factors such as VEGF and platelet remnants and microparticles are found at sites of angiogenic sprouts. Both, the inhibition of platelet function, and of platelet numbers, reduce retinal angiogenesis by half. This is compatible with a significant contribution of platelets to retinal neovascularization. Inflammatory cytokines share proangiogenic activities with angiogenic cytokines, and vessel stabilization includes anti-inflammatory activities. Therefore, inflammation is considered a crucial part in vascular remodelling, and angiogensis in diabetes. The receptor for AGEs, RAGE, is central in mediating regressive, and inflammatory signals to the diabetic vessel. One of its ligands, HMGB1 is present in the angiogenic retina, suggesting that the RAGE-NFkB-axis could be involved in angiogenesis. However, RAGE-/- mice do not display reduced angiogenesis in the ROP model. In turn, mice with a genetic deletion of TNF-α show a reduction in neovascularization. Treatment of mice with the angiostatic triamcinolon acetonid reduces proliferative retinopathy likewise.
In summary, novel data provide evidence against long-held dogmas that pericytes are involved in sprouting angiogenesis, and that survival factors for endothelial cells play a crucial role in vascular resistance against regression. The multiplicity of cellular and humoral interactions still to be discovered, however, prevents us from fully understanding mechanisms, and best-suitable targets for intervention or treatment of proliferative retinopathy.

The Role of RPE cells in Neovascularisation of Choroidal Vessels

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Purpose: Choroidal neovascularisation (CNV) represents the most severe complication in age-related macular degeneration (AMD), the major cause for blindness in western countries and accounts for 90 % of vision loss in AMD. A variety of growth factors, such as insulin-like growth factor (IGF-1), basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF), have been identified to play a role in the induction of CNV in AMD. This was so far mainly deduced from histological studies of CNV membranes. How this network of different factors function in a line of events leading to neovascularisation is unclear. Known as the most important factor for neovascularisation in general, VEGF has also been identified to play this role in CNV. Furthermore, the role of the accessory growth factors IGF-1 and bFGF is unclear.

Methods and Results: We used a combination of cell physiological methods and immune histological analysis of CNV membranes to identify the role of IGF-1 and bFGF as signalling pathways in the network of growth factors causing CNV. Patch-Clamp recordings from freshly isolated cells isolated from CNV membranes indicate that these cells express voltage-dependent Ca^{2+} channels and are, thus, metabolic active. Primary cultures from these cells showed a higher VEGF secretion rate than those from RPE cells from healthy donors. IGF-1 induced a strong stimulation of VEGF secretion by RPE cells from CNV membranes, human RPE cells in primary culture or the RPE cell line ARPE-19. Analysis of the distribution pattern of IGF-1 receptors revealed that the RPE expresses IGF-1 receptors but not the photoreceptors. IGF-1 was found in CNV membranes as well in photoreceptors of AMD eyes. Thus, we could identify a signalling pathway in which photoreceptors under metabolic stress release IGF-1 which in turn induce the secretion of VEGF by the RPE to promote neovascularisation. The new blood vessels should then improve the metabolic situation. bFGF was found to function as an autocrine factor to modify RPE function. bFGF is released from the RPE cells which have been hurt. RPE cells themselves express two bFGF receptors, FGFR-1 and FGFR-2. Stimulation of FGFR-2 leads to direct stimulation of L-type Ca^{2+} channels which in turn leads to an increased secretion of VEGF. In contrast stimulation of FGFR-1 does not stimulate L-type channels and does not lead to increased VEGF secretion. However, stimulation of FGFR1 induced an increased expression rate of the expression factor c-fos. Since it was shown that over-expression of VEGF alone is not sufficient to induce CNV, it needs further events such as a break in the integrity of RPE cell layer. This could be enabled by the autocrine stimulation of RPE cells by bFGF.

Conclusions: In summary, we propose the following chains of events in the network of growth factors leading to CNV in which RPE cells represent the amjor source for VEGF. Early events in the pathology of AMD are the formation of Drusen, the thickening of Bruch's membrane and a loss of RPE cells leading to disintegration of the RPE. This results in metabolic stress of the photoreceptors which release IGF-1. Damaged RPE cells release bFGF. Both IGF-1 and bFGF induce the secretion of VEGF from healthy RPE cells which change their cell behaviour by c-fos-induced modulation of gene expression. The main targets of VEGF are endothelial cells which in turn proliferate and migrate

Osmotic swelling of glial cells in the diabetic rat retina: Implications for the development of retinal edema

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Purpose: The development of macular edema is a common complication of diabetic retinopathy, contributing to impaired vision. Vascular leakage and swelling of retinal cells may be implicated in edema formation. Since Müller glial cells normally mediate the fluid clearance from the inner retinal tissue, via transcellular ion and water transport, a swelling of Müller cells may indicate a disturbance of this function. The aim of the study was to investigate whether experimental diabetic retinopathy alters the osmotic swelling characteristics of Müller cells, whether this is associated with an altered expression of potassium and aquaporin water channels, and whether triamcinolone acetonide, which is clinically used to resolve retinal edema, may inhibit Müller cell swelling.

Methods: Diabetes was induced in rats by injection of streptozotocin. The osmotic swelling of Müller cell bodies was determined in retinal slices of 6 months-diabetic and control animals by application of a hypotonic bath solution. Potassium currents of isolated Müller cells were examined by patch-clamp recordings. The expression of Kir4.1 potassium and aquaporin-4 water channels was determined immunohistochemically in retinal slices.

Results: Experimental diabetes causes a significant alteration of the swelling characteristics of Müller cells. Müller cell bodies in diabetic retinas swell upon osmotic stress but no swelling is inducible in Müller cells of control retinas. The osmotic swelling in diabetic retinas is mediated by a downregulation of Kir4.1 potassium channels in Müller cells, by inflammatory mediators, and by oxidative stress. In the diabetic retina, Müller cells retract their aquaporin-4-containing membranes from the superficial vessels. Triamcinolone inhibits the osmotic swelling of Müller cells in the diabetic retina, via a mechanism which involves stimulation of the release of endogenous adenosine, A1 receptor activation, and opening of potassium and chloride channels in the Müller cell membrane. Triamcinolone also inhibits the swelling of glial cells in control retinas evoked by the inflammatory mediators arachidonic acid and prostaglandin E_2, or by oxidative stress.

Conclusion: We suggest a mechanism of edema development which includes accumulation of potassium ions in Müller cells after downregulation of Kir4.1 channels, and osmotic movement of water from the blood into the Müller cells facilitated by aquaporin-4. Inhibition of Müller cell swelling, and facilitation of the fluid clearance function of Müller cells, may contribute to the edema-resolving action of triamcinolone in the diabetic retina. A1 adenosine receptors may be a promising target for the development of new drugs for the resolution of macular edema.

AMD phenotypes and aspects of recruitment for case/control association studies

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Purpose: Age-related macular degeneration (AMD) is a complex disease and a prevalent cause of visual impairment in industrialized countries. The clinical phenotype of AMD is heterogeneous. Early forms of the disease are characterized by the presence of soft drusen, areas of hyperpigmentation and depigmented areas while later stages manifest as either choroidal neovascularization (CNV) or atrophy of photoreceptors and the retinal pigment epithelium (GA). A genetic predisposition conferring a defined risk to develop AMD is well established. As case-control association studies represent a powerful strategy to identify these genetic factors, a large number of age-, gender- and ethnically matched well-defined cases and controls are needed. Stratification of the clinically heterogeneous AMD population into defined subgroups based on functional-physiological aspects may help to enrich for functionally related risk alleles.

Methods: 1167 AMD patients (mean age: 77.7 ± 6.7 years) with at least one eye exhibiting a well-defined pattern of early AMD pathology and 796 normal age-related controls (mean age: 77.6 ± 5.2 years) were exclusively selected from the Lower Franconian area of Bavaria. Digital color fundus images, confocal infrared and autofluorescence images were used to identify AMD subgroups. To match for age with the AMD group, only AMD-free controls over 67 years were included.

Results: Features such as drusen of different sizes and shapes, pigmentary changes or incipient atrophy of the RPE were classified as early AMD. This group was further subdivided with regard to the risk of progression to late AMD (high or low risk). Other conditions that may mimic the common AMD phenotype like late-onset macular dystrophies or inflammatory macular diseases were excluded from the study. The exact definition of cases and controls, age-matching and the choice of an ethnically homogeneous population enabled the identification of common genetic variants (e.g. *CFH*, Tyr402His or *LOC387715*, Ala69Ser) strongly associated with an increased risk to develop AMD. Stratification for defined AMD subgroups revealed no significant differences between different forms of late stage AMD (GA/CNV) but showed that a significantly higher frequency of these variants was observed in early AMD cases classified as high risk for AMD compared to those with low risk. The frequency in low risk early AMD cases was similar to that observed in controls.

Conclusions: Accurate definition of cases and controls enables the identification of defined allelic variants associated with complex diseases like AMD. Clarification of the genetic factors contributing to disease etiology will help to identify pathophysiologically relevant mechanisms amenable to the development of novel therapeutic strategies.

Susceptibility factors in AMD

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Introduction: Age-related macular degeneration (AMD) is a genetically complex disease and a major cause of impaired visual acuity in the industrialized world. In recent years, tremendous efforts have been undertaken to identify the genetic factors predisposing individuals to develop AMD. This has included the search for common as well as rare gene variants although the identification of common variants with strong effects on disease etiology is more promising to succeed. Nevertheless, both approaches are relevant for our further understanding of the fundamental processes governing the susceptibility to AMD.

Methods: Mainly two approaches are being used to determine the contribution of genetics to AMD susceptibility. Genome-wide linkage/association studies in affected sib-pairs facilitate the identification of strong genetic effects while the candidate gene approach which investigates the contribution of functional gene candidates to AMD disease load may reveal weaker effects of risk factors.

Results: To date, two major susceptibility genes for AMD have been identified by genome-wide scans and refined association studies, namely the gene encoding the complement factor H (CFH) and the gene encoding a hypothetical protein LOC387715. Both contribute almost equally to a strikingly increased risk of AMD disease. CFH points to an involvement of inflammatory processes and the innate immune system in AMD pathology. This is further supported by candidate gene analyses including toll-like receptor 4 (TLR4), factor B (BF) and complement component 2 (C2). So far, the function of LOC387715 and implications toward disease etiology are entirely unknown. Similarly, the role in disease pathology of other associated genetic factors such as APOE, ELOVL4, and FIBL-5 are also elusive.

Conclusion: Genetic association studies in AMD have been shown to be most powerful to identify genetic risk factors modulating disease characteristics. This will provide novel insight into basic pathways leading to age-related disease formation and will help to design novel approaches to develop rational therapeutic treatments for this ailing disease.

The role of the complement system in age related macular degeneration (AMD)

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Complement is a central and important immune system that acts as an immediate first defense mechanism against microbes and surfaces of modified self cells. Complement activation is initiated spontanously by the alternative pathway or is induced by antibodies via the classical pathway. Once activated the complement system is highly destructive and generate toxic and inflammatory products. In order to prevent activation on host cell surfaces this defense system is tightly controlled. Complement regulatory proteins like Factor H, CR1, DAF and MCP protect self surfaces by inactivating complement C3 convertases. Absence or functional defects of complement regulators like Factor H result in severe diseases like Membranoproliferative Glumerulonephritis or Hemolytic Uremic Syndrom. Recently it has been proposed that a common variant Y402H in the complement Factor H gene increases the risk for the development of soft drusen and advanced age related macula degeneration (AMD). We purified Factor H from plasma of AMD patients and control persons with the genomic subtypes: homozygous H402, homozygous Y402 and heterozygous H/Y402. Functional tests with these proteins revealed that Factor H homozygous for the risk phenotyp H402 showed significant reduced cell binding activities compared to the other two subtypes Y402 and H/Y402. Thus the H402 variant may lead to decreased binding of Factor H to the cells of the choriocapillaries, the retinal epithelial cells and the drusen, leading to reduced protection and subsequent deposition and activation of complement pathway proteins

The animal models of AMD-A critical appraisal.

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Purpose: We wanted to analyse the different animal models of AMD in order to establish the role of imflammation in AMD.

Methods: We analysed various chemokine knockout mice strains, conducted comprehensive literature search and utilized immunohistochemistry, MALDI TOFF, FA, Westarn blotting and *insitu* degradation assays to assign functional role to macrophages in Ccl-2 and Ccr-2 mouse model of AMD.

Results: Various animal models have been used to simulate AMD through high fat diets and phototoxicity, senescence acceleration or candidate gene manipulation, resulting in clinical, histological and angiographic features of the human condition. Some of these have similarities with basal linear deposits but drusen formation, immune deposits and photoreceptor atrophy are lacking and provide little information about pathophysiology of AMD. The dystrophic RCS rat is a model that undergoes progressive photoreceptor degeneration (without CNV) due to primary defect in RPE cells and is useful model for stem cell transplantation studies. The VEGF overexpressing mouse model and the laser injury mouse models are good tools for analyzing inhibitors of choroidal growth. Some Belly spot and tail heterozygote knockout mice also develop CNV while TIMP-3 knockouts display early features of age related changes in Bruch's membrane and RPE but they take long time to develop such features or exhibit severe ocular disturbances. The VMD model, characterized by deposits in lipofuscin in Bruch's membrane with accompanying photoreceptor atrophy, can be used to study RPE function. The Ccl-2 and Ccr-2 mouse model leads to development of drusen and lipofuscin deposits in the RPE or spontaneously occurring CNV resembling that seen in patients with AMD. Such pathologies are absent in MIP-1a, B5 integrin, Ccr-5 and apo E knockout mice.

Conclusions: Through *invitro* and *invivo* studies we have demonstrated that choroidal macrophages migrate by Ccl-2 gradient, adhere to and degrade the immune deposits. As male Ccl-2 and Ccr-2 deficient mice have an impaired recruitment of macrophages, it has been suggested that there is accumulation of complement fragments that might damage RPE and induce VEGF production by these cells resulting in development of CNV. Combined with the role of complement participation in CNV and AMD, it has become possible to organize the inflammatory paradigm in AMD into a more cogent concept and devise strategies that ameliorate AMD pathology. With such developments, there is likely to be a major shift in our approach to the treatment of this disease.

POSTER ABSTRACTS

Mpp4 recruits Psd95 and Veli3 towards the photoreceptor synapse

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Purpose: Membrane-associated guanylate kinase (MAGUK) proteins function as scaffold proteins contributing to cell polarity and organizing signal transducers at the neuronal synapse membrane. The MAGUK protein Mpp4 is located in the retinal outer plexiform layer (OPL) at the presynaptic plasma membrane and presynaptic vesicles of photoreceptors. Additionally, it is located at the outer limiting membrane (OLM) where it might be involved in OLM integrity.

Methods: *Mpp4* knockout and wild-type retinas were histological, immuno-histochemical and electron microscopic examined at 1, 3, 6 and 12 months of age. The structural and functional integrity of the *Mpp4* knockout retina was investigated and compared to wild-type retinas using scanning laser ophthalmology (SLO) and electroretinography (ERG) at 9 and 18 months of age. Immunoprecipitation was used to study the interaction between Mpp4 and the MAGUK proteins Psd95, Veli3, Dlg and Cask.

Results: In the *Mpp4* knockout mice, loss of Mpp4 function only sporadically causes photoreceptor displacement, without changing the Crumbs (Crb) protein complex at the OLM, adherens junctions or synapse structure. Scanning laser ophthalmology revealed no retinal degeneration. The minor morphological effects suggest that *Mpp4* is a candidate gene for mild retinopathies only. At the OPL, Mpp4 is essential for correct localization of Psd95 and Veli3 at the presynaptic photoreceptor membrane. Psd95 labeling is absent of presynaptic membranes in both rods and cones but still present in cone basal contacts and dendritic contacts. Total retinal Psd95 protein levels are significantly reduced which suggests Mpp4 to be involved in Psd95 turnover, whereas Veli3 protein levels are not changed. These protein changes in the photoreceptor synapse did not result in an altered electroretinograph.

Conclusions: These findings suggest that Mpp4 coordinates Psd95/Veli3 assembly and maintenance at synaptic membranes. Mpp4 is a critical recruitment factor to organize scaffolds at the photoreceptor synapse and is likely to be associated with synaptic plasticity and protein complex transport.

Toward a gene therapy for dominant retinitis pigmentosa

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Purpose: RNA interference (RNAi) is a promising technology to silence the expression of genes of interest and may potentially be applied in the development of therapies for human disorders. Peripherin (rds) and rhodopsin (rho) are photoreceptor-specific genes in which a cumulative number of approximately 150 mutations have been described to be responsible for various forms of autosomal dominant retinitis pigmentosa (adRP), a degenerative retinopathy in humans. To test the feasibility of RNAi in photoreceptors, small hairpin RNAs (shRNAs) were used to knock-down mouse rds or rho expression. A possible route to treat adRP may be the suppression of mutated and wild-type rds or rho mRNAs, together with provision of a replacement gene which encodes wild-type protein that is protected from suppression by modification of the shRNA target sites using the degeneracy of the genetic code.

Methods: Potential shRNA sequences targeting rds or rho were tested and selected in COS7 cells. The most efficient sequences targeting rds or rho, as well as a non-targeting construct as a negative control, were tagged with an enhanced green fluorescent protein (EGFP) expression cassette. Additionally, cDNAs were constructed with degenerate nucleotide changes over the target sites for RNAi suppression that, in principle, still encode wild-type rds or rho protein. Suppression of endogenous rds/rho mRNA or protein and expression of the replacement construct was first tested in retinal explants electroporated with shRNA vectors and replacement cDNAs. Additionally, suppression and replacement constructs for rho were generated in recombinant adeno-associated virus (rAAV2/5) and injected into the subretinal space of mice to transduce photoreceptors *in vivo*. Results were evaluated by immunofluorescence microscopy and RT-PCR.

Results: The expression of shRNAs that target either rds or rho decreased the level of the corresponding mRNAs up to 90% when compared to non-targeting control shRNA. Immunohistochemical analysis of rho-targeted photoreceptors revealed a strong decrease in the number of endogenous rho expressing cells at the protein level. Furthermore, it was demonstrated that replacement constructs were expressed in transfected/transduced photoreceptors.

Conclusions: RNAi technology can significantly silence rds or rho expression in photoreceptor cells *in vitro* and *in vivo*. Thus, RNAi might be especially useful to knock-down mutant disease genes with dominant negative effects in a mutation-independent suppression and replacement strategy.

Zebrafish: a novel in vivo model to identify and characterise genetic determinants of retinal vasculature

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Purpose: The retinal vasculature is a complex capillary network that nourishes the photosensitive retina. Many severe human eye diseases (*e.g.* diabetic retinopathy, retinopathy of prematurity, familiar exudative vitreoretinopathy and Norrie disease) are linked with abnormalities or microvascular complications in the retinal blood supply. The advantages of zebrafish as a model for genetic, developmental and pharmacological studies has not been exploited in this area. Our objective is to characterise the morphology of the developing zebrafish intraocular vasculature in normal, mutant, hypoxic and hyperglycaemic states.

Methods: Vessels were visualised under bright-field and fluorescence microscopy after staining by the following techniques: A) *fli-1* transgenic line expressing EGFP in endothelial cells, B) antibodies specific for blood vessels (collagen IV, smooth muscle actin, FVIII, CD31 and GFAP) C) DAPI nuclear staining and D) endogenous alkaline phosphatase staining. For adult zebrafish, retinas were dissected and flat mounted for microscopy. For larvae, lenses were dissected and immersed in PBS/glycerol or methylcellulose for microscopy.

Results: Our preliminary data demonstrates that zebrafish have a retinal blood supply and that a transgenic line specifically expressing EGFP in blood vessels enables rapid and specific analysis of the morphology of these retinal vessels. Here we show a detailed analysis of retinal blood vessels in the developing and adult wild type zebrafish. The first hyaloid vessels can be distinguished at 2.5 dpf attached to the lens, and their growth can be followed through development as they progressively loosen their contact with the lens (between 20-30 dpf) and attach to the inner surface of the retina in the adult.

Conclusions: Zebrafish can be applied in a genetic approach to identify molecular regulators of intraocular vasculature development. We are currently screening for specific intraocular vasculature defects in zebrafish mutants to identify determinants of retinal vasculature.

Analysis of the sugar code in retinal development: galectin-16 affects formation of 3-dimensional retinal spheres

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Purpose: Carbohydrate moieties serving as ligands in biorecognition processes can by far surpass amino acids and nucleotides in information-storing capacity. Therefore, glycoconjugates play significant roles in embryonic development as well as in disease processes (Gabius et al., 2004). In particular, ß-galactosides of cell surface glycoconjugates are docking sites for endogenous lectins of the large galectin family. As a model for analysing the significance of galactose residues in retinal development, we applied galectin-16 in 3-dimensional reaggregated spheres from embryonic chicken retinae. This model is particularly suited, since it has become clear that differentiation processes in 3-dimensional environments are much more closely related to an *in vivo* situation as compared to conventional monolayer cultures (Layer et al., 2002; Abbot, 2003; Jacob et al., 2005).

Materials & Methods: The central parts of chick retinae were isolated at embryonic day 6 (E6), trypsin-digested and mechanically dissociated into single cells with a fire-polished Pasteur pipette. The resuspended retinal cells were rotation-cultured in 2 ml aggregation medium in 35 mm dishes, in absence or presence of increasing concentrations of galectin-16. The most effective concentration was 0.1 µg/ml. Medium was changed every 2 days. Reaggregated spheres were cultured for up to 14 days; normally, they were harvested after 10 days i.c. Cryosections from treated and non-treated spheres were prepared, with which immunostainings characterizing several retinal cell types were performed (antibodies were CERN901 and rho4D2 for rods, CERN906 for red/green cones, Pax6 and calretinin for amacrine and ganglion cells, vimentin and glutamine synthetase for Müller glia cells, etc.). In addition, Western blots and determination of AChE activity by the Ellmann assay established quantitative changes of spheroid growth and differentiation.

Results: Galectin-16 induced a prominent reduction in spheroid diameter size (up to 25%), indicating that galactose residues are essential for cell proliferation. Moreover, the organisation of Müller glia cells and their scaffold were severely altered (side protrusions into IPL shortened, processes on sphere surface diminished). While ratios of rods and cones were almost normal, the morphology of photoreceptors was deteriorating; in particular, their outer segments were not well developed. Similarly, the spatial organisation of amacrine and ganglion cells as visualised by Pax6 staining was decreased. AChE activities as visualized by the Karnovsky-Roots staining were decreased, particularly in cell groups entering apoptosis. These changes became most obvious in rosetted spheroids as they matured.

Conclusions: Taken together, this study establishes the validity of the 3D-spheroid approach to analyse the significance of the sugar code under defined *in vitro* conditions.

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Neurotransmitter induced currents in retinal bipolar cells of the rd mouse

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Purpose: The rd mice have been widely used as animal models of retinal degeneration (Chang et al, 2000). In the rd animals a natural mutation of the β subunit of the rod phosphodiesterase determines the degeneration and death of rod photoreceptors along their postnatal period. Structural modifications in rod isolated rod bipolar cells (RBCs) of the rd mouse retina have been documented after degeneration of rod photoreceptors, both at the dendrites and axon terminal (Strettoi et al, 2000,2002; Varela et al., 2003). Our purpose is to test the functionality of BCs along photoreceptor degeneration.

Methods: For our study, we selected two types of rd mice: the rd1 (kindly provided by the "Fight for Sight Foundation") and the rd10 (kindly donated by Dr. B. Chang, at Jackson Laboratories). Standard ERG and immunocytochemsitry were performed at different postnatal days on control and rd animals. Experiments using the patch clamp technique in the whole cell configuration were done on BCs enzimatically dissociated from the rd mouse retina and retinal slices.

Results: In the rd1 mouse, a reduction in number of rod photoreceptor and ERG wave amplitudes are observed after eye opening (p12 to p30), however in the rd10 photoreceptor degeneration is observed from p20 to p40 (tested both by immunohistochemistry and ERG). The RBCs dissociated from rd1 mouse retina showed a significant reduction in their glutamate responses but an increase in GABA induced currents (Varela et al,2003). An increase in glycine induced responses is already observed by one month of age in the rd1 mouse. A huge increase in the glycine induced responses is observed in the rd10 at two months of age. Currents activated by glutamate, GABA and glycine were also tested in BCs from retinal slices from the rd10 at two months of age. Conclusion: Our work analyzes the neurotransmitter induced currents recorded in retinal BCs from rd1 and rd10 animals both in dissociated cells and retinal slices. Data from BCs of rd animals were compared with those recorded from BCs of wt mice.

Conclusion: Our work analyzes the neurotransmitter induced currents recorded in retinal BCs from rd1 and rd10 animals both in dissociated cells and retinal slices. Data from BCs of rd animals were compared with those recorded from BCs of wt mice.

A protein interaction network linked to the mammalian GPCR rhodopsin

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Purpose: Mutations in the light receptive GPCR rhodopsin and in proteins linked to its downstream signalling account for numerous blinding diseases. The mechanistic interpretation of the majority of rhodopsin mutations is not straightforward, suggesting that at least some of them may affect its function indirectly by altering the correct wiring of a large and still poorly understood protein network. With the aim of establishing a protein interaction map centred on rhodopsin, we systematically investigated protein interactions from isolated photoreceptor outer segment discs.

Methods: We combined biochemical native separation methods with mass spectrometric identification of the isolated (super-) complexes and present here an interaction map of rhodopsin based on the analysis of protein complexes recovered in their native form.

Results: By mass spectrometry, 70 different proteins were identified, of which 52 were selected for further analysis. From these, 249 binary putative interactions could be defined mainly by analyzing co-migration within separated protein complexes. A total of 148 of these interactions have been reported to date, of which 100 were confirmed, while 48 were not detected by our approach. Out of all interactions, 101 from our dataset are putatively novel. The authenticity of isolated protein interactions was independently cross-validated by either data-mining or by a second alternative native separation method. Four novel candidates were used as baits in coimmunoprecipitation assays, leading to detection of more than 20 interactions confirming the comigration data. The dataset suggests that in addition to its role as a light transducing GPCR, rhodopsin plays a role in regulating photoreceptor structure and polarity and is likely to orchestrate a biological network with diverse functional diversifications.

Conclusion: By using the genome wide protein interaction data deposited in the MINT database we have extended the protein network obtained by our approach thereby gaining information on the topology of the complexes. This analysis has also offered a view of the peripheral links between the main rhodopsin pathway and the rest of the cell functional pathways.

Keywords: rhodopsin, protein interaction network, native fractionation, systems biology

RPGR mutation g.ORF15+650delAG causes retinitis pigmentosa with largely variable manifestation in females in a large German family

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Purpose: To identify the genetic defect in a large German family with retinitis pigmentosa (RP) in two subsequent generations.

Methods: DNA samples of family members were genotyped with polymorphic microsatellite markers specific for the twelve known loci for autosomal dominant RP (adRP) or by direct sequencing of the respective genes. A genome-wide linkage scan was performed. The coding region of the retinitis pigmentosa GTPase regulator gene (*RPGR*) was directly sequenced. The pattern of X-chromosome inactivation was defined by examination of methylation at the androgen receptor (AR) locus (genomic DNA from blood leukocytes).

Results: There was no obvious difference in disease severity between affected males and females, suggesting autosomal dominant inheritance. All 12 adRP loci known at the time of the study were excluded by linkage analysis. An 11.5 Mb region on the X chromosome comprising the *RPGR* gene segregated with the disease phenotype in the family. Sequence analysis of the *RPGR* gene revealed an exon ORF15 mutation (g.ORF15+650delAG) in all patients and heterozygotes. Remarkably, two of the carriers have typical RP although the onset was delayed (initial symptoms at age of 28 and 32 years), whereas two other carriers (age: 28 and 50 years) had no subjective visual complaints. X chromosome inactivation was shown to be random in all carriers.

Conclusions: The same *RPGR* mutation causes severe retinal degeneration in males and mild to severe ocular symptoms in female carriers in this family. Skewed X chromosome inactivation as an explanation of the largely variable disease manifestation in females was not supported by our data. Full expression of disease symptoms in females in case of an *RPGR* mutation may lead to a pitfall in the molecular analysis and genetic counselling. Thus, *RPGR* mutations should be excluded in families that seem to fit an autosomal dominant pattern of RP but lack male-to-male transmission.

Retinal degeneration in 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 retinitis pigmentosa (RP) due to a mutation in the gene encoding the retinitis pigmentosa GTPase regulator (*Rpgr*).

Methods: The mutation was bred into a pigmented (C57BL/6) and an albino (BALB/c) mouse strain. Functional and histological studies were accomplished by electroretinography (ERG) and light microscopy of retinal sections, respectively. In addition, localization of different *Rpgr* isoforms in wild type and mutant retinae was performed by immunostaining.

Results: Phenotypic changes in retinal function and morphology were apparent on both genetic backgrounds (C57BL/6 and BALB/c). However, disease progression was found to be different. In the BL/6 background, hemizygous mutant male mice showed a progressive decrease of the rod ERG amplitudes and loss of photoreceptor cells beginning at the age of 6 month. In contrast, the degenerative processes in the BALB/c background started as early as 1 month of age. In this strain the cone system was found to be affected first by a significant reduction of ERG b-wave amplitudes followed by rod photoreceptor dysfunction. Additionally, photoreceptor cell loss was more pronounced. There was no evidence of mislocalization of mutant *Rpgr* isoforms by immunofluorescence labelling.

Conclusions: Here we provide evidence that the genetic background has an impact on the progression of photoreceptor degeneration. Furthermore, our mouse mutant provides a potential model for studies of RPGR function in rods and cones, mechanisms of pathophysiology and finally for therapeutic approaches.

Investigation of the complement system in AMD pathogenesis. Outline of a project.

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Background: There is evidence from epidemiological studies that genetic factors play a substantial role in the etiology of AMD and associated macular characteristics, explaining about two thirds of the variation in the overall severity of the disease. The role of inflammatory processes in AMD pathogenesis recently gained new support by studies in molecular genetics, immunohistochemistry and cell biology. Combined analysis of the complement factor H (CFH) variants and factor B (BF) haplotypes showed that variation in the two loci can predict the clinical outcome in 74% of AMD patients and 56% of controls. Both factors play a pivotal role in the regulation of the alternative complement pathway activation. However, it has not been shown so far if the two variants of the CFH protein are different in biological function. The correlation between CFH polymorphism and blood concentrations of complement factors has not been investigated so far. Likewise, the impact of factors such as C3a and C5a on retinal pigment epithelial (RPE-) cells as well as the regulation of CFH-expression has not been determined.

Hypotheses: The Y402H CFH polymorphism may result in decreased activity of the protein. This results in measurable changes of complement factors in the blood that may reflect disease activity.
Proteins of the complement cascade have a regulatory effect on RPE cells.
The expression of CFH by RPE cells can be regulated.

Methods:
Functional characterisation of the two polymorphic CFH variants:
Purification of the two different CFH proteins from homozygous donors by immunoaffinity chromatography and in vitro characterisation of their regulatory activity.

Clinical study: AMD patients and matched controls will be phenotyped by established procedures (CRFs and SOPs), their blood samples will be genotyped and their serum samples will be tested for activation products of the complement system.

Cell culture: Cultured RPE cell lines will be incubated with complement factors C3a or C5a, triamcinolone or VEGF. The impact on phagocytose activity, complement receptor- or CFH-expression will be measured using flow cytometry, molecular biology and protein assays.

Systematic mutational screening of RPGRIP1 in glaucoma patients

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PURPOSE: Glaucoma is a clinically and genetically heterogeneous group of ophthalmologic disorders leading to visual impairment and a major cause of blindness worldwide. The most common form is primary open angle glaucoma (POAG), characterised in most cases by late onset and elevated intraocular pressure. In about half of the cases familial aggregation with autosomal dominant inheritance with reduced penetrance is observed. Several loci have been linked to POAG, but until now only 3 genes have been identified, MYOC, OPTN and WDR36, accounting for about 7% of the cases.
The aim of our study is to identify new glaucoma predisposing genes in previously described POAG loci. We report here the screening results on chromosome 14q11.

METHODS: Direct sequencing of exons, flanking intronic regions and 5'-3' UTRs of 10 candidate genes mapping to 14q11 (ZNF219, RPGRIP1, SALL2, DAD1, OXA1L, MMP14, BCL2L2, NRL, ISGF3G and ADCY4) was performed on genomic DNA samples from 46 unrelated German POAG patients. RPGRIP1 was further screened in a collective of altogether 307 patients. All novel variations found were resequenced in 96 controls.
RT-PCR was performed in human eye tissues (retina, sclera, choroids and cornea) and blood.

RESULTS: 37 variations were found within the coding regions of the 10 candidate genes in the initial study. Further screening of RPGRIP1 lead us to identify 13 novel variations, not present in 96 controls, and affecting most of them evolutionary conserved amino acid positions among human, mouse, rat, cow and chimpanzee. Most of the variants are located in important functional domains of the corresponding protein.
Expression of RPGRIP1 was observed in retina, sclera and blood and potential new isoforms were detected by RT-PCR analysis.

CONCLUSION: RPGRIP1 was previously described as a gene causing Leber Congenital Amaurosis (LCA6) and Cone-rod Distrophy (CRD9). The results of our study suggest implication of RPGRIP1 also in glaucoma. It is both positional and functional candidate, with expression in different eye tissues. Novel variants found in the POAG patient collective will be further characterised.
We are currently performing familiar segregation studies, protein structural modelling of these variants and further screening of new POAG patients and controls, in order to establish the role of RPGRIP1 in the aetiology of POAG.

Identification of a mutation in the VMD2 gene in a patient with autosomal dominant vitreoretinochoroidopathy (ADVIRC)

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ADVIRC (OMIM 193220) is a rare autosomal dominant condition with characteristic retinal and vitreous findings. This fundus dystrophy has been first described by Kaufman et al. in 1982 (Arch Ophthalmol. 100(2):272-8, 1982). It is characterized by peripheral circumferential retinochoroidal dystrophy with a sharp demarcation towards the central normal trophic area and vitreal disorganization. Further findings are an early cataract formation, retinal vascular abnormalities and cystoid macular edema.
In 2004, Yardley et al. described mutations in the VMD2 gene in patients with defects of ocular development, including nanophthalmos and microcornea, and ADVIRC (Yardley et al. IOVS 45:3683-9, 2004). They suggested that these mutations lead on the one hand to missense substitutions and on the other hand also to an altered the splicing behaviour. These particular mutations are suspected to disturb the early ocular development.
Missense mutations in VMD2 are usually associated with the clinical picture of juvenile onset vitelliform macular dystrophy (Best disease, OMIM 153700) which is clinically distinguishable from ADVIRC. Best disease is characterized by large deposits of lipofuscin-like material in the subretinal space.
Here we report on a female patient with the clinical features of ADVIRC including circumferential peripheral retinal hyperpigmentation, punctuate retinal opacities, oedema of the maculae and cataract. Sequence analysis of the coding exons of VMD2 has revealed a c256G>A mutation in exon 4 that should lead to the amino acid exchange from valine to methionine at position 86 (Val86Met). The fact that we could not find nanophthalmos or other developmental eye abnormalities in our patient does not agree with this hypothesis by Yardley et al. so far. The availability of a lymphoblastoid cell line of the patient will facilitate the further analysis of the in vivo splicing behaviour involving exon 4. This will clarify the consequences of the c256G>A mutation on the protein level and will help to further delineate the genotype-phenotype correlation in VMD2.

Fundus autofluorescence pattern in atrophic age-related macular degeneration - Impact on progression and phenotype-genotype correlation

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Purpose: We recently identified distinct patterns of abnormal FAF in the junctional zone of geographic atrophy (GA) in age-related macular degeneration (AMD) (BJO 2005;89:874-8). Here, we investigated the impact of these FAF phenotypes on GA progression rates over time. To test the hypothesis that the classification reflects heterogeneity of underlying genotypes we screened for mutations in the ABCA4 gene.

Methods: In the prospective, multi-center natural history *FAM* study, standardized digital FAF images were recorded with a confocal scanning laser ophthalmoscope (cSLO). In 195 eyes from 129 patients GA areas were analysed with digital image analysis. The impact of distinct patterns of abnormal FAF in the junctional zone of GA on progression rate was evaluated. Mutations in the ABCA4 gene were analysed in patients with the FAF phenotype fine granular with peripheral punctate spots.

Results: Areas of GA showed a mean enlargement of 1,74±1,29mm^2/year. Eyes with "diffuse" and "banded" FAF pattern had a significantly higher mean rate of progression (1,91±1,12mm^2/year) compared to eyes with no FAF abnormalities or a "focal" pattern outside the GA (0,65±0,47 mm^2/year; p<0,0001). Mutations in at least one allel in the ABCA4 gene were identified in 100% (7/7) of the tested individuals with the particular FAF phenotype. Onset of visual impairment in these individuals was beyond 50 years of life.

Conclusions: Based on cSLO FAF phenotyping we identified specific patterns of FAF in the junctional zone of GA that are associated with high and low progression rates of GA, respectively. Consideration of high-risk characteristics and, thus, prognostic factors will be helpful to design future interventional trials in patients with atrophic AMD to slow down spread of atrophy and, therefore, prevent severe visual loss. A phenotype-genotype correlation was identified for a distinct FAF phenotype which obviously represents late-onset Stargardt macular dystrophy mimicking atrophic AMD. Further genetic analyses are ongoing in our cohort to evaluate the impact of specific genetic variants on phenotypic features of late atrophic AMD.

A proteomic approach to identify MPP4-associated proteins in the bovine retina

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Purpose: Membrane-associated guanylate kinases (MAGUKs) are typically characterized by several conserved protein domains which mediate the assembly and maintenance of large multiprotein complexes at specialized cellular compartments. We have recently identified MPP4 as a retina-specific MAGUK abundantly present in the photoreceptor synapse. In addition, MPP4 is a component of the non-motile primary cilium of rods and cones. To further elucidate the diverse functions of this molecule we aim at the identification of interacting proteins.

Methods: Bovine retinal extracts were subjected to immunoaffinity chromatography using a specific monoclonal MPP4 antibody covalently linked to sepharose beads. Bound proteins were separated by 1D- and 2D-gel-electrophoresis and identified by mass spectrometry. Putative binding partners were further analyzed by immunoprecipitation and GST-pull down assays.

Results: Several proteins were co-purified with MPP4 from the cytoplasmic fraction of bovine retina including various cytoskeleton proteins and components of the phototransduction cascade. Two prominent proteins of 25 and 100 kDa were repeatedly found in all preparations and were identified as Veli3 and PSD-95. Both proteins co-localize with MPP4 at the photoreceptor synaptic terminals in the OPL and interact with MPP4 by L27 domain heterodimerization.

Conclusions: Using a proteomic approach we have identified adaptor proteins Veli3 and PSD-95 as major binding partners of MPP4 at photoreceptor synaptic junctions. This indicates that MPP4 is part of a large protein network involved in synapse function and development.

Assessment of transcriptional inhibition by the photoreceptor-cell specific nuclear receptor (NR2E3) and of the effects of mutations leading to enhanced S-cone syndrome

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Purpose: To investigate functional consequence on NR2E3 transcriptional activity of frequent ESCS mutations localized in the ligand binding domain (LBD).

Methods: Point mutations were introduced into the LBD of full length and Gal4 chimeric NR2E3 receptor and transcriptional activity was investigated by using transient co-transfection assay on corresponding luciferase reporters. Expression and DNA binding properties of transfected mutant and wild-type receptors were tested by Western-blotting and gel-shift assay.

Results: This analysis show that two ESCS mutations, R385P and M407K, abolish NR2E3 repressive activity in the context of full-length and Gal4 chimeric receptors, while W234S and R311Q mutants retain their repressive activity in both assays. All mutant receptors retain their stability and DNA binding ability.

Conclusion: These results show that NR2E3 mutations localized in LBD induces ESCS disease without affecting inhibitory activity as recorded *in vitro*. This demonstrates the absence of correlation between transcriptional inhibition and ESCS phenotype. This analysis suggests that NR2E3 has other transcriptional properties yet not identified.

Glutamate uptake of Müller glial cells during simulated pathological conditions

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Purpose: Extracellular glutamate is mostly taken up by glial cells via high affinity Na^+-dependent glutamate transporters. The uptake capacity of these transporters is influenced by the resting membrane potential and by the extracellular K^+ ion concentration. Müller glial cells (MC) respond to pathological changes of the retina with distinct changes of their membrane properties (e.g. reduced inward K^+ conductance and depolarized resting membrane potentials). These MC changes should disturb the spatial buffering and may lead to increased extracellular K^+ concentrations and/or neurotoxic glutamate levels. Aim of the study was to investigate the influence of these pathological changes on the retinal glutamate uptake and glutamine release.

Methods: By means of HPLC analysis we evaluate the extracellular concentrations of glutamate and glutamine in the incubation solution of isolated retinas and MC from guinea pig eyes. The pathological conditions were experimentally simulated by blocking the K^+ inward conductance by Ba^+ or by increasing the extracellular K^+ concentration or both.

Results: Under control conditions retinas and MC did not release glutamate, but released continuously moderate amounts of glutamine. Additionally applied glutamate (100µM) were taken up by retinas as well MC and increased the glutamine release 3-4 fold. Increased K^+ concentrations or blocked inward K^+ conductances reduced the glutamate uptake into retinal tissue (by 10-15%) as well as into MC (by 20-30%). Application of Ba^{2+} and high K^+ simultanously revealed an additive effect of reducing the glutamate uptake of retinal tissue (by 20%) and isolated MC (by 40%). Furthermore, the glutamine release was reduced by 50-75% in retinas and by 40-70% in MC under these conditions. Blocking the glial glutamine synthetase by methionine sulfoximine reduced only the glutamate uptake by MC (by 40%), but not of the retinal tissue. Removal of the extracellular Na^+ ions reduced the glutamate uptake by only 30% in retinas and by 50% in MC.

Conclusion: The simulated pathological conditions reduce the capacities of retinal glial glutamate uptake and glutamine release. Therefore, the glutamate-glutamine cycle and the retinal signal transduction might be disturbed under such pathological conditions. Nevertheless, this reduction do not necessarily result in chronically increased concentration of extracellular glutamate, because there might be other transport systems (e.g. neuronal glutamate transporter, Na^+-independent glial transport system) to compensate the reduction

Differential Modification of Phosducin Protein in Degenerating rd1 Retina is Associated with Constitutively Active CaMKII in Rod Outer Segments

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Purpose: Retinitis pigmentosa (RP) comprises a heterogeneous group of incurable progressive blinding diseases with unknown pathogenic mechanisms. The rd1 mouse is an RP model that carries a mutation in a rod photoreceptor specific phosphodiesterase gene, leading to rapid degeneration of these cells. Elucidation of the molecular differences between rd1 and healthy retinae is crucial for explaining this degeneration and could assist in suggesting novel therapies.

Methods: We used high-resolution proteomics to compare the proteomes of the rd1 mouse retina and its congenic, wild-type, counterpart at postnatal day 11 (PN11), when photoreceptor death is profound. Over 3000 protein spots were consistently resolved by 2-dimensional gel electrophoresis and subjected to a rigorous filtering procedure, involving computer-based spot analyses. Significantly differentially expressed proteins were excised from gels and identified by mass spectrometry (MALDI-TOF). Results were confirmed by immunostaining on retinal sections.

Results: Five proteins were accepted as being differentially expressed in the rd1 model on PN11. The difference in one such protein, phosducin, related to an altered modification pattern in the rd1 retina rather than to changed expression levels. Additional experiments showed phosducin in healthy retinae to be highly phosphorylated in the dark but not in the light-adapted phase. In contrast, rd1 phosducin was highly phosphorylated irrespective of light status, indicating a dysfunctional rd1 light/dark response. The increased rd1 phosducin phosphorylation coincided with increased activation of calcium-calmodulin activated protein kinase II, known to utilize phosducin as a substrate.

Conclusion: Given the increased rod calcium levels present in the rd1 mutation, calcium-evoked over-activation of CaMKII may be an early and long sought for step in events leading to photoreceptor degeneration in the rd1 mouse.

Macular pigment density and distribution in patients with type II macular telangiectasia

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Purpose: To assess the macular pigment optical density (MPOD) and its topographic distribution patterns in type 2 macular telangiectasia (MT). In this rare disease of the parafoveal capillaries, it is unknown if the MPOD is reduced or if its distribution is changed. Therefore, MPOD was measured in patients with type 2 MT and its distribution was topographically correlated with abnormalities detected by other imaging methods.

Methods: 19 eyes of 10 patients (median age: 63 years; range 48 - 73 years) were included. Patients were examined by means of funduscopy, best-corrected visual acuity (ETDRS), fluorescein angiography, and optical coherence tomography (OCT3). MPOD measurements were performed using a modified confocal SLO (HRA, Heidelberg Engineering, Dossenheim, Germany). MPOD was calculated using the "two-wavelength-method" from fundus autofluorescence (FAF) images, which were acquired at two different excitation wavelengths (488nm, 514nm).

Results: All eyes exhibited the typical clinical features of type 2 MT with late hyperfluorescence temporal to the fovea without macular edema on fluorescein angiography. Visual acuity was reduced in all eyes (median 20/40; range: 20/200 - 20/25). MOPD distribution showed a consistent pattern in all patients with type 2 MT: in exact correspondence to the late hyperfluorescent areas temporal to the fovea, MPOD was significantly reduced.

Conclusions: There is good topographic correspondence between angiographically visible alterations and reduced MPOD in type 2 MT. These MPOD findings support the notion that in this disease there is apparent asymmetry in impairment between the nasal and the temporal parafovea. It remains to be determined why the temporal parafovea is primarily affected in this disease and by what mechanisms macular pigment is depleted in the inner neurosensory retinal layers in the presence of teleangiectatic retinal capillaries.

Differential regulation of Kir 4.1 and Kir2.1 expression in the ischemic rat retina

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Purpose: One of the important functions of Müller glial cells is K^+ clearance, a mechanism that involves the uptake of excess neuron-derived K^+ from the retinal extracellular space and release into the blood, vitreous, and subretinal space. The cells express a high density of different types of inward-rectifying K^+ (Kir) channels in their plasma membranes. Ischemia-reperfusion of the rat retina causes gliosis of Müller cells that is associated with a decrease of their K^+ conductance. However, it is unknown whether the glial expression of various subtypes of Kir channels is differentially regulated in the course of retinal ischemia-reperfusion. Here, we compared the expression of Kir4.1 and Kir2.1 in the post-ischemic retina.

Methods: Transient retinal ischemia was induced in one eye of adult Long-Evans rats (250-350 g) for 60 min by elevation of the intraocular pressure. At 7 days after reperfusion the animals were killed and the eyes were removed. The untreated eye served as control. Kir^+ channel mRNA expression was investigated by semi-quantitative real-time PCR. Protein expression was analyzed by Western bloting and immunohistochemical staining. Electrophysiological investigations were carried out with acutely isolated Müller cells using the Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA).

Results: In control retinas, Müller cells prominently expressed both Kir4.1 and Kir2.1 proteins. At 7 days after reperfusion, the expression of Kir4.1 protein was strongly downregulated, while the Kir2.1 protein expression remained unaltered. The expression of Kir4.1 mRNA was reduced by 55% after ischemia while the expression of Kir2.1 mRNA was not altered. Retinal ischemia-reperfusion caused a strong reduction of the whole-cell K^+ currents in Müller cells.

Conclusions: The expression of various Kir channel subtypes of Müller cells is differently regulated during ischemia-reperfusion. The downregulation of Kir4.1 may disturb retinal K^+ clearance and, therefore, may contribute to ischemic injury. The unaltered expression of Kir2.1 in postischemic retinas may favor K^+ accumulation within the Müller cells, resulting in water influx and cytotoxic edema.

Towards the function of *AIPL1* splice variants

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Purpose: *AIPL1* is the fourth gene involved in Leber Congenital Amaurosis, the most frequent cause of congenital blindness in children, due to very early onset retinal degeneration. To elucidate the still vague role of *AIPL1* in photoreceptor viability we are investigating gene expression on the transcriptional and translational level.

Methods: Full length transcripts of *AIPL1* were amplified by RT-PCR from human and mammalian neuroretina. Samples were obtained from male and female human donors aged 20 to 90 years, who underwent surgery for trauma of orbital bones or extended facial bone tumors. RT-PCR products were subcloned and sequencing confirmed the completeness of the plasmid inserts. Evolutionary conservation of splice variations was tested by RT-PCR using retinal RNA preparations from other mammals.

Results: 6 different human transcripts of *AIPL1* were identified at various amounts by RT-PCR. The major variant corresponded to the full-length transcript. The minor variants were present in approximately 20% of the gene's transcripts and lacked either the entire exons 2 or 3 or the first parts of exons 2 and/or 5 respectively. Examination of *AIPL1* transcription in several mammals demonstrated alternative splicing in all those species with both splice variants either identical to or different from human variants.

Conclusions: The identified splice variants present transcriptional in-frame deletions of the protein coding region, interestingly affecting important functions like the peptidyl-prolyl-isomerase domain and the binding site of cell cycle regulator NUB1. We expect the transcriptional modifications to tune the respective AIPL1 isoforms for individual functions, some of those being conserved throughout evolution, as human, pig, and rodents have the most frequent splice variants in common.
To further clarify the role of alternative splicing in *AIPL1* expression, protein interaction studies and evaluation of isoform-specific cellular expression patterns are underway.

Long-term follow-up of AAV-mediated gene replacement therapy in a mouse model for X-linked juvenile retinoschisis (RS)

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Objectives: The Rs1h-deficient mouse reveals several features characteristic of human RS, thus representing an ideal model to test the potential of AAV-mediated gene replacement therapy for this condition. A construct containing the human RS1 cDNA under control of the mouse opsin promoter in the adeno-associated virus (AAV) serotype 5 was injected into the subretinal space of one of the eyes of 15 day old knockout mice. The uninjected eyes served as internal controls. With the present follow up study we report on the long term efficiency (over 20 months post-injection) of this therapeutical approach.

Materials and Methods: Scotopic and photopic ERGs were performed at different time points. Synaptic plasticity of rod and cone pathways in treated and untreated eyes were analyzed by immunolabeling techniques and electron microscopy (EM).

Results: ERG measurements showed nearly normal b-wave amplitudes in injected eyes suggesting preservation of retinal function for up to 20 month post-injection. Untreated control eyes already completely lacked b-wave responses by the age of 12 month. Consistent with these data immunohistochemistry with several retinal markers revealed intact OFF cone and rod bipolar cells in 20 month old RS1-AAV treated eyes. In the treated eyes, EM shows intact but slightly abnormal morphology of both cone pedicles and rod spherules. Control eyes presented with severe disorganisation and disintegration of bipolar cells with an overall destruction of integrity of photoreceptors and its synapses.

Conclusions: Our study demonstrates that AAV-mediated gene replacement therapy can restore retinal tissue integrity and visual function to nearly normal levels for almost the entire life span of the RS1-deficient mouse. This is a first report on the efficacy of gene therapy during the physiological life span of a treated mouse model.

Identification of mutations in the CRX gene in cone- and cone-rod-dystrophy

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Purpose: Cone and cone-rod dystrophies are a genetically and clinically heterogenic group of retinal dystrophies. Only few reports exist describing the causative mutations in this disorder. Here we describe the identification of gene mutations in patients with cone (CD) and cone-rod dystrophy (CRD) whose detailed phenotype has been established using clinical and electrophysiological methods.

Patients and Methods: DNA of 24 unrelated patients with cone or cone-rod dystrophies was screened for mutations in the cone rod homeobox gene (CRX) by PCR of the three coding exons including exon/intron boundaries and subsequent DNA sequencing. DNA of family members of an affected individual was analysed by mutation specific RFLPs of the mutation-bearing PCR-amplified exon. Results were assured by DNA sequencing. All patients underwent a detailed ophthalmological examination and electrophysiological testing including visual acuity, color vision, visual field testing, dark adaptation and ISCEV-ERG.

Results: A novel frame-shift mutation in exon 4 of the CRX gene leading to a premature stop codon predicted to cause a truncation of the CRX polypeptide was found in one family with cone dystrophy. The predicted protein truncation abolishes the important transactivation domain of the transcription factor, situated in the otx-tail. Family carriership analysis confirmed segregation of the mutation with the disease phenotype within the family, but also identified additional mutation carriers. Detailed clinical phenotyping of available family members revealed that all mutation carriers showed clinical signs of CD. Yet affection varied from severe visual disturbances with morphological and functional changes to minor disturbances with no significant visual impairment but reduced cone ERG.

Conclusion: We identified a novel disease causing mutation in the CRX gene. In one out of 24 (4%) patients with CD/CRD a mutation in the CRX gene was found. This supports high genotypic heterogeneity in this group of disorders and emphasises the necessity to identify novel / additional genes for CD/CRD.

Identification of the genetic defect in the original Wagner syndrome family.

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Purpose: The aim of this study was to determine the genetic defect in Wagner syndrome, a rare autosomal dominant disorder belonging to the group of hereditary vitreoretinal degenerations. This disease had been genetically mapped to chromosome 5q14.3.

Methods: The progeny of the original pedigree described by Wagner in 1938 was subjected to DNA sequence analysis of *EDIL3* and *CSPG2* exons and their immediate flanking intron sequences. *CSPG2* transcripts were analyzed by RT-PCR.

Results: While no alterations were detected in *EDIL3* several changes were identified in the *CSPG2* gene. Only one of these, a heterozygous G to A substitution of the first nucleotide in intron 8, co-segregates with the disease phenotype. This change disrupts the highly conserved GT splice donor sequence. In blood cells of an index patient we found three different *CSPG2* transcripts: as expected, one of them has a normally spliced exon 8/9 junction while the others lack either the entire exon 8 or only the last 21 bps of exon 8.

Conclusions: *CSPG2* encodes versican, a large extracellular matrix proteoglycan, which is expressed as four tissue-specific variants distinguishable by the presence or absence of exons 7 and/or 8. We hypothesize that, in the vitreous, the splice defect results in haploinsufficency of V0 and V1 variant and ectopic expression of the V2 variant, thereby affecting interactions with other extracellular matrix components and consequently disturbing the ultrastructural organisation of the vitreous gel. This may cause accelerated liquifaction of the vitreous and rendering the physiological properties to the observed pathology of vitreoretinopathy. Tests to verify our hypothesis and investigate the role of the two aberrant *CSPG2* transcripts are in progress.

Electrophysiological characterization of mutant CNGA3 channels associated with achromatopsia

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Purpose: Mutations in the *CNGA3* gene have been associated with complete and incomplete forms of autosomal recessive achromatopsia, a disorder that is characterized by reduced visual acuity, lack of color discrimination, photophobia and nystagmus. *CNGA3* encodes the A-subunit of the cone cyclic nucleotide-gated (CNG) channel. The CNG channel is an essential component of the phototransduction cascade that generates the membrane hyperpolarization signal upon light stimulation. The objective of this study was the electrophysiological analysis of mutant CNGA3 channels in a heterologous expression system.

Methods: Mutant CNGA3 expression constructs based on the pcDNA3.1 vector were generated by means of in vitro mutagenesis using a wildtype full-length cDNA construct as template. Purified plasmid DNA was transiently expressed in HEK293 cells and the function of the resulting channels analyzed in membrane patches with inside-out configuration. Dose-response curves for the cGMP- and cAMP-activation of mutant channels were established and the primary biophysical parameters such as the ligand concentration of half-maximal activation ($K_{1/2}$) and the hill coefficient (h) were determined and compared to the reference values of wildtype channels.

Results: A3-channels with the point mutations R427C and R563C were analyzed as homooligomers and also as heterooligomers with the wildtype B-subunit present in native channels. In general, maximum currents were profoundly reduced in mutant channels, in homooligomers more so than in heterooligomers. Overall, the R563C mutant showed $K_{1/2}$ and h values for the activation by cGMP which are essentially the same as for wildtype channels while the cGMP-sensitivity of homooligomeric channels with the mutation R427C was increased by a factor of 10 and cAMP maximum currents were quadrupled. These effects were almost completely abrogated when coexpressing $A3_{R427C}$ with B-subunits, which supports the hypothesis that the B-subunit in some cases partially compensates for the negative impact of an A3 mutation on the functionality of the channel (rescue effect).

Conclusion: The CNGA3 mutations R427C and R563C have been found to occur only in patients with incomplete achromatopsia or patients who have not been classified yet, which is consistent with the observed functionality of heterologously expressed channels with these mutations. The observed, significantly reduced maximum currents could be due to a decrease of channel density in the membrane which might also explain the pathogenic effect of these mutations.

Subcellular localization of bestrophin encoded by vitelliform macular dystrophy gene *VMD2*

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Introduction: Vitelliform macular dystrophy also known as Best macular dystrophy (BMD) is an autosomal dominant disorder characterized by typical egg-yolk macular lesions of retina, decrease of light peak in the electro-oculogram (EOG), and juvenile age of onset. Progressive macular degeneration in patients with vitelliform macular dystrophy is associated with abnormal accumulation of lipofuscin-like material in retinal pigment epithelium (RPE) cells. The *VMD2* gene encodes bestrophin-1 and is expressed in RPE cells. Bestrophin-1 has been proposed to function as chloride channel or to have regulatory function on voltage-dependent Ca^{2+} channels. In this study, we investigated the expression and localization of endogenous and heterologously expressed bestrophin.

Methods: Human RPE cells were isolated from adult donor eyes. After removing the vitreous and retina the RPE cells were carefully brushed off and grown in cell culture. These cells were subsequently used for immunocytochemistry. As a control we used HEK293 and CHO cells transiently transfected with a bestrophin-eGFP fusion construct. Additionally, we have used the human RPE cell line ARPE-19. Furthermore, western blots were performed with equal amounts of total cell lysates and protein extracts from different cell compartments. These protein extracts we obtained from freshly isolated RPE cells of adult human donor eyes. Western blot and immunocytochemistry were performed with different bestrophin-specific antibodies.

Results: The subcellular distribution of heterologously expressed human bestrophin was determined using immunofluorescence microscopy. The human bestrophin-eGFP construct was detected within cytoplasmic vesicles of HEK293, CHO and in ARPE-19 cells as well. Immunocytochemistry on primary RPE cell cultures revealed comparable subcellular expression pattern. Transient transfection of HEK293 and CHO cells with mouse and human bestrophin and subsequent western blot analysis support the data obtained from immunocytochemistry by showing that bestrophin is expressed in a fraction containing cell organelles.

Conclusions: The results of this study show that large amounts of mouse or human bestrophin-1 protein are localized in fractions containing cell organelles.
The additional localization of bestrophin-1 in cytoplasm implicates that bestrophin-1 might provide a regulatory pathway between cell organelles and the cell membrane.

Detection of HNE- and MDA- modifications on lysosomal cathepsins in human retinal pigment epithelial cells

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Purpose: A hallmark of early-stage age-related macular degeneration (AMD) is the progressive accumulation of abnormal material in the retinal pigment epithelium (RPE), finally forming lipofuscin granules and drusen. These intra- and extracellular deposits are thought to result from incompletely digested material secondary to impaired lysosomal capacity. Here we study the effect of the lipid peroxidation products 4-hydroxynonenal (HNE) and malondialdehyde (MDA) on RPE lysosomal enzyme activities.

Methods: Pure intact lysosomes were isolated from primary human RPE cells. Following incubation with HNE and MDA, activities of the lysosomal proteases cathepsin B and L were analysed in isolated lysosomes as well as in cultured cells. In parallel, lysosomal proteins of HNE- and MDA-treated cells were separated by 2D-gelelectrophoresis. Western blotting was utilised to detect cathepsin B and L as well as HNE- and MDA-modified proteins. Matching of the Western blots to the protein gel resulted in the identification of spots representing HNE- and MDA-modified cathepsin B and L. Tryptic peptides obtained from these spots were further characterised by mass spectrometry.

Results: Treatment of isolated lysosomes with concentrations of 1 µM HNE or MDA resulted in an inhibition of both cathepsin B and L activities by about 90%. Likewise, HNE- and MDA-treated cultured RPE cells demonstrated reduced cathepsin activities. Mass spectrometric analysis of cathepsin B and L isolated from these cells detected covalent HNE- and MDA-modifications of critical active site residues in both proteases.

Conclusion: Products of lipidperoxidation like HNE and MDA cause covalent modifications in the active centre of RPE lysosomal cathepsins. This may result in the observed inhibitory effect of lipidperoxidation products on lysosomal proteolytic activities and may contribute to RPE lysosomal dysfuntion in early-stage AMD.

Expression of Gas6 protein and the anti-coagulant factor, protein S, in the rat retina

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The shedding allows the daily renewal of the retina photoreceptor outer segments in a diurnal cycle. A null mutation in the MER gene coding for the receptor tyrosine kinase Mer is responsible, in both RCS Rats and Humans, of defects in photoreceptor outer segments phagocytosis leading to retina degeneration and subsequently to blindness. Mer is a member of the Axl/Mer/Tyro3 subfamily of tyrosine kinase receptors whose major ligand is Gas6 (Growth-Arrest-Specific-Protein 6). Gas6 is the structural homologue of the anticoagulant factor, protein S. Recent studies showed that Gas6 and Protein S play a major role in the activation of the phagocytosis of photoreceptor outer segments by the retinal pigment epithelium. However, the source of Gas6 in the retina has not been documented. We have used immunohistochemistry, real time PCR, in situ hybridation to localize either Gas6 or protein S signals in rat retina.
Our on going immunohistochemistry experiments demonstrate the presence of both Gas6 and protein S signals in retinal Müller cells and in photoreceptor inner segments. We further investigated possible regulation of the Gas6 and protein S signals in the retina by various parameters. Gas6 and protein S signals seem to be regulated by the nycthemeral rhythm, light exposure and mechanical lesions. Blood samples measurements for coagulations factors, did not show a significant variation by the nychtemeral rhythm. Northern blotting and Real time PCR analysis, revealed the presence of Gas6 mRNA signal in the retina. However, attempts to localize Gas6 mRNA by in situ hybridization have so far remained inconclusive possibly because Gas6 mRNA levels in the retina were below to the detection limit of our in situ hybridation procedure.

In conclusion, our work revealed the presence of both Gas6 and protein S proteins in the retina. The source of these two proteins in the retina as well as the relation between their localization within the retina (Müller cells and photoreceptor inner segments) and their putative function (regulation of photoreceptors outer segments phagocytosis by the retinal pigment epithelium) remain to be investigated.

Mutation in Factor H (H402Y) associated with age related macular degeneration (AMD) results in reduced cell binding activities

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PURPOSE: Age related macular degeneration is the most frequent cause of irreversible blindness in developed countries. Recently several groups reported that the mutation H402Y in the complement Factor H gene predisposes for the development of soft drusen and advanced age related macula degeneration (AMD). However, at present it is unclear if this mutation results in functional changes of Factor H that contribute to the occurrence of drusen and the progression to AMD.

METHODS: In order to define a molecular role of Factor H in AMD we purified Factor H from plasma of AMD patients and control persons with the genomic subtypes: homozygous H402, homozygous Y402 and heterozygous H/Y402. Functional tests were performed to compare these Factor H subtypes.

RESULTS: While C3b binding was similar to all Factor H proteins, the risk phenotyp H402 showed significantly reduced cell binding activities as compared to the other two subtypes Y402 and H/Y402.

CONCLUSION: Thus the H402 variant may lead to decreased binding of Factor H to the cell surface of the choriocapillaries, the retinal epithelial cells and the drusen. As a consequence reduced local protection results in local complement activation and deposition of complement activation products.

Effects of a missense mutation in the splicing factor hPrp4 on its biochemical activity

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RP mutations in pre-mRNA splicing factors do not conform with the presumption that only alterations in genes with retina-specific function cause RP. Instead, the RP-linked splicing factors hPrp3, hPrp8 and hPrp31 are ubiquitous and essential to all tissues. It remains to be shown how mutations in splicing factors evoke cause the retinal degenerationspecific RP symptoms.

Purpose: To resolve the tissue-specificity paradox, we have begun started to characterize the biochemical effects of RP mutations in splicing factors. Genetic screening for other RP-linked mutations in splicing factors is carried out in parallel. In zebrafish, we want intend to evaluate the effects of protein insufficiency on eye development.

Methods: Splicing factors carrying RP mutations are tested in vitro for binding to their interactors. In addition, in vivo the integration of mutated proteins into spliceosomal subunits in vivo is analyzed by immunoprecipitation from cell lysates. Recombinant proteins are challenged investigated for their effect on splicing in cellular extracts. Morpholino oligonucleotides are injected into zebrafish embryos to reduce the levels of individual RP-linked splicing factors and to examine eye development.

Results: In a 65-year-old female RP patient, we identified a novel heterozygous mutation in the splicing factor hPrp4 that substitutes a residue conserved across species. A heterozygote daughter of this patient, however, does not show any signs of retinal degeneration. This phenomenon recalls the incomplete penetrance of splicing factor mutations observed in some RP families. The hPrp4 mutation abrogates prevents binding of hPrp4 to its interactor hPrp3 in vitro whereas binding to Snu-Cyp20, a second interactor, remained unaffected. Loss of the hPrp4-hPrp3-interaction was further demonstrated by lack of co-immunoprecipitation from cell lysates. Moreover, the hPrp4 mutant was not co-immunoprecipitated with hPrp31 (not a direct interactor, but, as hPrp4, a member of the tri-snRNP subunit), indicating an impaired uptake of hPrp4 into spliceosomal subunits. Reduction of the Prp4 paralogue in zebrafish leads to defective development of the eyes.

Conclusion: A Mmutation of hPrp4 identified in an RP family patient specifically impairs its protein binding and its integration into the tri-snRNP subunit of the spliceosome. Since mutant hPrp4 also does not confer a trans-dominant negative effect on splicing in vitro, these effects appear to result from loss of the protein's function. High sensitivity of the zebrafish eye to Prp4 reduction supports this view.

Binding properties of retinoschisin, a secreted protein defective in X-linked juvenile retinoschisis (RS)

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Purpose: X-linked juvenile retinoschisis (RS) is caused by mutations in the RS1 gene which encodes a protein termed retinoschisin. Retinoschisin is a 24 kDa protein that is secreted from photoreceptor and bipolar cells as a large disulfide-linked multisubunit complex. The monomer consists of a leader sequence with a putative signal peptidase cleavage site and a discoidin domain spanning over 80% of the protein. Functional properties of the discoidin domain are not well understood although in some proteins the discoidin domain has been implicated in cell-adhesion and cell signalling processes through protein-protein, protein-carbohydrate, or protein-lipid interactions. In RS, deficiency of retinoschisin has been established as the underlying disease mechanism, however little is known about the molecular events leading to the degenerative manifestation in the neural retina. In this study, we focused on the binding mechanism of retinoschisin to the outer membrane surface.

Methods: Tissues including lung, brain, liver, heart, kidney and retina were isolated from 3-week old Rs1h-deficient mice, homogenised and tested for binding to recombinant RS1. Soluble and membrane bound fractions were assessed by SDS-PAGE separation and Western blot analysis. The binding of recombinant RS1 to artificial phospholipid membranes (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine) and different types of Collagen (I-IV) was analysed by ELISA.

Results: Recombinant RS1 binds strongly to retinal membranes but not to membrane fractions of other tissues indicating that membrane binding of retinoschisin is tissue-specific. Furthermore, our data show that recombinant RS1 fails to bind to artificial phospholipid membranes of various compositions. Finally, no binding of recombinant RS1 to the collagens tested was evident.

Conclusion: Our data suggest that RS1 membrane binding is mediated by a thus far unknown binding partner specific to the retinal outer membrane surface.

Morphology and gene expression studies in different brain regions of wildtype and *Ndph* (Norrie disease pseudoglioma homolog) knockout mice.

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Purpose: To quantitatively assess mRNA expression of the ligand Norrin (*Ndph*) and its receptors Frizzled 4 (*Fzd4*) and LDL-related protein receptor 5 (*Lrp5*) in different brain regions of wild type and *Ndph* knockout mice and to correlate the respective transcript levels with morphological findings. This allows us to evaluate Norrin-dependent versus Norrin-independent *Fzd4/Lrp5* signalling in the brain.

Methods: Six brain regions (cerebellum, cortex, hippocampus, olfactory bulb, brain stem and pituitary) of 6 – 8 month old *Ndph* knockout and wild type mice were analysed. We used histological stainings, immunohistochemistry for the vascular marker collagen IV; Real Time RT-PCR (Taqman) for *Ndph, Fzd4, Lrp5* and three angiogenic factors *Itgb3, Tie1 and Vegfa*.

Results: *Ndph* expression levels were significantly different in various brain regions of wild type mice. In contrast, expression of the Norrin-receptors *Fzd4* and *Lrp5* was more or less evenly distributed in these brain areas. In comparison to *Fzd4, Lrp5* is about 10fold lower expressed. Transcript levels of both genes were not altered in brain regions of *Ndph* knockout mice. Furthermore, we quantified the number of cells in the cerebellum, where *Ndph* mRNA levels were highest and where degenerative changes had been described for *Fzd4-/-* mice from the age of 3 weeks onwards. However, in *Ndph* knockout mice we did not detect significant reduction of Purkinje and granule cells. We determined the transcript levels of three angiogenic factors (*Vegfa*, *Itgb3* and *Tie1*) and did not observe significant differences in the six brain regions. Also, blood vessel density seemed unaffected in cerebellum, cortex and hippocampus.

Conclusions: Our data suggest that there is no major effect of Norrin-deficiency on brain vasculature and cerebellar morphology until the age of 6 - 8 month and in contrast to retina, hypoxia does not develop in the brain.
Interestingly, degenerative and vascular changes have been described in *Fzd4-/-* mice in the cerebellum. Compared to our data, these changes are most likely consequences of Norrin-independent Frizzled4 signalling. This suggests the existence of additional Norrin signalling pathways in the brain to explain the mental retardation phenotype in Norrie disease patients.

Role of OSBP2 gene in age related macular degeneration

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Purpose: Allelic variants of several genes are increasingly recognized as susceptibility factors in the genesis of AMD. Owing to its metabolic characteristics, the macula is sensitive to oxidative damage and supplementation with antioxidants has been shown to be effective in slowing the progression of disease in a subset of AMD patients. The oxysterol-binding-protein2 (OSBP2) gene is expressed mainly in the retinal pigmented epithelium underlying the macular region. Its product specifically binds oxysterols whose cytotoxic effects may be involved in macular damage. We evaluated the presence of allelic variants of OSBP2 as a susceptibility gene in patients withAMD.

Methods: 24 patient with AMD had genetic analysis of OSBP2 gene and dosage of total cholesterol, HDL cholesterol, vitamin A and vitamin E. OSBP2 gene analysis was done with PCR, SSCP and sequencing.Vitamin A and E were analyzed with HPLC and total and HDL cholesterol by enzymatic methods.

Results: Total cholesterol was elevated in 66% of the patients, HDL cholesterol was reduced in 12%, vitamin A or vitamin E deficiency was not observed. OSBP2 gene analysis showed 8 allelic variants: two apparently non polymorphic exonic variants (c.232G>T and c.2133G>A), six polymorphic variants: two exonic (c.129A>G and c.870T>C), two intronic (IVS12-14insGCCACC and IVS13+69C>G) and two at 3' UTR (c.2876G>C and 3415A>G). 110 control subjects (220 chromosomes) aged 60 years or more were also analyzed.

Conclusions: Two non polymorphic exonic variants were observed: c.232G>T –> p.A78S and c.2133G>A –> p.S711S. The patient with c.232G>T –> p.A78S has high total cholesterol and a positive family history of low vision. Evaluation of the segregation of this variant with the disease is not feasible due to the family structure and to the late onset of the disease. However wildtype and mutated aminoacid have different chemical characteristics. Patient with c.2133G>A –> p.S711S has high cholesterol and no family history. This variant does not result in an aminoacid change but produces a binding site for protein SF2/ASF. This protein has a role in Nonsense Mediate Decay. Since OSBP2 has high level of expression only in the retina, fetal liver and testis, transcript analysis in these two patients could not be done. These data indicate a possible role for oxidative damage to the macula induced by oxysterols in the pathogenesis of AMD in a subset of patients.

Assessment of major age-telated macular degeneration susceptibility loci in age-related ophthalmic disorders

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Purpose: Two major AMD susceptibility loci have recently been identified as contributing significantly to the manifestation of AMD. We seek to assess the genetic influence of these loci in AMD and cataract cohorts derived from Northern Ireland.

Methods: 593 samples were investigated. Sporadic AMD cases (N=285), age-related cataract (N=166) and control cohort (N=142) were collected from the Retinal Clinic at the Department of Ophthalmology, Royal Group of Hospitals, Belfast. All AMD patients were graded in accordance with the criteria established by the Rotterdam study. Ascertainment of genotypic status was undertaken in all cohorts at the following SNP sites: *CFH* rs800292, rs3766404, rs1061170, rs203674 and at *LOC387715* rs10490924 using ABI validated SNP assays and an ABI 7900HT genetic analyser. Genetic influence and allelic interaction at both loci were assessed between cohorts using logistical regression.

Results: Preliminary statistical analysis suggests that the genetic influence of these loci on AMD manifestation are similar to those previously reported in other investigations. *LOC387715* would also appear to be associated with age-related cataract formation and additional samples are currently being assessed.

Conclusions: This study confirms that in Northern Ireland, as has been reported in other populations, *CFH* and *LOC387715* are major susceptibility loci associated with the manifestation of AMD. Logistical regression suggests that both contribute independently but additively to AMD progression. The reduced influence of *CFH* reported in relation to AMD is most likely because the control cohort used in this investigation is derived from the general population and not selected specifically to be free from any AMD disease symptoms. While *CFH* protein function and its role within the immune response is widely known, the specific contribution of the significant genotypic variation observed in terms of disease progression has yet to be elucidated. Nothing is known about the protein function of *LOC387715*, its role in AMD disease manifestation or its interaction, if any, with additional unidentified proteins or environmental risk factors that lead to the progression of age-related ophthalmic disease such as AMD or cataract.

SANS (USH1G) – a scaffold protein in the Usher interactome of photoreceptor cells

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Purpose: Human 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. The USH1G gene encodes the scaffold protein SANS (scaffold protein containing ankyrin repeats and SAM domain), which is composed of ankyrin repeats, a central domain and a sterile alpha motif which are capable of protein-protein interaction. The knowledge of protein function is a necessary prerequisite for development of founded therapeutic strategies. Since the precise cellular localization of a protein is necessary to determine protein function, we analyzed the expression and subcellular localization of SANS in the developing and maturated mouse retina. Furthermore, we searched for novel binding partners to specify SANS function.

Methods: Antibodies against SANS were generated, affinity purified and applied in Western blot analysis, immunoelectron microscopy and indirect immunocytochemistry. Tangential cryosections of photoreceptor cells were analyzed by Western blotting. Yeast-two-hybrid screens were performed to identify novel SANS interaction partners. Organotypic retina culture was treated with the microtubule destabilization drug thiabendazole.

Results: Pre-adsorption of antibodies with the antigen used for immunization revealed high specificity of the anti-SANS antibodies. Analysis of SANS expression showed gradual increase of protein expression during postnatal development and maturation the mouse retina. Immunocytochemistry revealed SANS localization at ribbon synapses and in the ciliary apparatus of photoreceptor cells. The subcellular distribution of SANS was confirmed by immunoelectron microscopy and Western blot analysis of tangential sections. Destabilization of microtubules by thiabendazole induced mislocalization of SANS in photoreceptor cells indicating a linkage of SANS to the microtubule cytoskeleton. Yeast-two-hybrid screens revealed the PDZ-protein whirlin as a novel binding partner of SANS which was confirmed by immunocytochemistry.

Conclusions: The USH1G protein SANS is an integrative component of the USH interactome. SANS supports the scaffolding features of the PDZ-protein harmonin in certain compartments of photoreceptor cells. We also provide first direct evidence that SANS links the USH

network to the microtubule cytoskeleton. The present study supports our hypothesis that defects of one component of the interactome will lead to dysfunction of the entire USH network and cause sensory degeneration.

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Ca^{2+} homeostasis in murine RPE cells and its influence on the light peak in the EOG

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In *VMD2*-deficient mice the light-peak in the DC-ERG shows larger amplitudes at low light-intensities compared to wild-type mice. This study aims to investigate the underlying mechanism of intracellular Ca^{2+}-signalling in freshly isolated murine RPE cells of *VMD2*-deficient and wild-type mice compared with the human ARPE-19 cell line. Therefore, RPE cells were isolated from murine eyes under Ca^{2+}-free conditions and held over night prior Ca2+ measurement to recover from isolation procedure. Intracellular free calcium ($[Ca^{2+}]_i$) was monitored using the Fura-2 Ca^{2+} imaging method. In ARPE-19 cells application of ATP led to a tri-phasic change in $[Ca^{2+}]_i$: an initial fast peak increase was followed by a slight transient decrease below resting $[Ca^{2+}]_i$ from which a sustained increase followed. Freshly isolated RPE cells showed completely different characteristics in response to ATP: a heterogeneous pattern of ATP responses caused by a possible functional differentiation could be seen. In those cells showing an increase in $[Ca^{2+}]_i$ after application of ATP, the responses had higher amplitudes in *VMD2*-deficient mice than in non-deficient mice. These results seem to support our theory in that Ca^{2+} plays an important role in signal transduction causing the light-peak. Furthermore, these data suggest that disturbances in Ca^{2+}-homeostasis might play an important role in the etiology of Best's disease.

Retinitis Pigmentosa Assessment Severity Scale (RPASS) for use in scientific analysis and classification of disease progression

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Keywords: 681 retinal degenerations: hereditary, 687 retinitis, 489 degenerations/dystrophies

Purpose: Retinitis Pigmentosa (RP) is a group of genetic disorders exhibiting wide phenotypic expression which makes classification for scientific evaluation difficult. The aim of this study is to establish a clinical assessment scale to objectively evaluate the disease state of patients with RP in Northern Ireland and subsequently correlate disease severity and progression with genotype.

Methods: 50 patients with a clinical diagnosis of RP, confirmed with ISCEV, were selected from the Northern Ireland Retinitis Pigmentosa Database. Each participant was assessed using LogMar distance acuity charts, Farnsworth Dichotomous test for colour blindness series D15 and Humphrey 24-2 for visual fields. The fundus was imaged using the Topcon TRC 50EX. A grading scale was constructed using a combination of subjective clinical findings such as optic disc pallor, extent of retinal pigmentary changes and vascular attenuation. Objective parameters such as visual acuity assessment, visual field loss and electrodiagnostics were also included. The clinical grading scale was then applied to the 50 selected patients and assessed for appropriateness in terms of reflecting clinical severity using the Statistical Package for Social Sciences.

Results: 34% of the participants had Autosomal Dominant RP, 34% had Autosomal Recessive RP, 4% had X-linked RP and 28% were sporadic. The fundus assessment scale demonstrated a strong correlation with the visual function scale of distance acuity and contrast sensitivity and when both scales were combined they correlated highly with disease duration. However the scale also illustrates the varying severity which exists between inheritance patterns when cross tabulated with genetic type. Higher mean assessment scores were recorded for patients who had X-linked RP in comparison to those who had similar disease durations with dominant, recessive or sporadic inheritance patterns.

Conclusion: Classification scales are difficult to apply to disorders such as RP because of the variety of manifestations of disease. However this method of applying a score to each clinical sign and objective test parameter, has allowed us to categorise this group of patients into a mild, moderate or severe set with reference to age and disease duration. This simplistic method of classifying RP can be used as a scale to assess disease progression over a period of time and could be used to inform genotypic-phenotypic studies. It is anticipated that this scale would form the basis of a phenotyping system that could be applied consistently by independent clinicians.

Alterations of Müller glial cells during diabetic retinopathy

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Purpose: Diabetic retinopathy is a leading cause for visual impairment and aquired blindness. The presence of a macular edema may be responsible for the decreased vision. Although vasogenic and cytotoxic mechanisms may contribute to edema formation, a swelling of retinal Müller glial cells seems to be involved in this process. We investigated whether osmotic swelling characteristics of Müller cells are altered in the course of experimental diabetes, and whether such changes are accompanied by alterations in K^+ channel expression.

Methods: Experimental diabetes was induced in Wistar rats by injection of streptozotocin. This resulted in an increase in the blood glucose to 28 mM (control: 5 mM). At 4 and 6 months after treatment, retinal tissue and isolated Müller cells were used for immunohistochemistry, fluorescence microscopy, and patch-clamp recordings.

Results: The immunoreactivity for glial fibrillary acidic protein, a marker for reactive gliosis, was clearly increased during diabetes. Müller cells from diabetic retinae displayed a downregulation of inward K^+ currents to 53% after 6 months. Moreover, transient voltage-dependent (A-type) currents could be recorded in more than half of the cells from diabetic retinae, but not in control cells. Similar effects had been demonstrated before in several other models for retinal diseases. Whereas the inwardly rectifying K^+ channel, Kir4.1, is enriched at the inner limiting membrane and in perivascular membrane domains of Müller cells in control retinae, this prominent expression was not found in diabetic retinae, which displayed a more even distribution of the Kir4.1 protein. This corresponded with an even distribution of the K^+ conductance observed during focal ejection of a solution with 50 mM KCl. Application of a hypotonic solution did not affect the somata of control Müller cells, which were selectively stained with a vital dye. However, after 6 months of diabetes, osmotic stress caused a swelling of Müller cells. Pharmacological data suggest, that arachidonic acid and oxidative stress may be involved in the induction of Müller cell swelling. The expression of the water channel aquaporin-4 was not strongly altered in diabetic retinae.

Conclusions: Experimental diabetes in rats induces alterations of the swelling characteristics under hypotonic challenge and a downregulation of functional Kir4.1 channels in Müller cells. It is suggested that these alterations may be implicated in the development of diabetic retinal edema.

Down-regulation of CREB and calpastatin correlates with increased calpain activity and photoreceptor cell death in the rd1 mouse

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Keywords: retinitis pigmentosa, calpain, calpastatin, calcium, CREB

Purpose: The rd1 mouse displays an inherited retinal degeneration which allows studies of the molecular mechanisms behind the blinding disease retinitis pigmentosa (RP). Activation of the calcium dependent protease calpain has been suggested to play an important role in cell death in various tissues, however, little is known about the expression and activity of calpain during inherited retinal degeneration. Calpain activity is regulated by its endogenous inhibitor calpastatin, calpastatin gene transcription in turn is controlled by cyclic AMP response element binding protein (CREB). Methods: Using micro-array techniques, transcript levels of CREB, calpastatin and of various calpain genes were analysed in the rd1 mouse and compared to its wild-type control. Expression of distinct calpain isoforms and calpastatin was investigated using immunofluorescence and immunoblot. Gene transcription and protein expression levels were compared to calpain activity using an enzymatic assay that allowed to monitor calpain activity at the cellular level. Finally, calpain activity was compared with terminal dUTP nick-end-labelling (TUNEL).

Results: We show here that the transcription and expression of the major calpain isoforms are unchanged in rd1 mouse retinae when compared to corresponding wild-type (wt) mice. However, CREB and calpastatin expression is reduced while calpain activity is drastically increased and co-localizes to a large extent with TUNEL staining. Calpain activity peaks at post-natal day 13, together with rd1 photoreceptor cell death. Calpain specific inhibitors decreased calpain activity on retinal sections *in situ*.

Conclusion: These results indicate that activation of calpains correlates with rd1 photoreceptor cell death, which raises the possibility to use calpain inhibitors to prevent or delay photoreceptor degeneration. The down-regulation of CREB might be an important hallmark in

Implication of the most frequent mutation in Bardet-Biedl syndrome in Spanish families

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PURPOSE: Bardet-Biedl syndrome (BBS) is a complex genetically heterogeneous disorder. BBS patients manifest a variable phenotype that it includes early retinal dystrophy, polydactily, mental delay, obesity and hipogonadism. Until now, nine BBS genes have been described (*BBS1-BBS9*), with evidence for at least one more gene. *BBS1* is the gene with major involvement in the disease. In some families, the BBS has been proposed as a triallelic inheritance trait, in which three mutant alleles in two genes are involved in the disorder.
In this study we analyze the implication of the commonest *BBS1* mutation, M390R (c.1169T>G), in a pool of Spanish BBS patients.

METHODS: The DNA of sixty eight Spanish patients and their relatives were recruited by the retinal dystrophy investigation Spanish network (EsRetNet), after informed consent was obtained. Among the 49 affected families, 26 were sporadic cases and the remaining families had at least two affected individuals. In eleven families consanguinity were proven.
To screen the common M390R mutation of *BBS1* gene in the patients and controls, we perform two restriction tests, in order to ensure the results, with the NlaIII and BspHI restriction enzymes. In both cases, 7 μL of the exon 12 PCR product was digested with each enzyme in the appropriate buffer at 37ºC overnight.
Segregation analyses of M390R mutation in homozygous patients were performed when the DNA from the patient's relatives was available.

RESULTS: Twenty three of sixty eight (33.8%) of the patients have at less one M390R mutation, being 47.8% of them homozygous. The M390R allele frequency was estimated in 25%. No healthy M390R homozygous relatives were found in the segregation analysis performed in three M390R homozygous families.
The M390R mutation was not found in 96 unrelated control chromosomes.

CONCLUSIONS: In our series of SBB patients M390R has an elevated incidence, as has been reported in other studies. Furthermore, it is implicated in the 33.8% of our patients and that is the hightest value reported to now.
In the analyzed families, homozygous M390R mutation has not been found in healthy relatives, so it appears to follow a mendelian inheritance model and do not participate in trialelism.

Amacrine cells expressing connexin 45 in the mouse retina

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Purpose: Electrical synapses, or gap junctions, are cell-cell contacts formed by two hemichannels, or connexons. Each connexon is composed of six proteins termed connexins (Cx). Cx45 has been shown to be expressed in seven types of bipolar cell (Maxeiner et al, 2005) and two bistratified ganglion cells (Schubert et al, 2005) in the mouse retina. It is known that some amacrine cell types express this connexin but their morphology and characteristics are unknown. In this study we wanted to classify the Cx45-expressing amacrine cells according to their horizontal and vertical stratification patterns, general morphology, dendritic field size, soma size, coupling patterns and neurotransmitter content.

Methods: Transgenic mice, in which EGFP is expressed under the control of the connexin 45 promotor, were used (Maxeiner et al, 2005).
EGFP-positive amacrine cells were filled with the tracer Neurobiotin. Intercellular spreading of Neurobiotin was visualized by incubation with streptavidin-indocarbocyanine-Cy3.
Analysis was done by confocal microscopy.

Results: Morphologically, two major groups of amacrine cells expressing Cx45 were found. One of them corresponds to the rod A17 cells which are located in the INL and GCL. The second type, a small wide-field amacrine cell (SWFAC), was found only in the INL. This study also shows that A17 cells present homologous tracer coupling patterns and the SWFAC type shows homologous and heterologous coupling patterns when injected with Neurobiotin. These tracer coupling patterns are totally absent in mice deficient in Cx45. Labelling with antibodies against neurotransmitter candidates showed that Cx45-expressing amacrine cells do not contain glycine or acetylcholine and are most likely GABAergic.

Conclusions: Two amacrine cell types express Cx45: A17 cells and a type of SWFAC. The rod A17 cells are homologously coupled with other rod A17 cells, most likely by homotypic gap junctions, and A17 cell somata are either located in the INL or displaced in the GCL. The SWFAC type is homologously coupled to other SWFACs and heterologously coupled to an unknown amacrine cell type. All amacrine cells expressing Cx45 are GABAergic neurons.

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Cx36/Cx45 heterotypic gap junctions in the rod pathway

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Purpose: The primary rod pathway in mammals contains gap junctions between AII amacrine cells and ON cone bipolar cells which relay the rod signal into the cone pathway under scotopic conditions. Two gap junctional proteins, connexin36 (Cx36) and connexin45 (Cx45), seem to play a crucial role in this pathway because lack of either protein leads to an impairment of visual transmission under scotopic conditions. To investigate whether these two connexins form heterotypic gap junctions between ON cone bipolar cells and AII amacrine cells we used a newly-developed anti-Cx45 antibody and studied its cellular and subcellular distribution in the mouse retina.

Methods: To test the specificity of the Cx45 antibody we performed western blotting with samples of mouse retina, heart, lens and recombinant fusion proteins of Cx45. We also compared the Cx45 immunolabeling of retinas from wildtype mice with retinas from a conditional Cx45-deficient mouse line (Cx45fl/fl:Nestin-Cre, Maxeiner *et al*., [2005]). A detailed distribution of Cx36- and Cx45-immunoreactivity (IR) was studied in bipolar cells and AII amacrine cells by double immunofluorescence combined with intracellular dye injections. Data were analyzed using confocal microscopy.

Results: On western blots the anti-Cx45 antibody, directed against part of the cytoplasmic loop of mCx45, detected a protein with a molecular mass of ~52 kDa in membrane samples of mouse heart and retina. Furthermore, the antibody strongly labeled a GST-Cx45loop fusion protein and revealed no cross-reactivity with lens connexins Cx46 and Cx50. In wildtype retinas strong Cx45-labeling was found in the ON sublamina of the IPL. This staining was strongly reduced in Cx45-deficient retinas. The combination of dye injection and Cx36/Cx45-double labeling experiments revealed that Cx45-IR is located within axon terminals of type 5 and 6 ON bipolar cells. At these sites Cx36-IR appeared attached to Cx45-IR, suggesting that Cx36 hemichannels provided by AII amacrine cells represent the coupling partner for Cx45 hemichannels on type 5 and 6 ON bipolar cells.

Conclusion: Since lack of either Cx45 or Cx36 disrupts the coupling of ON bipolar cells and AII amacrine cells, and the two connexins are localized in close proximity at the terminals of ON bipolar cells and AII amacrine cells, we believe that Cx45 and Cx36 form heterotypic gap junctions between these cells.

Melanosome movement in the retinal pigment epithelium requires a functional Rab27a, Myrip and MyoVIIa complex

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Introduction: Rab proteins are small GTPases that interact with cytoskeletal elements and influence the movement of intracellular vesicles. It has been proposed that in retinal pigment epithelium (RPE), Rab27a recruits Myrip, which then recruits MyosinVIIa for the movement of melanosomes into the apical processes. The role of this complex might have a crucial function in the physiology of the retina, supported by the fact that MyosinVIIa is mutated in Usher syndrome type 1B. However, the functionality of this complex has never been demonstrated and is the goal of the present study.

Materials and Methods: In order to understand the role of the tripartite complex, primary RPE cultures were transduced with adenovirus expressing GFP or RFP-tagged versions of Rab27a and Myrip, or specific mouse Myrip-RNAi. Time-lapse microscopy of melanosome dynamics was performed on RPE primary cultures isolated from Rab27a deficient (*ashen*), MyoVIIa deficient (*shaker-1*) and control littermate 15 day old mice.

Results: Primary cultures presented a characteristic phenotype, with small, hexagonal shape, and a heavily pigmented cytoplasm, while maintaining some polarization. The analysis of melanosome dynamics revealed a clear difference in the distance moved by individual melanosome between *ashen, shaker-1* and the induced absence of Myrip (around 30 µm), compared with control values (10 µm), also showing a greater fluctuation in their orientation. Kymograph analysis showed that in control RPE cells the melanosomes moved slowly, or not at all, having bursts of rapid movement whilst in any of the mutants the movement bursts are longer, resulting in a wider displacement. The results obtained for the Myrip-induced depletion with specific siRNAs are in perfect agreement with the ones obtained for the proposed partners, Rab27a and MyosinVIIa.

Conclusions: Knocking-down the expression of the linker protein, Myrip, leads to the same melanosome displacement phenotype as observed in *ashen* and *shaker-1*, indicating the functional requirement of Myrip for melanosome movement.

Oligomerisation properties of bestrophin and its family members

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Purpose: The VMD2 gene is mutated in Best vitelliform macular dystrophy (BMD), an early-onset autosomal dominant retinal disorder. VMD2, a member of the TM-RFP protein family, encodes bestrophin and has been suggested to form a Ca^{2+}-dependent chloride channel. With this study, we aim to identify the essential structural properties for channel formation of the TM-RFP proteins and to investigate the pathomechanism of VMD2 mutations associated with BMD.

Methods: The potential for homo-dimerisation of the human VMD2 transmembrane domains (TMD) was tested by the ToxR-two hybrid system (Langosch et al. J Mol Biol. 8;263(4):525-30, 1996).

Results: Applying the ToxR-assay, 3 of 6 TMDs of bestrophin were observed to contribute to oligomerisation. These TMDs with high dimerisation potential were further analysed by introducing patient related mutations. In several instances, significant differences between wild type and mutated TMDs were observed.

Conclusion: We have identified TMDs of bestrophin with high dimerisation potential. In addition, selected TMD bestrophin mutations known to occur in BMD reveal significant differences to the normal TMD sequences. This strongly suggests that an impaired oligomerisation of bestrophin may be part of the pathomechanism in this disorder.

Early steps in photoreceptor synaptogenesis: ribbon assembly from "Precursor Spheres"

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We have recently shown that the mature photoreceptor ribbon complex consists of two separate molecular compartments: the ribbon and the active zone/arciform density compartment; both are composed of a set of proteins of the cytomatrix at the active zone. The current concept of conventional chemical synapse formation suggests that "active zone precursor vesicles", which contain multiple active zone components, lead to the rapid formation of new synaptic sites. Here, we have tested this hypothesis for the formation of the photoreceptor ribbon complex.

We have examined with immunocytochemistry and laser-scanning microscopy the molecular assembly of the presynaptic ribbon complex, and with electron microscopy the ultrastructural appearance of the ribbon during photoreceptor synaptogenesis.

At birth, immunofluorescent aggregates of the proteins Bassoon, Piccolo, RIBEYE and RIM1 are present in the neuroblast layer of the postnatally developing retina. The protein aggregates are transported in a complex along the growing axons to the developing photoreceptor terminals, which form the outer plexiform layer at the end of the first postnatal week. In contrast to the ribbon proteins, the active zone/arciform density proteins Munc13-1, RIM2, ERC2/CAST1 and a Ca2+ channel alpha1-subunit are detected earliest at postnatal day 4 (P4), at the time when the first photoreceptor ribbon synapses form. Active zone/arciform density proteins colocalize with the ribbon proteins as soon as they are detectable. At P4, many of the differentiating photoreceptor terminals contain round, electron-dense profiles, which are surrounded by smaller, synaptic vesicle-like structures. Only rarely, ribbon-like structures are found at this stage. Serial sectioning and 3D-reconstruction shows that most of the electron-dense profiles are spheres with up to 200 nm in diameter. With increasing age a continual decrease in the number of spheres is accompanied by an increase in the number of ribbons.

From these data we conclude that the mature photoreceptor ribbon is formed from non-membranous "precursor spheres" which are transported separately from the proteins of the active zone/arciform density compartment to the future photoreceptor ribbon synaptic site. The 80 nm "active zone precursor vesicles", as described for conventional synapses, could not be identified so far in photoreceptor synaptogenesis.

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Cytoskeletal elements participate in light-dependent translocations of arrestin and transducin in photoreceptor cells – A study in organotypic retina cultures –

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Purpose: Arrestin and the visual G-protein transducin move between the inner and outer segment of the photoreceptor cell in a light-dependent way. Their compartmented distribution under the diverse light conditions is assumed to participate in long term light adaptation. The transport mechanisms and their regulation underlying these intracellular movements still remained largely elusive. In the present study, we investigated the light-dependent movements of transducin and arrestin in organotypic retina culture and evaluated their dependency on actin filaments and the microtubule cytoskeleton.

Methods: Maturated mouse retinas were cultured and kept under cyclic light. Cultures were treated with cytoskeletal drugs, namely with cytochalasin D or thiabendazole. The cytoskeleton was analyzed by light and electron microscopy. Light-dependent movements of transducin and arrestin were visualized by immunocytochemistry.

Results: Light-dependent movements of arrestin and transducin, described in vertebrate eyes were confirmed *ex vivo* in organotypic retina culture. Treatments with cytoskeletal drugs affected actin filaments and the microtubule system of photoreceptor cells. These treatments interfered with the translocation of arrestin and transducin from the outer segment to the inner segment or vice versa, respectively, during dark adaptation of photoreceptors. Furthermore, the translocation of arrestin was slowed during light adaptation after microtubule depolymerisation by thiabendazole treatments.

Conclusions: The physiological conditions of the organotypic retina culture are suitable for analysis of light-dependent molecular translocations in vertebrate photoreceptor cells. Our present study revealed that during dark adaptation, the translocation of arrestin and transducin is dependent on actin filaments as well as on the microtubule system of photoreceptor cells. In contrast, the molecular movements associated with the light adaptation are not fully dependent on both cytoskeletal systems. Our data strengthen the hypothesis that different mechanisms are responsible for the oppositional translocation of arrestin and transducin under different light conditions.

Keywords: molecular translocation, light adaptation, retina culture, cytoskeletal drugs

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Retinospheres derived from neonatal Gerbil retinae: towards tissue engineering of mammalian retina

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Introduction: Transplantation of fetal retina sheets including retinal pigmented epithelium (RPE) can recover sight (1). Especially the correct laminated 3d structure of the retina implant seems to be important for the integration and regeneration process. Previous work has shown that reaggregation of cells from the E6 chick retina leads to cellular spheres including constituents of all retinal layers (2). To analyse the reorganisation ability of mammalian retinal cells, a reaggregation system by using the gerbil retina (*Meriones unguiculatus*) were established to analyse cellular processes of rodent retinal histogenesis.

Materials and Methods: 3D-spheres derived from dissociated retinal cells of neonatal (P1-P3) gerbils were grown in control reaggregation medium (DMEM plus 10% fetal calf serum, 0,1% penicillin/streptomycin, 0,01‰ gentamycin) or in same medium conditioned by RPE-monolayers. Reaggregates were collected after 10-15 days in rotation culture, then the spheres were fixed (PFA 4%) and cryosectioned. Differentiation and reorganisation were documented immunohistochemically with antibodies against specific cell-markers.

Results and Discussion: Distinctive histotypic reorganisation occurred in 3D-spheres of mammalian retinal cells, whereby most major retinal cell-types could be identified. Formation of an inner plexiform layer (IPL)-like matrix, and sorting out of amacrine and displaced amacrine cells in spheres grown in unconditioned medium was shown by the early differentiation marker calretinin. Moreover, signs of neuropil sublamination emerged. Advanced lamination occurred when spheres were grown in RPE-conditioned medium. Amacrine cells, Müller glia and horizontal cells were located inside in a correct but inverted manner. Photoreceptors occur just in spheres derived from advanced stages.

Conclusions: Dissociated mammalian retinal cells are able to form histotypic laminated reaggregates. In contrast to spheres derived from birds or rats, this in vitro study shows that retinal histogenesis is possible in absence of photoreceptors, whereby self-organisation first takes place at the level of the IPL. Thereby, diffusible factors derived from the RPE influence lamination. Predetermination of photoreceptor differentiation in the Gerbil retina starts two days after birth.

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Pals1/Mpp5 is required for correct localization of Crb1 at the sub-apical region in Müller glia cells

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Purpose: Mutations in the human Crumbs homologue-1 (*CRB1*) gene cause retinal diseases including Leber's congenital amaurosis (LCA) and retinitis pigmentosa 12 (RP12). In the retina, the CRB1 transmembrane protein localizes at the sub-apical region (SAR) of the outer limiting membrane (OLM), just above the intercellular adherens junctions between photoreceptor and Müller glia (MG) cells. *Crb1-/-* mice revealed that Crb1 is essential for maintenance of the adherens junction complex to prevent retinal disorganization. We study the mechanisms of CRB1-mediated retinal degeneration and focus on cell polarity and adhesion control proteins in photoreceptor and MG cells: the Crb-family proteins (Crb1, Crb2, Crb3), the MAGUK-family proteins (e.g. Mpp3, Mpp4, Mpp5/Pals1, Veli-3), the PDZ-motif containing proteins (Mupp1, Patj), adherens junction proteins, and others.

Methods: Retinas of 1.5-days-old wild type (WT) and *Crb1-/-* mice were isolated without RPE and maintained in culture up to 28 days in vitro (DIV). An *in vitro* electroporation method was used for both gain- and loss-of-function studies to introduce CMV-driven human CRB1 and to downregulate Pals1/Mpp5 using RNAi.

Results: Primary wild type (WT) retinas developed normal with all retinal layers formed but with underdeveloped photoreceptor outer segments. In *Crb1-/-* primary retina cultures, the *Crb1-/-* phenotype, which is characterized by OLM interruptions, upregulation of GFAP immunoreactivity in MG cells, formation of retinal folds, and photoreceptor half-rosettes and pseudorosettes, was accelerated and intensified. Electron microscopic immunohistochemistry showed strong Crb1 immunoreactivity at the SAR in MG cells but barely in photoreceptor cells. Reintroduction of CMV-driven CRB1 into *Crb1-/-* primary retinas resulted in human CRB1 localization at the SAR in MG cells. RNAi induced silencing of the Crb1 interacting protein Pals1 in MG cells, resulted in proportional loss of Crb1, Crb2, Mupp1, Veli-3 protein localization and a partial loss of localization of Crb3.

Conclusions: The phenotype of Crb1-/- mice is significantly accelerated and intensified in primary retina cultures. Pals1/Mpp5 is required for correct localization of Crb family members and its interactors at the SAR of MG cells.

Changes inAnalysis of Wnt/beta-catenin signaling in the retina of a mouse model for Norrie's disease.

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Purpose: To evaluate signaling differences in the Wnt/beta-catenin signaling pathway that may be involved in incomplete development of the retinal vasculature in a mouse model for Norrie's disease, a rare form of bilateral, congenital blindness.

Methods: Retinae of wild- type and Norriein disease pseudoglioma homolog (*Ndph*) knockout mice were collected at developmental stages p5 (postnatal day 5) 5, p10, p15 and p21. Signaling differences were studied on the transcriptional and on the protein level. GDifferential gene expression of the Wnt-regulated genes *Tcf1*, *Lef1*, *Tcf3* and *Bmp4* has beenwas determinedexamined by real-time PCR using SYBR-Green®. Expression differences on the pProtein levels of the Wnt signal mediator were studied by Western-Blot analyses for the Wnt signal mediator beta-catenin wasere studied by Western-Blot analyses. Subsequently, the TotalLab TL100 Software was used to quantify the blots.

Results: Protein analyses revealed no significant alterationsdifferences in beta-catenin levels through p5 – p21 comparingbetween wild type and *Ndph* Norrin knockout mice from p5 until p21. In accordance withto these findings, the transcript levels of *Tcf1*, *Lef1*, *Tcf3* and *Bmp4* didwere not show any significantly differencesaltered in knockout mice.

Conclusions: Since it has been shown recently that Ndphorrin is a high affinity ligand for Frizzled-4 (a receptor of the Wnt pathway) and can activate Wnt/beta-catenin dependent transcription in cell culture, it seems mandatory towe looked for differentially expressed Wnt/beta-catenin target genes in the (living organism) oder: affected tissue. Yet, our data suggest that Norrindph is not involved in the transcriptional regulation of *Tcf1*, *Lef1*, *Tcf3* and *Bmp4*. Still, it could be that Though, we cannot exclude a highly specific Norrindph-dependent signaling in a minority certainof cells that was masked by Wnt/beta-catenin signaling processes in other cells, because RNA and proteins were obtained from whole retina samples. Further cell type-specific studies might help to localize Norrindph dependent Wnt/beta-catenin signaling in the retina.

Hydroxyl radical induced Ca^{2+} response in the human Retinal Pigment Epithelium cell line ARPE 19

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Background: In RPE cells the second messenger Ca^{2+} is involved in the regulation of ROS phagocytosis, growth factor secretion, epithelial Cl-transport and apoptosis. Thus a disturbance of the Ca^{2+}-homeostasis in RPE cells may lead to serious functional impairments leading to cell death, and therefore participating in the genesis of age-related Macular Degeneration (AMD).

Methods: We used the human RPE cell line ARPE 19 to study the effects of extracellular application of OH^- radicals (Fenton) on $[Ca^{2+}]_i$. Intracellular Ca^{2+} was measured and calculated using the Fura/2 method and the Grynkiewicz formula. The presence of two potential Ca^{2+} transport systems, NCX 1 and L-type Ca^{2+}-channel, was examined with RT-PCR. Nifedipine, KB-R7943 and Thapsigargin were used to block L-type Ca^{2+}-channels, the reverse modus of NCX 1 and intracellular Ca^{2+}-stores, respectively. Additionally a life dead assay was performed to proof that we used sub-lethal and threshold concentrations of OH^- radicals.

Results: Extracellular application of sub-lethal concentrations of OH^- radicals resulted mostly in a monophasic transient $[Ca^{2+}]_i$ increase that peaked 150% higher as the baseline. This peak was still present under extracellular Ca^{2+}-free conditions and could partially be inhibited by adding 10µM of the L-type Ca^{2+}-channel blocker Nifedipine. Thapsigargin (2 µM) and KB-R7943 (10 µM) showed no effect on the initial Ca^{2+} increase. In about 20% of the experiments a second continuous $[Ca^{2+}]_i$ increase could be seen only when the extracellular Bath solution contained Ca^{2+}. This second increase could be blocked by Nifedipine and to some extent by KB-R7943, but not by Thapsigargin.

Conclusions: Our results suggest that a substantial part of the Ca^{2+} increase caused by OH^- is due to the activation of L-type Ca^{2+}-channels. The second sustained $[Ca^{2+}]_i$ increase was completely abolished by bathing the cells in Ca^{2+}-free solution and partially reduced by blocking the L-type Ca^{2+}-channel, thus indicating an additional Ca^{2+} transport system.

Antioxidant defences in LHON cybrids

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Purpose: Lebers Hereditary Optic Neuropathy (LHON) is an acute or subacute, bilateral central vision loss due to optic nerve degeneration and occuring predominantly in young males. The mechanism of the underlying retinal ganglion cell loss is still uncertain, although mutations in mtDNA encoded subunits of respiratory chain complex 1 were identified as the primary cause of the disease. Lowered ATP/ADP ratio and oxidative stress induced apoptosis have been discussed as potential mechanisms. In the present study, H_2O_2 sensitivity and antioxidant defence were characterized in teratoma derived LHON cybrids.

Methods: The line NT2/D1 and three cybrids of NT2/D1 origin (G.A. Cortopassi, USA) were compared: two contained the most common LHON mutation at np11778, while one was a revertant. The lines were analyzed either as proliferating tumor cell cultures or after 4 day retinoic acid (RA) treatment in neurobasal/B27, leading to loss of mitotic activity but not yet to neuronal differentiation (which requires longer RA treatments). The lines were analyzed for H_2O_2 sensitivity (MTT assay), total glutathione per mg protein, specific activities and mRNA abundance of antioxidant enzymes.

Results: Only after 4 day RA differentiation, 11778 cells became significantly more H_2O_2 sensitive as compared to NT2/D1, suggesting that differentiation generates a stress situation, in which the effects of the mutations may become measurable. This stress situation may be supported by Real-Time-PCR data of antioxidant enzymes. RA differentiation generally increased SOD2 mRNA 10 to 40-fold and also GR and GPx mRNA ($p<0.05$), suggesting higher protein turnover. However, no LHON specific effects were visible at the level of enzyme activities. Glutathione reductase (GR) activity did not differ significantly between clones. A siginificant decrease of glutathione peroxidase (GPx) activity, if compared with NT2/D1, occured in all three undifferentiated cybrids, including the revertant. It was paralleled by GPx1 mRNA downregulation and suggested a cybridization effect. On the other hand, total glutathione content was reduced to 49% of the NT2/D1 level in 11778 LHON cybrids only after differentiation ($p<0.05$), which may be a true effect of the LHON mutation in our cybrid model.

Conclusions: Many questions are open, including SOD2 activity and ATP/ADP ratios, which are currently measured in our system. However, a potential glutathione reduction caused by LHON mutations would offer an interesting link between energy crisis and oxidative stress.

Microperimetric assessment of patients with type II Macular Telangiectasia

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Purpose: Type 2 macular telangiectasia (MT) is a rare disease of the parafoveal capillaries with angiographically late hyperfluorescence temporal to the fovea without macular edema. To assess retinal light increment sensitivity (LIS) of both the angiographically hyperfluorescent and the adjacent areas at the posterior pole we performed fundus controlled static threshold microperimetry in patients with type 2 MT.

Methods: 35 eyes of 18 patients (mean age: 61 years; range: 48 - 73 years) MT were included. Patients were examined by means of funduscopy, best-corrected visual acuity (ETDRS), fluorescein angiography, and optical coherence tomography (OCT3). Fundus controlled static threshold perimetry was performed with the Nidek MP1 (Goldmann III stimuli, white background illumination, 4-2-test strategy, background illumination: 1,27 cd/m^2, maximal stimulus intensity: 127 cd/m^2, maximal attenuation: 20 dB).

Results: Visual acuity (VA) was reduced in all but two eyes (median 20/50; range: 20/200 - 20/20). LIS was reduced within the hyperfluorescent areas temporal to the fovea in all but four eyes. The areas with reduced LIS correlated well with the angiographically hyperfluorescent areas and showed a relatively sharp demarcation from areas with normal LIS. There was no apparent correlation between VA and parafoveal LIS-reduction. In eyes with additional hyperfluorecence of the nasal parafoveal region, LIS was considerably higher or within normal limits in the nasal compared to the temporal area. In 19 eyes there was no LIS detectable at least at some test locations temporal to the fovea. Retinal sensitivity of normal appearing areas on angiography at the posterior pole was within normal limits in all but two eyes.

Conclusions: These findings indicate that MT is associated with functional impairment of retinal light sensitivity. There is good topographic correspondence between angiographically visible alterations and parafoveal scotomas, which both are mainly located in the temporal parafoveal area. Abnormal retinal sensitivity represents a different feature from visual acuity loss, i.e. in eyes with profound parafoveal scotomas, VA can be relatively preserved. Therefore, testing for retinal light sensitivity should be included as an additional outcome measure for future interventional studies.

Horizontal cell receptive fields are reduced in connexin57-deficient mice

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Purpose: Horizontal cells are coupled by gap junctions and mediate lateral inhibition in the outer plexiform layer. Horizontal cell coupling is modulated by ambient light in an adaptive mechanism which enables enhancement of the signal-to-noise ratio without losing important basic visual information, such as object boundaries. Several neuromodulators reduce horizontal cell coupling, including dopamine, which is released in the retina under prolonged illumination. In order to understand the molecular mechanism underlying this adaptive process, we sought to identify the connexin subunits involved in gap junctional coupling of the horizontal cell network. Hombach et al. (2004, *Eur. J. Neurosci.* 19, 2633-2640) showed that connexin57 (Cx57) forms gap junctions coupling the horizontal cell network in the mouse retina. The aim of this study was to confirm the functional role of Cx57 in horizontal receptive field organization.

Methods: We used immunolabeling and dye injection to examine the morphology of the Cx57-deficient retina. We recorded intracellularly from horizontal cells in the inverted eyecup preparation to measure the receptive fields of horizontal cells from wildtype and Cx57-deficient retinae. Length constants were computed for each cell using the cell's responses to concentric light spots of increasing diameter.

Results: Horizontal cell spacing and dendritic field size were unaffected by Cx57 deletion. Horizontal cell receptive field size was dependent on stimulus intensity. Deletion of Cx57 significantly reduced receptive field size. Dopamine reduced the receptive field size in wildtype cells, but had no effect on Cx57-deficient horizontal cells. Dark resting potentials were strongly depolarized and response amplitudes reduced in Cx57-deficient horizontal cells compared to the wildtype, suggesting an altered input resistance. This was confirmed by patch-clamp recordings from dissociated horizontal cells.

Conclusions: Cx57 is expressed exclusively by horizontal cells: no other neuron in the retina or brain expresses this connexin. Deletion of Cx57 strongly reduces horizontal cell tracer coupling (Hombach *et al.*, 2004) and receptive field size. While deletion of Cx57 has little impact on horizontal cell morphology, it strongly influences membrane physiology. This study thus provides the first quantification of mouse horizontal cell receptive fields, and underscores the unique role of Cx57 in horizontal cell coupling and physiology.

Effects of a Ser163Arg *C1QTNF5* mutation on cell adhesion.

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Purpose: A Ser163Arg mutation in the short-chain collagen *C1QTNF5* gene results in late-onset retinal and macular degeneration (L-ORMD), which resembles age-related macular degeneration. The aim was to elucidate the function of C1QTNF5 and to examine the effect of the mutation on cell function and specifically on cell adhesion.

Methods: ARPE-19 and HEK 293-EBNA cells stably expressing wildtype and mutant C1QTNF5 were examined for C1QTNF5 secretion, by immunoblotting and cell adhesion assays and immunocytochemically for evidence of cytotoxicity.

Results: Wildtype C1QTNF5 was secreted while the mutant protein formed high molecular weight aggregates and was not secreted. The mutant protein was able to multimerise with wildtype protein. Cell adhesion assays showed reduced cell adhesion to laminin-coated plates in mutant-expressing cells, compared with wildtype and control cells. Evidence of cytotoxicity was also found in mutant-expressing cells, which showed aggregates co-localising with endoplasmic reticulum (ER) stress proteins.

Conclusions: Native C1QTNF5 protein is multimeric, so that heteromultimer formation may result in haploinsufficiency due to lack of secreted C1QTNF5 in L-ORMD heterozygotes. This is associated with changes in adhesion to laminin-coated plates, suggesting a role in cell adhesion. Cytotoxicity may also be present, as a result of protein aggregation and endoplasmic reticulum stress, in transient expression assays, but a true gain-of-function remains to be demonstrated in cells expressing normal levels of mutant C1QTNF5.

Characterization of the pathogenic mechanisms of two novel rhodopsin (*RHO*) mutations causing Retinitis pigmentosa.

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Purpose: To characterize two novel rhodopsin (*RHO*) mutations leading to Retinitis pigmentosa (RP) in patients.

Methods: Patients (n=78) were clinically characterized by visual acuity testing, fundoscopy, Goldmann perimetry, dark adaptometry and Ganzfeld electroretinography (ISCEV standard). Their DNA was screened for mutations in *RHO* by direct sequencing of PCR fragments. Computational analyses of RHO protein structure were performed with the Swiss-Pdb Viewer program (http://www.expasy.org/spdbv/). A putative splice site mutation was analyzed using a minigene approach. Wildtype and mutant *RHO* sequences, including exons 3 and 4 and intron 3, were cloned in the pSPL3 vector and transfected into COS-7 cells. Transcripts were analyzed by RT-PCR and sequencing.

Results: We identified two novel *RHO* mutations, c.269G>T (G90V) and c.1032G>A (R344R), in two distinct Swiss families. Patients carrying the G90V mutation showed phenotypic abnormalities compatible with the clinical diagnosis of classical RP. Interestingly, a mutation at the same amino acid position, G90D, leads to night blindness (NB) (Sieving et al., 1995). To separate the molecular mechanism that distinguishes between RP and NB, we performed 3D structure modeling of the G90V and G90D variants in comparison to the wildtype rhodopsin conformation. The results indicate that the orientation of the transmembrane helix 2 and the chromophore 11-cis-retinal are disturbed by both mutations. In contrast, only asparagine (G90D), but not valine (G90V), is capable to form hydrogen bonds that help to stabilize the active conformation of RHO and thus might distinguish between RP and NB. The silent mutation c.1032G>A (R344R) is located at the last base of exon 4 of *RHO* and might alter the splice donor site. Indeed, minigene assays revealed that this mutation interferes with normal splicing of *RHO in vitro* and generates transcripts that truncate the deduced protein sequence.

Conclusions: G90V and G90D represent the first case of two different phenotypes associated with mutations at the same amino acid position in RHO. Our data suggest that even small structural changes in RHO influence the human phenotype. The c.1032G>A (R344R) mutation induces mis-splicing of *RHO* pre-mRNA transcripts that lead to truncated proteins and thus are likely to cause RP in patients.

Retinal neuroprotection by hypoxic preconditioning: identification of possible neuroprotective targets using affymetrix gene chip technology.

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Purpose: Loss of vision in retinal diseases is often due to the apoptotic loss of photoreceptors. Hypoxic preconditioning stabilizes transcription factor HIF-1a and protect the visual cells from apoptosis in an established model of induced retinal degeneration. To identify genes, which might contribute to the protective effect of hypoxic preconditioning, gene expression patterns of normoxic and hypoxic preconditioned mice were compared using gene chip technology.

Methods: Mice were kept for 6h in hypoxia and retinal RNA was isolated immediately, 2h, 4h, 16h, after hypoxia. Gene chip (Affymetrix) hybridization was done in collaboration with the Functional Genomic Centre Zurich. To verify individual gene expression Real Time PCR and Western Blot were used.

Results: Cluster analyzes showed a highly different expression pattern immediately after hypoxia as compared to normoxic mice. During reoxygenation gene expression returned to normal levels. Immediately after hypoxia 120 genes with various biological functions were differentially regulated at least by a factor of 2, including 4 up regulated anti-apoptotic genes, which might contribute to the observed protection of photoreceptors.

Conclusion: During Hypoxia some anti-apoptotic genes (among others) are up regulated. Further analysis will show whether these genes might be responsible for the neuroprotective effect observed by hypoxic preconditioning.

Keywords: neuroprotection, hypoxic preconditioning, retinal apoptosis

Kir 4.1 channel subunit expression of Müller glial cells in experimental proliferative vitreoretinopathy

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The spatial buffering of extracellular K^+ ions is one important function of Müller glia cells in the retina. The glia cells mediate the clearance of K^+ ions released by excited neurons via a process called K^+ siphoning. K^+ flux through open inwardly rectifying K^+ channels (Kir) is the most important mechanism of keeping K^+ homeostasis, besides Na^+, K^+ -ATPase activity and passive co-transport with other ions. In particular, the Kir 4.1 channel subtype has been implicated in Müller cell-mediated K^+ buffering. Despite the significant role of glial K^+ channels, there is only restricted knowledge about their altered expression pattern in retinal pathology. It is known that gliotic Müller cells reduce their K^+- inward conductance and display depolarised resting membrane potentials. Therefore, we investigated the expression of Kir channel subtypes in a well-established rabbit model of proliferative vitreoretinopathy (PVR). Rabbit retinas were examined by immunohistochemistry, Western blotting and real-time PCR. The mRNA analysis of healthy and pathologically altered retinas revealed similar expression pattern for Kir 3.1, 3.2, 3.4, and 6.2. In contrast, the real-time PCR revealed an increased expression of Kir 2.1 and 7.1 channel subunits in pathological altered retinas. Surprisingly, we did not find a significant difference of dominant Kir 4.1 channel subunit expression in Müller glia cells of pathological altered and healthy retinas. To ensure that the commercially available peptide antibodies recognize the rabbit Kir 4.1 channel we sequenced the carboxy-terminal sequence of the Kir 4.1 channel subunit. Western blot analysis and immunohistochemical staining did not reveal obvious differences of Kir 4.1 expression at protein level. Electrophysiological experiments confirmed reduced inward K^+ currents and depolarized membrane potentials of gliotic Müller cells. Despite unmodified mRNA and protein expression of the Kir 4.1 subunit in pathologically altered retinas compared to controls the inwardly rectifying channels are not functional. Therefore, other regulatory mechanisms might be responsible for the distinct changes in channel function during pathological alterations in Müller glial cells.

Glial cell line-derived neurotrophic factor promotes differentiation and survival of rod photoreceptors in reaggregated spheres of the chicken retina

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Purpose: To investigate the role of glial cell line-derived neurotrophic factor (GDNF) on proliferation, differentiation, and apotosis of different retinal cell types – in particular, photoreceptors.

Methods: Reaggregated histotypic spheres, derived from retinal cells of the E6 chicken embryo were used. Under rotation conditions, so-called rosetted spheroids were formed by aggregation of dissociated retinal cells, followed by their proliferation, migration and differentiation. Rosetted spheroids were cultured for 10 days under serum-reduced conditions either in the absence or presence of 50 ng/ml GDNF.

Results: At early stages of culture, GDNF significantly increases and sustains the rate of proliferation. Possibly caused by this proliferative effect, we observed an increase in the number of rod photoreceptors, whereas cone photoreceptors were not affected. Moreover, in GDNF-treated cultures, rod photoreceptors differentiate earlier than in non-treated cultures. When spheroids were raised under serum-reduced conditions in the absence of GDNF, rod but not cone photoreceptors undergo apoptosis. By supplementing GDNF, apoptosis of rod photoreceptors was decreased (31% to 6% at 8 days in culture, 71% to 3% at 10 days in culture).

Conclusion: Our data indicate that GDNF regulates the proliferation, differentiation and survival of rod photoreceptors.

Identification of calcium transporting membrane proteins in the retinal pigment epithelium

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Introduction: The retinal pigment epithelium (RPE) fulfills many tasks to support normal retinal function. These include the light absorption, transepithelial transport, the recycling of 11-cis retinal, the phagocytosis of shed photoreceptor outer segments, and the secretion of cytokines. As all these processes are regulated by changes in intracellular free Ca^{2+}, the cells need a variety of signalling molecules leading to a distinct rise of intracellular free Ca^{2+}.
In this study, we investigated the expression pattern of Ca^{2+} transporting membrane proteins and we found a wide range of them expressed in the human RPE.

Methods: Human RPE cells were isolated from adult donor eyes. The cells were directly sampled in lysis buffer for RNA extraction. The isolated RNA was reverse transcribed and the resulting cDNA used for PCR amplifications with specific oligonucleotides. Human fetal brain cDNA was used for control reactions.

Results: We found a surprising large variety of genes encoding Ca^{2+} transporting membrane proteins expressed in freshly isolated human RPE cells. So far, the expression of only one TRP channel (TRPC1) and L-type voltage-gated Ca^{2+} channel (α1D) in the RPE cells has just been reported. The members of TRP channels identified in this study couples the intracellular free Ca^{2+} in RPE cells to diverse extracellular and intracellular signals. Additionally, we found the transcripts of six voltage-gated Ca^{2+} channels, two nucleotide-gated channels and five purinergic receptor channels. These channels couple the Ca^{2+} content in the RPE to membrane potential changes as they may occur by light changes, to additional intracellular and extracellular. For the termination of these Ca^{2+} responses the cardiac Na^{+}/Ca^{2+} exchanger and all four known plasma membrane Ca^{2+} ATPases are expressed in the human RPE cells.

Conclusions: The diversity in Ca^{2+} transporting proteins in the RPE reflects on the one hand the diversity of functions Ca^{2+} ions fulfill in these cells. On the other hand it may be an expression of the heterogeneity of RPE cells where some might be involved in the immune response while others might be responsible for the secretion of cytokines.

VEGF prevents osmotic glial cell swelling

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Purpose: In different diseases an edema develops in neural tissue. Swelling of glial cells causes a cytotoxic edema. Whereas the vascular endothelial growth factor (VEGF) can induce a vasogenic edema, it is not known whether it also influences cytotoxic edemas. The swelling of retinal Müller glial cells is involved in the development of a cystoid macular edema, an important complication of some ocular diseases. To investigate the effect of VEGF on the swelling behaviour of Müller cells, we used retinal slices from adult control rats, from rats during postnatal development, from diabetic rats, and from rats after retinal ischemia-reperfusion.

Methods: Freshly isolated slices were stained with the dye Mitotracker Orange which is selectively taken up by Müller cells. To determine volume changes of Müller cells evoked by hypotonic stress (extracellular ionic strength was decreased to 60%), the cross-sectional area of Müller cells somata was measured at their largest extension by using a laser scanning microscope.

Results: Müller cells in control retinae did not alter their soma area under hypotonic conditions. The dominant inward K^+ conductance of Müller cells is known to be small at early postnatal stages. Under pathological conditions (transient ischemia, 6 months diabetes), the K^+ inward currents were demonstrated to be downregulated. Moreover, these currents can be blocked by application of 1 mM Ba^{2+}. Under all conditions mentioned, the Müller cell soma area was significantly increased in the hypotonic solution, suggesting that the K^+ channels are involved in the volume regulation. This swelling was inhibited by application of VEGF in all experiments. Pharmacological data suggested the involvement of the VEGFR-2 receptor tyrosine kinase. VEGF acted via transactivation of metabotropic glutamate receptors, P2 nucleotide receptors and A1 adenosine receptors, as was shown by the effects of the respective antagonists.

Conclusions: The present data demonstrate that VEGF inhibits osmotic glial cell swelling in retinal slices and, thus, may have an influence on the formation of a cytotoxic edema in vivo. The effect was mediated by activation of VEGFR-2 receptors and a downstream signalling cascade involving different types of metabotropic receptors. We suppose that this cascade results in the opening of membrane channels for osmotic relevant ions or molecules under conditions where the main K^+ conductance of Müller cells is blocked or downregulated.

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