PRO RETINA DEUTSCHLAND E. V.

3rd PRO RETINA
Research-Colloquium Potsdam

CONFERENCE REPORT

Retinal Degeneration

Genes – Progression – Therapy

An Interdisciplinary Dialogue

March 30th/31st, 2007

Potsdam, Seehotel am Templiner See

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PRO RETINA – AN INTRODUCTION

WHO WE ARE

„Pro Retina Deutschland e.V." was founded in 1977 as „Deutsche Retinitis Pigmentosa-Vereinigung" by patients and their relatives intended to organize help for themselves. The three objectives mentioned in the constitution are to actively support research, to give psychological and social advice for its members and to strengthen public information.
Every member can join one of the 65 regional groups, which are distributed throughout Germany. At present (2007), PRO RETINA Deutschland e.V. counts approx. 6,400 members. The Board, the Counsellors, the leaders of the regional groups and all active members are working on a non-profit basis. They are supported by an official office which is located in Aachen (www.pro-retina.de).

WHAT WE DO FOR RESEARCH

We have created a stable network with researchers and ophthalmologists for joint information and advice. We support research projects with direct financial funding – since the 'Foundation for Prevention of Blindness" was established in 1996, more than one million Euro have been donated. We actively initiate research projects and therapy tests and contribute to their implementation.

Every year, we award two research prices and organize and support national and international seminars and conferences on relevant topics. We are financing PhD grants in order to foster research activities and networking between researchers.

We are consulted by a Scientific and Medical Advisory Board ("Wissenschaftlicher und Medizinischer Beirat", WMB) and a Working Group on Clinical Questions ("Arbeitskreis Klinische Fragen", AKF). In this Working Group scientists of different medical and other relevant disciplines are taking part.

PRO RETINA-STIFTUNG ZUR VERHÜTUNG VON BLINDHEIT

The "Foundation for Prevention of Blindness" was founded on January 1st, 1996, (since 2007 as own juridical person "Pro Retina Foundation for Prevention of Blindness"). The main objective is to secure a long-term support for research activities, e. g. by granting financial means for the development of new research projects or by financing the initial phase of relevant projects.

It is envisaged to increase the capital of the foundation to a minimum of Euro 5,000,000, which are to result in a steady source of funding for the support of research, independent from changing income from donations.
We guarantee that the benefits of the Foundation will only be dedicated to the research of retinal diseases, with the wider objective to develop applicable therapies for the patients.

PREFACE

Dear Colleagues,

Welcome to the ProRetina Research Colloquium in Potsdam, now known as the "Potsdam Meeting". Organised for the third time, the meeting appears to have developed into an accepted and highly regarded scientific platform in retinal research. From the feedback of the participants we learned that the Potsdam Meeting has become an important annual meeting point not only for German scientists but also for colleagues from its neighbouring countries. Despite the timely overlap with the German Neuroscience Meeting, this year we again welcome more than 150 participants from all over Europe. Part of our concept is to support young scientists, to combine presentations of established research and data "just from the bench". We also give presentations a scientific profile in order to raise ideas for new synergisms and cooperations. We hope that a novel addition to the program – the introduction of poster prizes – will further enhance the success of the meeting.

In continuation of the last meetings, this year's programm again will hopefully inspire new concepts and interactions. The stationary disease session will raise the question why some diseases remain stationary while others become progressive. Session 2 touches the complexity of gene and protein networks which is still a challenging problem for basic science as well as for those aiming for new therapeutic treatments. Examples of complex diseases associated with retinal degeneration such as Refsum disease or the Bardet-Biedl syndrome will be discussed, but also the impact of altered splicing mechanisms in Retinitis Pigmentosa will be addressed. The session on animal models aims to open our minds for such models which originally were not generated to study retinal degeneration but nevertheless turned out to be valuable for learning about the retina and its disease mechanisms – a source which awaits further and more in-depths exploration in the future. Never out of sight is the issue of therapy in retinal degeneration. Surprising new concepts have recently been developed and will be presented. Ultimately, this may also motivate scientists who are primarily interested in general mechanisms of neuronal degeneration which nonetheless provide the basis for treatment options.

It is a pleasure to welcome you all and to look forward to a meeting which brings together science, curiosity, enthusiasm, and friendship.

We wish you a fruitful and inspiring meeting,

Franz Badura, Klaus Rüther, Olaf Strauß and Bernhard Weber

PROGRAMME

Friday March 30th:

13:00 – 13:05 Franz Badura:
Welcome and opening remarks

13:05 – 13:15 Rainald von Gizycki:
"The patient's view in times of scientific progress "

13:15 – 13:30 Eberhart Zrenner:
"Retinal degeneration; genes – progression – therapy"

13:30 – 15:10 **Session 1: Stationary Diseases of the Retina**
Chairman: Prof. Olaf Strauß

1. Birgit Lorenz, Regensburg:
   "The clinics of stationary retinal disease"
2. Alexandra Koschak, Innsbruck:
   "Functional consequences of Ca(v)1.4 L-type Ca^{2+} channel mutations"
3. Christina Zeitz, Zürich:
   "Gene mutations leading to stationary or progressive defects in retinal signal transmission"
4. Peggy Reuter, Tübingen:
   "Functional analyses of mutant cone CNG channels"

15:10 – 15:45 Coffee break

15:45 – 17:25 **Session 2: Gene/Protein Networks in Retinal Degeneration**
Chairman: Prof. Bernhard Weber

1. Christian A. Hübner, Hamburg:
   "RDH12 and visual function"
2. Ronald Wanders, Amsterdam:
   "Molecular basis of Refsum disease"
3. Nicholas Katsanis, Baltimore:
   "Protein networks in Bardet-Biedl syndrome"
4. Bastian Linder, Würzburg:
   "Defects in splicing as a possible cause of Retinitis Pigmentosa"

18:30 Dinner

19:30 **Poster Session and "Swinging" Discussions**

PROGRAMME

Saturday March 31st:

08:30 – 10:10 **Session 3: Systemic Disease and Mouse Models for Retinal Degeneration**
Chairman: Prof. Klaus Rüther

1. Muriel Auberson, Berlin:
   "Retinal degeneration in CLC knockout mouse models"
2. Stefan Storch, Hamburg:
   "Retinal degeneration in lysosomal storage diseases"
3. Andreas Feigenspan, Oldenburg:
   "PKC – an ubiquitously expressed enzyme with crucial relevance for retinal function"
4. Tanya Tolmachova, London:
   "Retinal degeneration in choroideremia mouse models"

10:10 – 10:45 Coffee break

10:45 – 12:25 **Session 4: Approaches to Therapy**
Chairman: Prof. Shomi Bhattacharya

1. Naomi Chadderton, Dublin:
   "Strategies for therapeutical approaches in dominantly inherited retinopathies"
2. Marius Ader, Dublin:
   "Toward an RNAi-based gene therapy in dominant Retinitis Pigmentosa"
3. Marijana Samardzija, Zürich:
   "Rpe65-R91W knock-in mice: pathophysiology and consequences for therapy"
4. Stefan Linke, Hamburg:
   "Stem cell-based therapeutic approaches to retinal disease"

12:25 – 13:00 **Poster Awards and Short Presentations**

13:00 Lunch and end of meeting

SPEAKER ABSTRACTS

THE PATIENT'S VIEW IN TIMES OF SCIENTIFIC PROGRESS

Abstract for speech at scientific colloquium, Potsdam March 30th / 31st, 2007
Dr. Rainald von Gizycki, honorary president of Pro Retina Deutschland e. V.

What are the motivations and obstacles of patients for RD research funding?

The motivations of funding research into retinal diseases are different between patient organizations on one side and public funding organizations (like the European Commission) on the other:

- Patient organizations are **authentic carriers of patient needs** as determining force of research requirements;
- patient organizations depend on **small financial means** for promoting research, mostly provided by individual patients, relatives and friends;
- patient organizations usually provide **additional project funding** (e.g. seed money, research prices, travel grants etc.), as part of the overall project budget;
- patient organizations aim at **finding a cure** (e. g. for blinding eye diseases) as primary funding objective;
- patient organizations **do not dispose of a huge professional apparatus** for research promotion, like industry or government agencies.

What are the financial achievements of research promotion by Retina organizations?

Member organizations of Retina International are funding basic and applied research in all fields of retinal degenerative diseases (RD) by an estimated annual amount of 40 to 45 million Euros. European Retina Patient Organizations contribute about 25 % of this amount, i.e. ca. 10 million Euros per year. Pro Retina is about to establish a new independent foundation with a capital of about 2 million Euros.

How can patients remain aware of relevant progress in the field of RD research?

Retina patient and umbrella organizations need to document and compare their research funding achievements and funding instruments in order to establish mechanisms of self-control, to formulate common funding and publication strategies. Hence, they need to establish their own project database to provide information about patient sponsored RD research projects to all interested target groups and in order to avoid duplication of project funding. Also patient organizations need to monitor regularly results of projects presented on international conferences and documented in paper-bound and electronic media. This result must be broadly diffused to individual patients and eye doctors to ascertain their application for prevention, therapy and rehabilitation as quickly as possible.

THE CLINICS OF STATIONARY RETINAL DISEASE

Birgit Lorenz

Dept. of Paediatric Ophthalmology, Strabismology and Ophthalmogenetics, Regensburg University Medical Center

Objective: To describe the phenotypes of stationary hereditary retinal disorders most commonly associated with infantile nystagmus.

Stationary hereditary retinal disorders associated with infantile nystagmus may be due to reduced cone density (macular hypoplasia in albinism and aniridia), cone dysfunction (autosomal recessive forms of achromatopsia, X-linked blue-cone monochromacy), or inner retina transmission disorders (congenital night blindness, X-linked complete, X-linked incomplete, autosomal recessive, autosomal dominant). Absence of nystagmus does not exclude any of these diseases. The correct clinical classification requires a full eye examination including colour vision testing, visual field testing, dark-adaptation measurement, and electrophysiology. More sophisticated tests include 2-colour-threshold perimetry, spectral sensitivity measurements, and advanced electrophysiology e.g long stimuli to differentiate disturbances in bipolar on – and off-responses. Slow deterioration may be observed in some forms. Differential diagnosis comprises cone-rod dystrophies, rod-cone dystrophies, retinitis punctata albescens, and X-linked retinoschisis. A good clinical classification allows gene-directed mutational analysis in an increasing number of conditions. This is important due to significant locus and allelic heterogeneity of stationary retinal diseases.

FUNCTIONAL CONSEQUENCES OF $CA_V1.4$ L-TYPE CA^{2+} CHANNEL MUTATIONS – IMPACT FOR CALCIUM CHANNEL FUNCTION

A. Koschak[1], A. Singh[1], JC. Hoda [1], D. Hamedinger[2], M. Gebhart [1], C. Romanin [2] and J. Striessnig [1]

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[2]Institute of Biophysics, University, Linz, Austria

Purpose: Tonic neurotransmitter release at retinal synapses is mediated by Ca^{2+} influx through slowly inactivating $Ca_V1.4$ L-type voltage-gated Ca^{2+} channels (LTCCs) lacking Ca^{2+}-dependent inactivation (CDI). At least 48 mutations in the CACNA1F gene encoding retinal $Ca_V1.4$ L-type Ca^{2+} channels (LTCCs) have been linked to incomplete congenital stationary night blindness type 2 (CSNB2). A large number of these are missense mutations encoding full length $\alpha1$ subunits that can potentially form functional channels such as mutations G369D (GD), R508Q (RQ) L1069P (LP), L1364H and S229P (SP). Most CSNB2 truncation mutations lead to non-functional $Ca_V1.4$ channels but mutation K1591X (KX) is likely to form a functional channel because its C-terminus is removed after the IQ motif. The mutation's impact on calcium channel malfunction and function will be discussed.

Methods: For electrophysiological analysis in either two-electrode or patch-clamp configuration we expressed mutated $Ca_V1.4$ channel either in X. laevis oocytes or HEK-293 cells together with $\beta3$ and $\alpha2\delta1$ subunits. Currents were recorded using barium or calcium as charge carrier. FRET analysis allowed exploring an intra-molecular interaction of the $Ca_V1.4$ C-terminus.

Results: CSNB2 mutations confer their phenotype by different pathological mechanisms, such as change in $Ca_V1.4$ protein expression (e.g. RQ, LH), lower range of calcium current activation (e.g. GD, KX) or appearance of CDI (KX). Thereby a new regulatory mechanism in the $Ca_V1.4$ C-terminus that controls channel activation and inactivation gating was elicited. FRET experiments revealed that the last 55-122 amino acids of the proximal $Ca_V1.4$ C-terminus interact with physiologically important regions for CDI modulation also in other LTCCs.

Conclusion: Taken together our data provide insight into possible mechanisms underlying CSNB2. Moreover the absence of channel modulation by CDI in the CSNB2 mutant KX underlines its importance for normal retinal function in humans. Interestingly, similar Ca^{2+} and voltage-dependent properties were observed in a C-terminally truncated neuronal splice variant of $Ca_V1.3$ LTCCs suggesting a universal role of the C-terminus for LTCC gating modulation.

Support: FWF (P-17159), LFU Innsbruck and the Tiroler Wissenschaftsfonds.

GENE MUTATIONS LEADING TO STATIONARY OR PROGRESSIVE DEFECTS IN RETINAL SIGNAL TRANSMISSION

Christina Zeitz

Division of Medical Molecular Genetics and Gene Diagnostics, Institute of Medical Genetics, University of Zurich, Switzerland

Purpose: The Schubert Bornschein type of congenital stationary night blindness (CSNB) is characterized by a negatively shaped ERG. The mode of inheritance has been described as X-linked or autosomal recessive. To date, most of the mutations leading to this phenotype occur in two X-linked genes: *NYX* (complete CSNB) and *CACNA1F* (incomplete CSNB). Recently, mutations have been identified in three additional genes associated with either complete or incomplete autosomal recessive CSNB. We describe the genotype-phenotype correlations and possible pathogenic mechanisms.

Methods: Mutation analyses in candidate genes have been performed by direct sequencing of all coding exons and flanking splice sites. Some of these mutations have been studied on the transcript and protein levels.

Results: The three new genes are *GRM6, CABP4* and *CACNA2D4.* Mutations in *GRM6,* coding for the metabotropic glutamate receptor mGluR6, can lead to the complete type of autosomal recessive CSNB. Missense mutations abolish localization of the receptor to the cell surface. Mutations in *CABP4,* encoding a Calcium binding protein, lead to an incomplete type of autosomal recessive CSNB. Patients showed variable clinical features and a substantially reduced amount of *CABP4* transcript. In addition, a frameshift mutation results in cytosolic mislocalization of the respective protein. Mutations in *CACNA2D4,* encoding an L-type calcium-channel auxiliary subunit of the alpha-2-delta type, have been identified in patients previously suggestive for incomplete type of CSNB. However, the phenotype of these patients turned out to be more progressive and revealed a mild cone dystrophy.

Conclusion: Mutations in three novel genes lead to autosomal recessive CSNB or cone dystrophy with the typical Schubert Bornschein phenotype. The clinical features of the complete type of CSNB caused by *NYX* or *GRM6* mutations seem to be stationary. In contrast, patients originally classified with incomplete CSNB and mutations in *CACNA1F, CABP4* and *CACNA2D4* revealed a variable disease course from stationary to progressive.
Lack of the mGluR6 protein at the cell surface may abolish signal transmission from rods to bipolar cells in the retina. Mutated *CABP4* may interfere with proper function of the calcium channel and hence the continuous release of glutamate important for signal transmission is not warranted.

FUNCTIONAL ANALYSIS OF MUTANT CONE CNG CHANNELS FOUND IN ACHROMATOPSIA PATIENTS

Peggy Reuter, Katja Koeppen, Thomas Ladewig, Bernd Wissinger

Molecular Genetics Laboratory, University Eye Hospital Tuebingen, Germany

Purpose: Achromatopsia is a rare autosomal recessive retinal disease characterised by absent or strongly limited colour discrimination ability and severely reduced visual acuity due to defective cone function. In about 30 % of the patients the lack of cone function is caused by mutations in the *CNGA3* gene, which encodes the A3-subunit of the cone cyclic nucleotide-gated (CNG) channel, an essential component of the phototransduction cascade. The aim of this study was to analyse and characterise the properties of mutant channels at the molecular level and to correlate it with the patient's phenotypes.

Methods: The analysed mutations were introduced by *in vitro* mutagenesis of wild type *CNGA3* cDNA cloned into the pcDNA3.1/ZEO vector. HEK293 cells were transfected with mutant CNGA3 expression constructs alone or together with wild type CNGB3 constructs for formation of heterooligomeric channels. Expression of the channel protein was verified by immunocytochemistry. Mutant channels were assessed by calcium imaging and functional mutants were further analysed with the patch clamp technique.

Results: 25 CNGA3 mutations were analysed in total. Some of the mutants showed to be functional as homo- and heterotetrameric channels in calcium imaging experiments. In some cases we obtained signals with heterotetrameric channels only, indicating that the functionality of those channels was rescued by the B3-subunit. In other mutants we achieved a functional improvement by incubating the transfected cells at a lower cultivation temperature. A residual fraction of mutants did by no means generate functional channels. Patch clamp analyses demonstrated that some mutations increase the ligand sensitivity of the channels while others have the opposite effect. This shift of the dose-effect relationship could be rescued by the B3-subunit in some mutants. Other CNGA3 mutations had no effect on ligand sensitivity, but they resulted in a decrease of maximum macroscopic currents.

Conclusion: Besides the change of ligand sensitivity the strongest effect of CNGA3 mutations seems to be on channel ontogeny, resulting in impaired protein folding or trafficking to the plasma membrane. This was shown by the effects of the lower cultivation temperature which is assumed to impair the quality control in the endoplasmatic reticulum or to enhance protein folding so that finally a larger fraction of channels is integrated into the plasma membrane. The B3-subunit also seems to be able to partially rescue the function of some channel mutants.

RDH12 AND VISUAL FUNCTION

Ingo Kurth[1], Debra A. Thompson[2,3], Klaus Rüther[4], Kecia Feathers[2], Jared Chrispell[2], Jana Schroth[1], Christina McHenry[2], Michaela Schweizer[5], Andreas Gal[1], Christian Hübner[1]

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Purpose: *RDH12* codes for a member of the family of short-chain alcohol dehydrogenases/reductases (RDHs) proposed to function in the visual cycle that supplies the chromophore 11-*cis* retinal to photoreceptor cells. Mutations in *RDH12* cause a severe and progressive form of childhood-onset autosomal-recessive retinal dystrophy (arRD), including Leber congenital amaurosis (LCA). To elucidate the function of *RDH12*, we generated *Rdh12* knockout mice.

Methods/Results: *Rdh12* knockout mice exhibited grossly normal retinal histology at 10 months of age. Levels of all-*trans* and 11-*cis* retinoids in dark- and light-adapted animals, and scotopic and photopic electroretinogram (ERG) responses were similar to wild type, as was recovery of the ERG response following bleaching in animals matched for an *Rpe65* polymorphism (p.L450M). Lipid peroxidation products and other measures of oxidative stress did not appear to be elevated in Rdh12$^{-/-}$ animals. RDH12 was localized to photoreceptor inner segments and the outer nuclear layer both in mouse and human retina using immunohistochemistry.

Conclusion: The present findings, together with those of earlier studies showing only minor functional deficits in mice deficient for Rdh5, Rdh8, or Rdh11, suggest that the activity of any one RDH isoform is not rate limiting in the visual response.

PROTEIN NETWORKS IN BARDET-BIEDL SYNDROME

Prof. Dr. Nicholas Katsanis

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The ciliopathies are a group of genetically heterogeneous but clinically overlapping disorders that are caused by structural and/or functional defects in the cilium and the basal body. Recent progress in elucidating the genetic and cellular etiology of these disorders is emphasizing the importance of cilia to mammalian cells and providing sometimes unexpected clues about how they communicate with the extracellular environment. Using Bardet-Biedl syndrome as a model ciliopathy, we will discuss how improved genetic and functional models for this disorder are illuminating new roles for the mammalian cilium, which in turn equip us with improved tools to dissect epistatic interactions that govern the penetrance and expressivity of most disease phenotypes.

DEFECTS IN SPLICING AS A POSSIBLE CAUSE OF RETINITIS PIGMENTOSA

Bastian Linder[1], Bernhard Laggerbauer[1], Christoph Winkler[1], Inga Ebermann[2], Andreas Gal[3], Hanno Bolz[2] and Utz Fischer[1]

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The majority of Retinitis Pigmentosa (RP) relevant gene loci encode for proteins with roles in the visual process. However, RP can also be caused by mutations in housekeeping genes including hPrp3, hPrp31 and hPrp8. These genes encode pre-mRNA splice factors that are integral components of one functional subunit of the spliceosome, the tri-snRNP particle. RP-patients with mutations in this group of genes could hence share a common pathomechanism, i.e. the reduction of active spliceosomes. Therefore, a key question for the molecular understanding of RP is how the weakening of a general pathway translates into a tissue specific phenotype.

Purpose: We have started to characterize the biochemical effects of RP mutations in splicing factors and to screen for RP-linked mutations in other splicing factor genes. In addition we have begun to analyze the RP-associated splice factors in the zebrafish model system.

Methods: A biochemical approach is used to analyze whether splicing factors carrying pathogenic mutations exhibit defects in their interaction with other cellular partners. The zebrafish model is used to analyze the consequences of reduced expression of the RP-associated splice factors as well as to functionally characterize RP mutants.

Results: We have previously reported a RP patient that carries a missense mutation in the tri-snRNP component hPrp4. A detailed biochemical analysis revealed impaired association of this mutant with the tri-snRNP caused by its failure to bind its known interactor hPrp3. Knockdown of the hPrp4 zebrafish homologue zfPrp4 leads to severe developmental defects that can be rescued by the wild type protein, but not pathogenic zfPrp4. Similar studies are currently being performed with the other splice factors implicated in RP.

Conclusion: Our biochemical investigation revealed specific defects of mutant hPrp4 found in a RP-patient to form splicing complexes (i.e. tri-snRNP particle). Similar defects may be observed with the other RP-associated tri-snRNP-proteins. Because the tri-snRNP is a key component of the spliceosome, our data are consistent with the idea that reduced splicing leads to Retinitis Pigmentosa in patients with mutant hPrps. We speculate that this biochemical defect affects processing of certain pre-mRNA transcripts relevant for the retina and hence lead to tissue specific phenotype of RP.

RETINAL DEGENERATION IN CLC KNOCKOUT MOUSE MODELS

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Chloride channels are present in the plasma membrane and in membranes of intracellular organelles. They are involved in a broad range of functions, including the stabilization of membrane potential, synaptic inhibition, cell volume regulation, transepithelial transport, extracellular and vesicular acidification, and endocytotic trafficking. When studying knockout mouse model for different ClCs channels, ClC-2, ClC-3 and ClC-7 have been shown to be blind due to a loss of their photoreceptors. ClC-2 mediates plasma membrane chloride currents and is present in neurons and many epithelia where it has been involved as potential chloride exit pathways. Cell fractionation and transfection studies localized ClC-3 to an endosomal compartment, where it partially colocalized with rab4 and lamp-1 and, additionally, to synaptic vesicles. ClC-7 belongs to a third branch of the CLC family and resides in late endosomes and lysosomes. Both ClC-3 and ClC-7 provide electric shunt for the H^+-ATPase in acidic compartments. The primary causes of the loss of photoreceptors in ClC-2, 3 and 7 KO mice seem to be different as shown by micro-Ussing chamber measurements on retinal pigment epithelium. While RPE transport is normal in ClC-3 and ClC-7 KO, chloride transport is absent in ClC-2 KO RPE. Ussing chamber experiments reveal a reduction of transepithelial current and resistance across the retinal pigment epithelium of the ClC-2 KO mice.

These results indicate that the loss of the photoreceptors in the ClC-2 KO mouse is secondary to the RPE dysfunction whereas it is primary in ClC-3 and ClC-7 KO mouse, due to neuronal degeneration. The observed degeneration of photoreceptors in the ClC-2 KO mouse may be due to the defective transepithelial transport across the retinal pigment epithelium, which is important to provide an appropriate environment for the photoreceptors. The exact mechanisms through which ClC-2 deficiency leads to RPE dysfunction remain to be elucidated.

RETINAL DEGENERATION IN LYSOSOMAL STORAGE DISEASES

Stephan Storch [1], Arne Quitsch [1], Udo Bartsch [2] and Thomas Braulke [1]

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The neuronal ceroid lipofuscinoses (NCLs) represent a group of neurodegenerative disorders of childhood which are clinically characterized by the loss of vision, epilepsy, progressive mental retardation and a reduced lifespan. Depending on the onset of clinical symptoms the NCLs are classified into congenital, infantile, late-infantile, juvenile and adult forms of the disorder. One common feature of the diseases, which mainly affect the central nervous system, is the progredient accumulation of autofluorescent lipopigments in lysosomes and autophagosomes. Currently nine genes associated with human NCL diseases are identified which encode both soluble lysosomal enzymes and integral polytopic membrane proteins localized in late endosomes/lysosomes and in the endoplasmic reticulum (ER), respectively. Analyses of *Cln*-knock-out and mutant mouse models have confirmed retinal defects associated with CLN-deficiencies except for the *Cln*3 knock-out mouse. A variant form of the late infantile NCL is caused by defects in the polytopic CLN6 membrane protein localized in the endoplasmic reticulum. For analysis of retinal pathology we have examined a natural mutant mouse model, the CLN6-defective *nclf* mouse. Immunohistochemical analyses of the retina of *nclf* mice were performed at different ages between 1 and 9 months in comparison with age-matched wild type mice. Based on results from transcriptional profiling of brain from 1 month old *nclf* mice and western blot analyses of CLN6-deficient neurons, the expression of soluble and membrane proteins of late endosomes/lysosomes, cytosolic clathrin-adaptor proteins, ER-stress related-proteins and markers of astrocytosis in the retina was analyzed by immunohistochemistry. At postnatal day 40, *nclf* mice developed morphological changes in the photoreceptor layer. By 2-3 months of life loss of photoreceptor cells and atrophy of the photoreceptor layer of 20% was evident. At 7 months a massive degeneration of the photoreceptor layer was observed. In the time range between 8.5 and 12 months only 1-2 photoreceptor layers were detected. The data suggest that CLN6-defects in mice causes a progredient retinal degeneration with early onset resulting in a pronounced atrophy of the photoreceptor layer by 12 months of age.

PKC – AN UBIQUITOUSLY EXPRESSED ENZYME WITH CRUCIAL RELEVANCE FOR RETINAL FUNCTION

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[3] The Biotechnology Centre of Oslo, University of Oslo, Norway
[4] University Clinic Eppendorf, Experimental Ophthalmology, Hamburg, Germany

Purpose: The protein kinase C (PKC) isoforms α and β are expressed in almost all major cell types of the vertebrate retina. However, their physiological function and significance in the processing and modulation of visual information are currently unknown. Therefore, we have investigated retinal morphology and physiology in PKCα- and β- deficient mouse lines.

Methods: Immunocytochemistry and electroretinography (ERG) on knock-out models of the PKC α and β isoforms.

Results: Both PKC α- and β-deficient mouse lines were viable and showed no significant alterations in retinal morphology. ERG measurements of PKCα-/- mice revealed a delayed return of the scotopic b-wave to baseline. In addition, the implicit time of the ERG c-wave, originating from the retinal pigment epithelium, and recovery of photoreceptors from bleaching conditions were substantially faster in knock-out animals than in wild type controls. Deletion of PKCα did not effect the cone pathway. In contrast, the scotopic ERG of PKCβ-/- showed reduced amplitudes of both b-wave and c-wave. Although PKCβ is not expressed in photoreceptors, the scotopic a-wave reflecting photoreceptor activity was also significantly reduced.

Conclusion: These results suggest that both PKC isoforms play a crucial role in the processing of visual information in the inner retina, especially in rod and cone bipolar cells. PKCα appears to be important for the termination of the rod bipolar cell light response, which itself is apparently enhanced in the presence of functional PKCβ. In addition, PKCα appears to be involved in photoreceptor – retinal pigment epithelium interactions, whereas PKCβ might modulate a so far unknown feedback mechanism between bipolar cells and photoreceptors.

RETINAL DEGENERATION IN CHOROIDEREMIA MOUSE MODELS

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Purpose: Choroideremia (CHM) is an X-linked degeneration of the retinal pigment epithelium (RPE), photoreceptors and choroid, which is caused by loss-of-function of the CHM/REP1 gene. *CHM/REP1* is involved in lipid modification (prenylation) of Rab GTPases, key regulators of intracellular vesicular transport and organelle dynamics. To study the pathogenesis of CHM and to develop a model for assessing gene therapy, we have created several mouse models of CHM.

Methods: We used a conditional *Cre/loxP* knock-out strategy. We performed histological, electron microscopic, ophthalmoscopic, electroretinographic and biochemical analysis of generated mouse lines.

Results: We created two conditional alleles of the mouse *Chm* gene *(Chm^{3lox}* and *Chm^{flox})*, that we used in combination with two *Cre* transgenes: tamoxifen (TM)-inducible *MerCreMer* and *six3-Cre*. TM-treated *$MerCreMer^{+}$* males show signs of RPE abnormality without photoreceptors degeneration, while *six3-Cre^{+}* mice exhibit degeneration of photoreceptors, but no obvious RPE defects. Analysis of underprenylated Rabs in these lines by *in vitro* prenylation assay suggests that there are different subsets of Rabs in photoreceptors and in the RPE, which are underprenylated in the absence of Rep1. We also generated female carriers *($Chm^{null/WT}$)* that due to random X-chromosome inactivation contain 50% of KO cells and exhibit typical choroideremia signs. Patchy RPE depigmention was noticed as early as P7. Degeneration of the photoreceptor layer was progressive and lead to 50% reduction of photoreceptors by 8 months, presumably due to demise of KO cells. ERG showed reduction in scotopic a and b wave amplitude similar to CHM patients.

Conclusions: Female carriers exhibit characteristic hallmarks of the CHM: progressive degeneration of the photoreceptors, patchy depigmentation of the RPE and Rab prenylation defects. Using conditional *Chm* alleles together with *MerCreMer* and *six3-Cre* transgenes, we show that CHM pathogenesis involves independently triggered degeneration of photoreceptors and the RPE, associated with different subsets of defective Rabs.

RNAI-MEDIATED SUPPRESSION AND REPLACEMENT OF HUMAN RHODOPSIN IN VIVO

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Purpose: Mutational heterogeneity represents a significant barrier to therapeutic development for many dominantly inherited diseases. For example over 100 mutations in the rhodopsin (RHO) gene have been identified in patients with Retinitis Pigmentosa (RP). Development of therapies for dominant disorders which correct the primary genetic lesion and overcome mutational heterogeneity is challenging.

Method: A two component approach utilising gene suppression in conjunction with gene replacement has been proposed. Suppression is targeted to a site independent of the mutation and hence both mutant and wild type alleles are suppressed. In parallel with suppression a codon-modified replacement gene refractory to suppression is provided. Both *in vitro* and *in vivo* validation of suppression and replacement for RHO-linked RP has been undertaken in the current study.

Results: RNA interference (RNAi) has been utilised to achieve greater than 90% *in vivo* suppression of RHO in photoreceptors using adenoassociated virus (AAV) to deliver these RNAi molecules. Demonstration that codon-modifed RHO genes express functional wild type protein has been explored through the generation of a new transgenic mouse model, together with *in vivo* expression of AAV-delivered RHO replacement genes in the presence of targeting RNAi molecules. Observation of potential therapeutic benefit from AAV delivered suppression and replacement therapies has been obtained in Pro23His mice.

Conclusion: Suppression and replacement can provide a therapeutic solution for dominantly inherited disorders such as RHO-linked RP and can be employed to circumvent mutational heterogeneity.

TOWARD A RNAI-BASED 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 tested in organotypic retinal explant cultures electroporated with shRNA vectors and replacement cDNAs. Results were evaluated by immunocyto/histochemistry and RT-PCR.

Results: Electroporation of retinal explants *ex vivo* is a highly efficient method to express foreign genes in photoreceptors. 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 14 days following electroporation. Immunohistochemical analysis of rho-targeted photoreceptors revealed a strong decrease in the number of endogenous rho expressing cells at the protein level.

Conclusions: RNAi technology can significantly silence rds or rho expression in photoreceptors in organotypic retinal explant cultures *ex 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.

RPE65-R91W KNOCK-IN MICE: PATHOPHYSIOLOGY AND CONSEQUENCES FOR THE THERAPY

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Purpose: RPE65 is essential for the regeneration of 11-*cis* retinal - the chromophore of both cone and rod visual pigments. More than 80 disease-associated mutations have been identified in the *Rpe65* gene. Patients with R91W missense mutation have useful cone-mediated vision in the first decade of life suggesting that the mutant RPE65 protein is at least partially functional. All currently available animal models for visual defects associated with RPE65 are functional knock-outs. We generated an R91W knock-in mouse model to understand the mechanism of retinal degeneration caused by aberrant RPE65 function.

Methods: The animal strains used were: single mutant (R91W, Rpe65-/-, Rho-/-), double mutant (R91W;Rho-/-, Rpe65-/-;Rho-/-) and control wt (129S6) mice. The analyses were: RT-PCR, Western blotting and immunohistochemistry for gene/protein screening, HPLC for retinoid composition; spectrophotometry for rhodopsin content; light microscopy for morphology; and ERG for function. Injection of lentiviral vector expressing wild-type Rpe65 cDNA (LV-RPE65) was used to test the therapeutic window.

Results: The R91W mutation caused: 1) reduced RPE65 protein levels, 2) reduced levels of 11-*cis* retinal, 3) disturbed rhodopsin regeneration, 4) progressive loss of photoreceptors, 5) severely reduced retinal function. Selective ablation of the rod opsin remnant function showed that residual vision is mediated by cones in young R91W mice. The phenotype of R91W knock-in mice was distinctive from that of the Rpe65 knock-out mouse as evidenced by: 1) presence of 11-cis retinal and rhodopsin, 2) slower degeneration 3) prolonged survival of cones, 4) preserved cone function, which is lost early postnatally in Rpe65-/- mice. As opposed to gene transfer in Rpe65-/-, injection of LV-RPE65 at 1 month in R91W resulted in detectable optomotor response 1 month postinjection.

Conclusion: The R91W knock-in mouse mimics many aspects of the human pathology caused by this mutation and is used as a model for pre-clinical investigations. Particularly, the relative longevity of cones in the R91W knock-in mice may have dramatic impact on upcoming clinical trials with regard to the therapeutic window in the corresponding patients.

STEM CELL-BASED THERAPEUTIC APPROACHES TO RETINAL DISEASES

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Purpose: Stem cells are undifferentiated cells with the capability to self-renew and to give rise to various specialized cell types. Furthermore, stem cells are amenable to genetic manipulations. They are thus candidate cells to develop *ex vivo* gene therapies and cell replacement strategies. Because neural stem cells (NSCs) effectively integrate into developing and adult retinas, these cells might be used to target therapeutic gene products to diseased retinas. We therefore established protocols that allow efficient genetic manipulations of NSCs. In addition, we analyzed the fate of subretinally grafted primary retinal cell suspensions in adult host retinas to address the question whether cell transplantation is a feasible approach to replace dysfunctional or degenerated retinal cell types.

Methods: NSCs were isolated from the CNS of mouse embryos, and expanded as free-floating neurospheres or adherent cultures. Cells were either nucleofected with multigenic expression vectors or transduced with bicistronic lentiviral vectors, both allowing simultaneous expression of a gene of interest and a reporter gene. Primary retinal cell suspensions were prepared from young postnatal EGFP-transgenic mice and grafted into the subretinal space of adult wild-type mice. Recipient retinas were analyzed by immunohistochemistry and electron microscopy.

Results: Successfully nucleofected or transduced NSCs were selected by their expression of reporter genes using FACS. Selected cells were again expanded either as bulk cultures or cloned, giving rise to NSC cultures with homogenous expression levels of the transgenes. A fraction of subretinally grafted primary retinal cells integrated into the outer nuclear layer of adult host retinas and differentiated into cells that displayed a photoreceptor-like morphology. Immunohistochemical and electron microscopic analysis identified the grafted cells as rod photoreceptor cells with synaptic terminals ending in the outer plexiform layer and with inner and outer segments extending above the external limiting membrane.

Conclusions: We have established protocols that allow efficient genetic manipulations of NSCs. Whether genetically modified NSCs can be used to target functionally relevant quantities of therapeutic gene products to diseased retinas will now be tested in appropriate animal models. Integration of subretinally grafted primary retinal cells into the outer nuclear layer of adult host retinas and differentiation of donor cells into rod photoreceptor cells suggests that cell transplantation might be a feasible approach to replace dysfunctional or degenerated retinal cell types.

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POSTER ABSTRACTS

CHARACTERIZATION OF A MUTANT *OPA1* MOUSE – A MODEL FOR AUTOSOMAL DOMINANT OPTIC ATROPHY

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Purpose: Autosomal dominant optic atrophy (adOA) is caused mainly by mutations in the *OPA1* gene. Here we characterize for the first time a mouse mutant with a pathogenic *Opa1* mutation.

Methods: A sperm archive of ENU-mutagenized mice was screened for mutations in *Opa1* and a mutant strain revitalized by in vitro fertilization. The mutated *Opa1* allele, its transcript as well as its gene product were well studied in various tissues by qualitative and quantitative analyses (i.e. RFLP, Q-PCR, western). Mitochondrial DNA content was assessed by real time PCR. Embryonic lethality of homozygous mutant mice was followed in vivo by magnetic resonance imaging (MRI). Furthermore, the retina and the optic nerve were investigated by histology and electron microscopy and in addition by quantitative analysis of time dependent retinal ganglion cell (RGC) loss.

Results: We identified and established a mouse mutant with a heterozygous splice mutation (c.1065 + 5G>A) in the *Opa1* gene. This mutation leads to complete skipping of exon 10 and results in an in-frame deletion of 27 amino acid residues (p.329-355del) in *Opa1*'s GTPase domain. On transcript levels mRNA from both alleles are equal abundant, while *Opa1* protein levels are reduced to 50%. Protein from the mutant allele is in all probability not degraded via the proteasome. The mitochondrial DNA content was not altered. Homozygous mutant mice are not viable and die during embryogenesis around E8.5 - the stage when the neural fold starts closing. Histological analysis revealed an erratic loss of RGCs in *Opa1* mice most prominent at the age of 17 months. A progressive loss of RGCs was confirmed and quantified by retrograde labelling with hydroxystilbamidine. Electron-micrographs of the optic nerve revealed a reduced number of axons, swelling and distortion of the remaining axons. Furthermore, alterations in the mitochondrial cristae structure of mutant mice were more frequently as of controls.

Conclusions: Our *Opa1* mouse displays typical adOA features of RGC loss and degeneration of optic nerve axons.

PROPOSED APPROACH FOR THE MOLECULAR DIAGNOSIS OF RECESSIVE RETINITIS PIGMENTOSA PATIENTS

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Introduction: One of the main characteristics of Retinitis Pigmentosa is its high heterogeneity. The number of known genes and loci involved in autosomal recessive forms of RP are 17 (RetNet), but they only explain around 50 % of the cases. We used current methodologies to study proved or possible recessive cases of RP, in order to optimise the molecular diagnosis of the disease.

Patients and Methods: We gathered 118 uncharacterised RP Spanish families: 60 autosomal recessive RP (ARRP) and 58 sporadic cases (SRP). DNA was obtained from affected individuals and their healthy relatives. Two complementary methods were employed in parallel: 1) a haplotype analysis using STRs strongly linked to 13 major recessive RP genes (RPE65, CRB1, USH2A, MERTK, SAG, PDE6B, CNGA1, PDE6A, TULP1, RGR, PNR, RLBP1 and CNGB1), with a custom computer program that analysed the genotypes and was able to automatically rule out genes and show homozygosity alerts for each gene; and 2) a direct approach using an arRP genotyping array (Asper Biotech), which detects 518 mutations in 16 recessive RP genes, including those aforementioned, plus CERKL, RDH12 and USH3A.

Results: The 118 families did not include 7 families which had homozygous mutations in CERKL, because this gene was not initially included in the haplotype analysis. Only 2 families (1.7 %) were fully informative with both methods (the microarray found one mutation in homozygosis and the haplotype analysis showed a homozygosity alert for the same gene). Likewise, in another 2 families (1.7 %), the microarray found the two causative mutations, and although the indirect method did not give a homozygosity alert, the genes involved could not be ruled out. In 12 families (10.1 %), the microarray found one mutated allele, but the indirect method did not show any homozygosity alert. In a further group of 63 families (53.3 %, 28 ARRP and 35 SRP), we could not find any mutation with the direct method, but the haplotype analysis showed homozygosity alerts for at least 1, 2 or 3 genes. And finally, another 39 families (33 %, 23 ARRP and 16 SRP), were not informative at all with neither of the two methods.

Conclusions: Using both approaches, we only obtained the responsible mutations in 16/118 families. Even though the mutation rate (13.5 %) found with the microarray was low, we could still narrow down the number of future candidate gene/s to study in 63/118 families (53.3 %). We propose a three step study for the SRP and ARRP cases: they should be first studied with the genotyping microarray, followed by a haplotype analysis; and finally and if necessary, a specific additional candidate gene screening in many cases (~50 %). However, ~30 % of the cases will remain undiagnosed with this approach.

DIFFERENT EFFECTS OF VEGF ON THE SWELLING OF MÜLLER GLIAL CELLS AND GANGLION CELLS IN THE RAT RETINA

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Purpose: One major cause for the vision loss in the ischemic and diabetic retina is the presence of a macular edema. Vascular leakage as a main cause of edema is evoked predominantly by the vascular endothelial growth factor (VEGF). Another cause of edema is an impairment of the fluid absorption from the retinal tissue normally carried out by Müller glial and pigment epithelial cells which may be associated with cellular swelling (cytotoxic edema). It is known that the osmotic volume regulation of Müller cells is altered in the postischemic and diabetic retina. Retinal neurons swell when they are overstimulated by glutamate. We investigated the effect of VEGF on the osmotic swelling of Müller cells and on the cell size of retinal ganglion cells.

Methods: Transient retinal ischemia was induced in one eye of rats by elevation of the intraocular pressure for one hour. Diabetes was induced in rats with intravenous injection of streptozotocin. The animals were killed three days after transient ischemia and after six months of hyperglycemia, respectively. Swelling experiments were carried out in acutely isolated retinal slices or wholemounts.

Results: Administration of a hypotonic solution had no effect on the size of Müller cell somata in control retinas whereas Müller cells in postischemic and diabetic retinas displayed a swelling of their cell bodies. VEGF inhibited the osmotic swelling of Müller cell bodies. The swelling-inhibitory effect of VEGF was mediated by activation of KDR receptors which caused a release of glutamate from Müller cells. Subsequent activation of glutamate receptors evoked an autocrine swelling-inhibitory purinergic signaling cascade that involved the consecutive release of endogenous ATP and adenosine, resulting in the opening of ion channels, and inhibition of cellular swelling. In contrast, VEGF caused a swelling of ganglion cell bodies, via activation of ionotropic glutamate receptors. Glial cell-derived glutamate may contribute to the swelling of ganglion cell bodies since metabolic poisoning of Müller cells inhibited the VEGF-evoked cell swelling.

Conclusion: VEGF may have a dual effect on cytotoxic edema: it inhibits the swelling of Müller cells and evokes swelling of ganglion cells. Both effects are mediated by glutamate released from Müller cells.

CONE VERSUS ROD DISEASE IN A MOUSE MODEL FOR X-LINKED RETINITIS PIGMENTOSA

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Purpose: To understand the influence of the genetic background on retinal degeneration in a mouse model carrying a mutation in the gene encoding the retinitis pigmentosa GTPase regulator *(Rpgr)*.

Methods: The *Rpgr$^{del-Ex4}$* mutation was bred into a pigmented (C57BL/6) and an albino (BALB/c) mouse background over several generations. Retinal function was monitored by electroretinography (ERG). Mice were measured at different time points under scotopic and photopic conditions for discrimination of the rod and cone system, respectively.
Results: Phenotypical changes in retinal function 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 photoreceptor ERG amplitudes starting at the age of 3 month. Interestingly, there were no alterations in the photopic responses at any time point examined. 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. Later, a functional impairment of the rod photoreceptor system also became evident.

Conclusion: The genetic background has an impact on the photoreceptor cell type and disease progression. Our data also indicate a role for *Rpgr* in rods as well as in cones, which is in accordance with the observed retinal phenotypes in humans carrying mutations in *RPGR*. These mutant mouse lines provide suitable models to study *Rpgr* function in rods and cones, as well as molecular disease mechanisms.

PRPF 31 KNOCK-IN MOUSE AS A MODEL OF RETINITIS PIGMENTOSA 11

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Purpose: Pre-mRNA processing factor 31 (PRPF31) is a ubiquitous protein, needed for the assembly of the pre-mRNA splicing machinery. It has been shown that mutations in this gene cause autosomal dominant retinitis pigmentosa 11 (RP11), which is characterised by rod-cell degeneration. Interestingly, mutations in this ubiquitously expressed gene do not lead to phenotypes other than retinal malfunction. Furthermore, the dominant inheritance pattern has shown incomplete penetrance which poses interesting questions about the disease mechanism of RP11.

Methods: In order to characterise the specificity of *PRPF31* function in the rod cells, an animal model has been generated. This is a knock-in (KI) mouse carrying a point mutation A216P, which has been previously identified in RP11 patients. Degeneration of the KI mouse retina was monitored by electroretinography (ERG) and histology. Additionally, apoptotic assays on the retinal sections were performed.

Results: Generation of the mouse model will be presented as well as results of ERGs, retinal structure and apoptotic assays. There was no significant difference observed between the KI and WT retinas up the age of 1 year. However, in ERG measurement there is a tendency towards a minor decrease in b-wave amplitude comparing the 10 and 12 months old animals. These findings suggest that there might be a late onset degeneration starting at 12 months. The A216P mutation of *PRPF31* in the homozygous state was found to be lethal, therefore heterozygous KI mice were analysed.

Conclusions: This work shows that the KI A216P *PRPF31* mouse model for retinitis pigmentosa is a slow retinal degeneration model, which is a common feature for the haploinsufficiency animal models.

INTRAVITREAL BEVACIZUMAB IN TYPE 2 IDIOPATHIC MACULAR TELANGIECTASIA

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Background: Type 2 idiopathic macular telangiectasia (type 2-IMT) is a rare bilateral macular disease of unknown origin which may cause macular atrophy or secondary neovascular membranes. Deterioration of visual acuity starts within the 5^{th} to 7^{th} decade and the most striking functional deficit is development of parafoveal scotomas. A genetic predisposition has been suggested since affected monozygotic twins have been reported. So far there has been no therapy with proven benefit for type 2-IMT. The hallmarks of the disease are incompetent parafoveal capillaries seen in fluorescein angiography with low grade leakage most apparent in the late angiographic phase. Vascular endothelial growth factor (VEGF) may cause a breakdown of the blood-retina barrier. Therefore, we hypothesized a potential role of VEGF in the pathogenesis of the disease and treated a study cohort with the anti-VEGF drug bevacizumab.

Method: Seven eyes of six patients received two doses of intravitreal bevacizumab (1,5 mg) at four weeks intervals into the study eye. Serial examinations included funduscopy, digital fundus photography, best corrected ETDRS visual acuity (VA), fluorescein angiography and optical coherence tomography (OCT).

Results: VA showed a mean increase of eight ETDRS-letters at eight weeks ($p<0.05$). VA improved more than 15 letters in one patient, 10 to 15 letters in two patients and remained stable (-1 to +10 letters) in another four patients compared to baseline. In all eyes, there was a reduction in extension and intensity of late stage parafoveal hyperfluorescence in fluorescein angiography. In OCT-imaging, mean retinal thickness showed a statistically significant reduction in the foveal and in the parafoveal zones.

Conclusion: These short-term results indicate that VEGF-inhibition by intravitreally administered bevacizumab is associated with a reduction in angiographic leakage and a decrease in retinal thickness in type 2-IMT. VA may be stabilized and in a subset of patients, may be improved. The results support a pathophysiological role of VEGF in type2-IMT.

CADHERIN CAD99C, THE *DROSOPHILA* ORTHOLOG OF USHER SYNDROME PROTEIN PROTOCADHERIN 15, IS REQUIRED FOR NORMAL MICROVILLI MORPHOLOGY

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Usher syndrome is the most common form of combined deafness and blindness in humans. A number of genes have been identified that are etiologically associated with this disease, including two genes encoding the cell-cell adhesion molecules Protocadherin 15 and Cadherin 23. Both cadherins localize to stereocilia, microvilli-derived extensions of cochlear hair cells important for auditory perception. Stereocilia normally display an ordered, staircase-like arrangement on the surface of hair cells and are held together by several molecular links. In the absence of Protocadherin 15 or Cadherin 23, stereocilia do not display their normal ordered arrangement, and are instead splayed. Cadherin 23 has been shown to be part of the molecular link connecting stereocilia. However, the function of Protocadherin 15 is not well understood. The fruit fly *Drosophila melanogaster* has been successfully used as a model system for a number of human diseases. Here, we have identified the *Drosophila melanogaster* cadherin Cad99C as the ortholog of vertebrate Protocadherin 15. We show that Cad99C localizes in the fruit fly to microvilli of epithelial follicle cells. In the absence of Cad99C, microvilli are disorganized. Overexpression of Cad99C results in the bundling of microvilli, suggesting that this cadherin can provide molecular links between adjacent microvilli. Our results suggest that insects and vertebrates use related cadherins to organize microvilli-like cellular extensions. These data furthermore raise the possibility that the follicle cells could be used as a model tissue for the study of Usher syndrome related proteins in the fruit fly.

EUROPEAN APPROACH FOR THE PROMOTION OF YOUNG SCIENTISTS IN VISION RESEARCH: THE TÜBINGEN MODEL

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Purpose: Highly qualified and motivated young talents are the only resource to guarantee further development of scientific knowledge for research and clinics. Thereby scientific knowledge in basic research as well as well educated and competent human resources become for the European Community more and more indispensable to provide, at least, for the actual standard of living.
In order to ensure the competitiveness and the quality of research in Europe and to prevent a dramatic penury of researchers and physicians in a near future, as the EU predicts, the Unit for Research Management (URM) at the University Eye Hospital Tübingen (UEHT) initiated over 20 applications and was/or is still involved in the coordination of 12 research projects.

Methods: Most of the projects (9) coordinated by the URM are exclusively dedicated to transfer of knowledge and training for young researchers. Already during the FP5 (1998 - 2002) up to the brand new FP7 (2007 -2013), the Marie Curie Programme of the European Union still represents the best implement to promote and encourage young talents to join the scientific community and start a scientific career. All the young fellows taking part to our research projects have the possibility to enjoy an outstanding training programme going from an intense in-lab-training over several lab-exchanges to professional soft skills training. Over more, together with the own Principal Investigator, they have also the possibility to compile an individually tailored career development plan.

Results: All in all, from 1998 to 2002, the URM together with the best research host-institutions, spread all-over Europe, took successfully care for the scientific transfer of knowledge and training of 23 research fellows, mostly coming from East Europe. During the still ongoing projects (5) about 75 research fellows are obtaining or will obtain, amongst others, an excellent scientific and research training.
Nevertheless for the URM there is no reason to take a rest, the motivation is not broken, the "goal" is still not reach. Looking ahead, the just starting FP7 of the EU delivers to the URM the best prerequisites to develop new ideas and to realize new projects.

IDENTIFICATION OF GDNF-INDUCED RETINAL MUELLER GLIAL CELL-DERIVED NEUROPROTECTIVE FACTORS

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Purpose: Glial cell line-derived neurotrophic factor (GDNF) was found to rescue PR function during inherited retinal degeneration in rd1 mice and is thus one of the most effective neurotrophic molecules for PR rescue in retinal degeneration. Recent evidence has proposed that the GDNF-induced neurotrophic rescue of PR is indirect, mediated by interaction of GDNF with retinal Mueller glial cells (RMG) (Hauck et al., 2006). RMG are proposed to release as of yet unidentified factors in response to stimulation with GDNF. These factors are good candidates for directly supporting PR rescue during retinal degenerations. Therefore we aim at identifying those GDNF-induced RMG-derived factors.

Methods: The strategy is to obtain pure populations of primary RMG by using hGFAP::eGFP mice that express GFP exclusively in RMG. Retinas were disrupted enzymatically and RMG were sorted and collected by FACS. Freshly sorted RMG populations were then stimulated with GDNF or left unstimulated. Verification of induced signal transduction in response to GDNF stimulation was performed by Western blot analysis with antibodies against phosphorylated ERK. For analysis of mRNA changes induced by GDNF stimulation, pure populations of RMG were stimulated for 24h with GDNF and total RNA was prepared.

Results: Retinal specimen of hGFAP::eGFP adult mice show expression of GFP exclusively in RMG as demonstrated by co-staining with RMG-specific marker glutamine synthetase (GS). However, not all GS-positive RMG are also GFP positive, the percentage of GFP-positive RMG can be estimated at 50%. FACS sorting of GFP-positive cells from the pool of total retinal cells after enzymatic trituration yielded on average 4.34 % GFP-positive cells equalling approx. 11771 RMG per retina. Direct GDNF stimulation of FACS-sorted GFP-positive cells resulted in increased phosphorylation of ERK, thus confirming that intracellular signalling cascades are induced by GDNF in freshly sorted primary RMG from mouse retina. Affymetrix array experiments are currently underway from GDNF-stimulated and unstimulated FACS-sorted RMG.

Conclusions: The analysis of murine GFP-positive RMG from hGFAP::eGFP verifies that freshly sorted primary RMG are reactive to GDNF stimulation. With the aim to identify secreted candidate proteins that have survival promoting activity on PR, this experimental setting can be used to study GDNF-induced changes in the mRNA expression.

ANALYSIS OF *IN VIVO* PRENYLATION OF RABGTPASES: EVIDENCE OF A PRENYLATION HIERARCHY WITH RELEVANCE TO CHOROIDEREMIA?

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Protein prenylation is a post-translational form of lipid modification in which geranylgeranyl or farnesyl groups are conjugated to the C-terminal cysteins of a variety of proteins. The most commonly prenylated proteins are the geranylgeranylated Rabs, required for the regulation of vesicle trafficking.

In order to be prenylated, Rab proteins are bound by the Rab escort protein (REP) which presents the Rab to the Rab Geranylgeranyl transferase (RabGGTase), this prenylates the Rab on (usually) two C-terminal cysteins. The prenylated Rab is now insoluble however; the REP binds the two geranylgeranyl groups in a hydrophobic pocket. The prenylated Rab:REP complex now disengages from RabGGTase and REP delivers the Rab to the appropriate membrane where the Rab can undergo GDP/GTP exchange and carry out its roles in membrane trafficking.
Defects in prenylation can result in vesicle transport pathologies such as Choroideremia or Hermansky-Pudlak syndrome.

Choroideremia is an X linked disease, characterised by the degradation of retinal cells and blindness by middle age. It is caused by the knock out of REP1. REP2 can almost compensate however, large amounts of unprenylated Rab27A build up in the lymphoblasts of choroideremia patients. The connection between a reduction in prenylation capacity and retinal degeneration is not known.

The dissociation constant for REP2:Rab27A is four fold higher than for REP1:Rab27A. However, as this is comparable to a 2.7 fold difference for Rab1 and a 6 fold difference for Rab7, it does not explain the build up of unprenylated Rab27A. In absolute numbers however, the dissociation constant for Rab27A (with either REP1 or REP2) is four fold higher than for Rab1 and over 150 fold higher than for Rab7. The difference in dissociation constants for different Rabs may establish a prenylation hierarchy. Therefore if total REP activity becomes limiting Rab27A will be unable to compete favourably with other Rabs for prenylation.

We use microinjection of fluorescently labelled Rab1, Rab7 and Rab27A to show that, when Cherry-Rab27A is microinjected, it is prenylated and delivered to cell membranes on a significantly longer timescale than YFP-Rab7 and CFP-Rab1A. Co-microinjection of the prenylation machinery, and injection of in vitro prenylated Cherry-Rab27A suggests that prenylation is the major limiting factor in localisation. This suggests that a prenylation hierarchy does exist and that Rab27A is indeed at a disadvantage in the competition for limited prenylation activity.

A NOVEL GENE FOR USHER SYNDROME TYPE 2 *(USH2D)*: MUTATIONS IN THE LONG ISOFORM OF WHIRLIN ARE ASSOCIATED WITH RETINITIS PIGMENTOSA AND SENSORINEURAL HEARING LOSS

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Purpose: To identify the causative mutation in a German USH2 family unlinked to known gene loci for Usher syndrome.

Methods: Patients underwent detailed ophthalmological and audiological investigations. The seven proteins that have been identified for Usher syndrome type 1 (USH1) and type 2 (USH2) interact in a large protein complex. We assumed that mutations in proteins interacting with this "USH network" may cause Usher syndrome as well. *DFNB31* encodes whirlin, a PDZ scaffold protein with expression in both hair cell stereocilia and retinal photoreceptor cells. Whirlin binds to Usherin (USH2A) and VLGR1b (USH2C). Genotyping of microsatellite markers specific for the known USH loci and the *DFNB31* gene locus on chromosome 9q32 and direct sequencing was performed.

Results: Two individuals in the family investigated herein are affected by USH2. Hearing loss is mild in the younger patient and moderate in the older sister. Both individuals exhibited a phenotype of a rod-cone dystrophy with preserved visual acuity, concentric visual field loss, and typical intraretinal pigment clumping in the mid periphery. Mutations in known USH genes could be excluded by linkage analysis and direct sequencing. Patients showed common haplotypes for the *DFNB31* locus. Sequence analysis of *DFNB31* revealed compound heterozygosity for a nonsense mutation, p.Q103X, in exon 1, and a mutation in the splice donor site of exon 2, c.837+1G>A. No mutations were identified in an additional 96 USH2 patients.

Conclusions: We describe a novel genetic subtype for Usher syndrome, which we named *USH2D* and which is caused by mutations in whirlin. Moreover, this is the first case of USH2 that is allelic to non-syndromic deafness. While mutations in the C-terminal half of whirlin have previously been reported in non-syndromic deafness (DFNB31), both alterations identified in our USH2 family affect the long protein isoform. We propose that mutations causing Usher syndrome are probably restricted to exons 1 - 6 that are specific for the long isoform and probably crucial for retinal function. *DFNB31* mutations appear to be a rare cause of Usher syndrome.

PHENOTYPIC AND GENETIC STUDIES IN A STARGARDT DISEASE FAMILY SEGREGATING FOUR DIFFERENT ABCA4 MUTATIONS

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Stargardt disease is the most frequent cause of juvenile macular dystrophy characterized by decreased visual acuity, atrophic macular lesions and attenuated electroretinographic (ERG) responses. It is caused by recessive mutations in the ABCA4 gene encoding a photoreceptor-specific ATP-binding cassette transporter, which functions as an outwardly directed flippase for N-retinylidene-phosphatidylethanolamine. Mutations in ABCA4 are also associated with cone rod dystrophy and inverse retinitis pigmentosa. As established by van Driel (1998) there is an inverse correlation between the ABCA4 residual activity and the severity of the retinal dystrophy with the most severe phenotype, RP, caused by two null/severe ABCA4 mutations. If ABCA4 activity is partially retained on at least one allele, patients will develop either cone rod dystrophy or Stargardt disease.

We have now identified a family affected with Stargardt disease in two generations including two parents and their two adult children. Molecular testing identified four different mutations in the ABCA4 gene segregating in this family. Two mutations should lead to an amino acid change (His1838Asp and Arg2077Trp), one small insertion is predicted to cause a frame-shift (3211insGT) and one nucleotide change is known to alter the splicing behaviour (768G>T) in RNA maturation. Since the two missense mutations are regarded as mild mutations and the splice / frameshift-mutations represent null-alleles with a likely loss of protein function, at least three different phenotypes are feasible depending on the combination of disease alleles (mild/mild, mild/severe, severe/severe). The variable expressivity of ABCA4-asociated retinopathy should call the counsellor's attention to the possibility that clinically different phenotypes may be caused by separate disease alleles of the same gene. This is most crucial for providing accurate recurrence risks.

LATE-ONSET STARGARDT MACULAR DYSTROPHY MIMICKING ATROPHIC AGE-RELATED MACULAR DEGENERATION

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Purpose: We have recently identified distinct patterns of abnormal fundus autofluorescence (FAF) in the junctional zone of geographic atrophy (GA) in patients with AMD (BJO 2005; 89:874-8). A high degree of intra-individual symmetry in the FAF pattern, along with a high degree of inter-individual variability suggests genetic influence with underlying heterogeneity. Here we screened for mutations in the *ABCA4* gene in patients displaying a distinct FAF pattern that shares phenotypic similarities with FAF findings in juvenile Stargardt macular dystrophy.

Methods: In the prospective, multicenter natural history *FAM* study (ClinicalTrials.gov Identifier: NCT00393692), FAF images were recorded with a confocal scanning laser ophthalmoscope (cSLO, exc 488 nm; em >500 nm) in a total of 590 patients with AMD. Abnormal FAF patterns outside GA areas were classified by two independent readers. Seven patients exhibiting the FAF pattern "fine granular with peripheral punctate spots", and 11 patients exhibiting other FAF patterns were screened for mutations in the *ABCA4* gene by gene chip analysis of known mutations (ASPER Biotech, Tartu, Estonia). All changes were confirmed by direct sequencing.

Results: All affected patients reported an onset of visual impairment beyond 50 years of age. In the group with the FAF pattern "fine granular with peripheral punctate spots", all patients had at least one mutated allele in the *ABCA4* gene. In two patients both disease alleles were detected. In the control group of 11 AMD patients with GA, but a different pattern of abnormal FAF, only two patients showed one mutated allele.

Conclusions: These preliminary results indicate that the classification system of abnormal FAF has a genetic base and that the FAF pattern "fine granular with peripheral punctate spots" is caused by mutations in the *ABCA4* gene implying that this distinct FAF phenotype represents late-onset Stargardt macular dystrophy rather than late atrophic AMD. Based on these findings expanded genotype-phenotype correlations are planned in our study population. The results reflect the importance of refined phenotyping using novel imaging methods for addressing genetic factors in AMD and related disorders.

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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. We investigated 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 $Ba2^+$ 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 does not necessarily result in chronically increased concentrations of extracellular glutamate, because there might be other transport systems (e.g. neuronal glutamate transporter, Na^+-independent glial transport system) to compensate the reduction.

RETCHIP 1.0 – A NOVEL DIAGNOSTIC TOOL FOR A CHIP-BASED MUTATIONAL ANALYSIS OF RETINAL DEGENERATIONS

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Currently, approximately 150.000 legally blind and 500.000 visually impaired people are registered in Germany. Of these, individuals affected with one form of hereditary retinal degeneration (including those with additional systemic manifestations) represent a large fraction. A hallmark of the hereditary retinal degenerations is their striking genetic heterogeneity, i.e. a single clinical phenotype may be caused by mutations in many different genes and even more specific mutations. Consequently, to date genetic testing in hereditary retinopathies is time-, labor- and thus highly cost-intensive. Nevertheless, the availability of genetic diagnosis in retinal dystrophies is an essential part of the clinical diagnosis. It is indispensable to clarify the individual mechanism of pathogenesis and to propose targeted treatment options.

The array based re-sequencing technology now offers a cost-efficient high-throughput solution to establish comprehensive mutational analyses in genetically heterogeneous conditions such as the hereditary retinopathies. This technology qualifies to simultaneously analyze a large number of disease genes and provides a capacity to re-sequence up to 300,000 bases of double-stranded DNA on a single array. Besides clearly pathogenic mutations, such analyses will also reveal a number of nucleotide variations with unclear pathogenicity.

Our current concept for the design of a 300K re-sequencing chip includes 68 genes hitherto associated with generalized RP (excluding stationary RP), Stargardt disease, Bardet-Biedl syndrome or Usher syndrome. The 68 genes comprise 1142 corresponding coding exons which need to be individually amplified before the chip analysis. This prior amplification represents a crucial step and warrants a significant reduction of required PCRs. We therefore aim to establish a multiplex strategy which should reduce the number of PCR reactions by approximately two third. Overall, the testing strategy needs to be established on a platform with a high degree of automatization.

The genetic testing will be done in selected centres in Germany where clinicians, genetic counsellors and molecular geneticists work closely together. In a first phase, we plan to develop a structured setting for the clinical/genetic counselling and the establishment of a quality-controlled DNA diagnostic set-up. This phase will be closely accompanied by statistical/biometrical evaluation. This approach will ensure the development of standardized structures for the DNA testing of retinal dystrophies and will facilitate an official introduction of this novel technology into the German health care system.

NEURONAL GLUTATHIONE SUPPLY BY MÜLLER CELLS DURING OXIDATIVE STRESS

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Purpose: Glutathione plays an important role in the defense of cells against oxidative stress. Astrocytes, in co-culture with neurons, can support the neuronal glutathione metabolism by supplying glutathione precursors to neurons. We examined the metabolic glutathione support of Müller (glial) cells in acutely isolated mammalian retinae during oxidative stress.

Methods: Pieces of acutely isolated retinae from guinea pigs were incubated for various time periods with different concentrations of hydroperoxide (H_2O_2) or tert-butylhydroperoxide (t-BHP). The thiol-reactive vital dye, CellTracker Green CMFDA, was used to detect intracellular levels of reduced glutathione (GSH). The cellular distribution of the fluorescent dye (corresponding to the GSH distribution) was visualized by means of a confocal laser scanning microscope.

Results: In control retinae, the GSH-sensitive-dye was exclusively detected in Müller cells (MC), whereas the ganglion cells (GC) were devoid of fluorescence. After application of H_2O_2 or t-BHP to the retinal pieces, the GSH level in MC decreased and the GC became fluorescent. This shift of fluorescence from MC to GC depended on the duration and intensity of the oxidative stress. This GSH shift from Müller cells to GC is not affected by an inhibition of the gamma-glutamyl-transpeptidase or by a block of the GSH synthesis with buthioninesulfoximine. Removal of the extracellular sodium ions during the peroxide application prevents the increase of the intracellular GSH in GC, but not the decrease of the intracellular GSH in MC suggesting an involvement of a sodium-dependent uptake system. Multidrug resistant proteins as possible GSH transporters were detected in MC by immunohistochemistry.

Conclusions: During oxidative stress the GSH content in MC was decreased, whereas in GC increased levels of GSH could be detected. Because this increase is not dependent on a de-novo synthesis of GSH, a transfer of intact glutathione (oxidized and/or reduced) molecules from Müller cells to GC is suggested.

THE ROLE OF SPLICING IN THE PATHOGENESIS OF RETINITIS PIGMENTOSA AND A THERAPEUTIC STRATEGY TO TREAT SPLICE SITE MUTATIONS

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Introduction: Retinitis pigmentosa (RP) constitutes a major cause of blindness worldwide. The disease is clinically and genetically heterogeneous and mutations in RP-associated genes lead to death of photoreceptors often resulting in complete blindness.
Purpose: We identified and characterize a novel exon in the Retinitis Pigmentosa GTPase Regulator *(RPGR)* and aimed to develop a strategy to treat splice site mutations in RP patients.

Methods: A combination of database analyses, quantitative RT-PCR, homology modeling, fluorescence microscopy on human retina slices, mutation screening of 78 RP patients, and minigene assays in cell culture systems were used.

Results: Bioinformatic approaches predicted novel exons and splice isoforms in 60-70% of all RP-associated genes. We identified and characterized the novel exon 9a of *RPGR* and showed that this new *RPGR* protein isoform has unique structural properties, distinct interaction partners and a specific localization within photoreceptor cells of the human retina. Furthermore, we found a mutation in an RP patient upstream of exon 9a. As shown by quantitative RT-PCR, the mutation leads to increased expression levels of exon 9a transcripts in the RP patient, whereas non-affected family members show significantly lower levels. This suggests that the mutation affects regulated alternative splicing of *RPGR* exon 9a.
Additional mutation screenings of candidate genes in RP patients revealed a novel mutation in rhodopsin *(RHO)*. The mutation is located at the last base of exon 4. Using a minigene approach, we showed that the mutation interferes with normal splicing of *RHO* and generates aberrant transcripts. To test therapeutic strategies to treat splice donor site mutations, we mutated snRNA-U1 to adapt to the *RHO* mutation. Quantitative analysis of co-transfections of the minigene and the mutated snRNA-U1 suggested a rescue of normal splicing of *RHO*.

Conclusion: These findings help to understand the relevance of splicing in the pathogenesis of RP and confirm the feasibility of a therapeutic strategy to treat splice donor site mutations in RP patients.

GENE TRANSFER INTO NEURONS OF ORGANOTYPIC RETINA CULTURES BY ELECTROPORATION AND BALLISTIC TRANSFECTION

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Purpose: The highly specialized neurons in the network of the vertebrate retina survive and maintain their function only in the connection to their neighbouring and co functioning retinal cells. Because of this essential network, retinal cells, especially photoreceptor cells, can not be studied in primary cell cultures. For the analyses of protein functions in retinal cells the organotypic retina culture provides an accessible *ex vivo* system, devoid of animal experiments.

Methods: Retinas of maturating mice (PN8-PN19) were cultured and cell death was subsequently analyzed. Cultured retinas were transfected with eGFP constructs either by electroporation or ballistic transfection (Gene Gun™). The expression of eGFP fusion proteins in retinal cells were analyzed by fluorescence microscopy in whole mounts and cryosections. Transfection efficiencies were assessed for different ages of the explants as well as for different transfection methods. Furthermore, transfected cells were identified by co-labelling with antibodies against cell type specific marker molecules.

Results: Retina cultures transfected via electroporation showed a higher amount of eGFP fusion protein expressing cells, especially in younger stages. In these younger explants (PN8), transfected cells were localized in the outer nuclear layer and in the inner nuclear layer, where as in the adult explants only cells of the retinal pigment epithelium could be transfected successfully. In contrast gene transfer by the Gene Gun™ method showed lower efficiency of transfection, but did deliver gene constructs also to cells of the inner retina. Ballistic transfection experiments also revealed that the transfection of specific retinal cell types correlated with the pressures of DNA gold practical acceleration applied.

Conclusions: The organotypic retina culture proofed as vital and suitable system for gene transfers to different cell types of the retina *ex vivo*. The electroporation method is appropriate for transfection of retinal cells in the outer and inner most layers in juvenile retinas. By application of the Gene Gun™, fewer cells were transfected, but DNA constructs were successfully delivered to all retinal cell types, including cells of the inner retina which are commonly unreachable for external applied transfection reagents and gene constructs.

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THE MOLECULAR ARRANGEMENT OF AN USHER SYNDROME PROTEIN NETWORK AT THE PHOTORECEPTOR CILIUM AND ITS ROLE IN THE INTERSEGMENTAL TRANSPORT IN PHOTORECEPTORS

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Purpose: The Human Usher syndrome (USH) is the most common form of combined deaf-blindness. USH is clinical (type 1 to 3) and genetically heterogeneous and 10 USH genes encode proteins of diverse protein families. All identified USH type 1/2 proteins can be connected by the scaffold protein harmonin (USH1C) within a USH interactom. Here, we address the question whether USH1/2 proteins are also qualified to form a protein network at the ciliary apparatus of retinal photoreceptors in the absence of harmonin.

Methods: Subcellular localization of proteins: Western blots of retinal tangential sections and subcellular fractionation, immunofluorescence and immunoelectron microscopy of mouse and *Xenopus* retinas; protein-protein interaction assays: yeast-two-hybrid, co-transfection of cell lines, GST-pull down assays of recombinant expressed polypeptides; pharmacological microtubule destabilization in organotypic retina cultures.

Results: In retinal photoreceptors, the subcellular distribution of SANS (USH1G) was dependent on the microtubule cytoskeleton. SANS co-localized with USH2A, VLGR1 (USH2C), and the scaffold protein whirlin in the ciliary apparatus - especially in the periciliary ridge complex of photoreceptor cells. In this complex, USH2 proteins provide molecular linkages between the plasma membrane of the cilium and the inner segment (calycal processes) of photoreceptor cells. Whirlin directly bound SANS and the USH2 proteins via its PDZ-domains.

Conclusions: In the ciliary specialization of photoreceptor cells, USH proteins are integrated into a protein network, associated with the cytoskeleton. As molecular components of the periciliary ridge complex this network may contribute to the handover of cargos between the inner segment and the transport through the connecting cilium. Defects of complex partners may lead to dysfunction of the entire USH network and cause photoreceptor degeneration in USH patients.

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POSITIVE FEEDBACK REGULATION BETWEEN MMP-9 AND VEGF IN HUMAN RPE CELLS

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Purpose: The proteolytic activity of matrix metalloproteinases (MMPs) is involved in pathological angiogenesis in the eye. However, it is unknown whether MMPs may stimulate the production of the major angiogenic factor, vascular endothelial growth factor (VEGF). There-fore, we determined the effects of MMP-2 and MMP-9 on the expression of VEGF by retinal pigment epithelial (RPE) cells. Furthermore, we investigated the effects of these MMPs on cellular proliferation and migration, and the effect of triamcinolone acetonide on the MMP-9 evoked cellular responses.

Methods: Human RPE cell cultures were stimulated with MMP-2 or MMP-9. The gene ex-pression and secretion of MMP-9 and VEGF were determined by real-time PCR and ELISA, respectively. Cell proliferation was investigated by a bromodeoxyuridine immunoassay, and chemotaxis was examined with a Boyden chamber assay.

Results: RPE cells *in vitro* express mRNAs for MMP-2 and MMP-9. Chemical hypoxia caused an upregulation of the gene expression of both MMPs, and VEGF increased the gene expression and secretion of MMP-9. Exogenous MMP-9 increased the gene expression of VEGF-A, and had no effects on the gene expression level of VEGF-B, -C, -D, flt-1, and KDR. The secretion of VEGF-A was dose-dependently increased by MMP-9 at higher con-centrations. MMP-2 and -9 did not alter the proliferation but stimulated dose-dependently the migration of RPE cells. The motogenic effect of MMP-9 was mediated by activation of the p38 mitogen-activated protein kinase and phosphatidylinositol-3 kinase. Triamcinolone fully inhibited the stimulatory effect of MMP-9 on the secretion of VEGF while it had no effect on the motogenic action of this proteinase.

Conclusion: There is a positive feedback regulation between MMP-9 and VEGF in RPE cells. The hypoxic expression of MMP-9 in RPE cells may stimulate production and secretion of VEGF under pathological conditions. Triamcinolone may inhibit neovascularization via blockade of the MMP-9 mediated upregulation of VEGF, and by decreasing the gene expres-sion of MMPs.

OPTICAL AND ELECTROPHYSIOLOGICAL RECORDING OF GANGLION CELL ACTIVITY IN THE MOUSE RETINA

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Purpose: The retina provides a neuronal network that performs the first steps in visual information processing. The retinal ganglion cells (RGCs) serve as output neurons that relay the information to the brain. We employed two approaches to study neuronal activity of RGCs *in vitro:* **A)** electrophysiological recording of light responses, and **B)** calcium imaging to visualize changes in the intracellular calcium concentration, that accompany or underlie neuronal activity.

Methods: A) RGCs of dark adapted retinae were patch-clamped in the whole cell mode. Light stimuli were generated on a computer screen and projected through the microscope optics onto the retina. Receptive fields of RGCs were mapped using small spots of light. During recording, RGCs were filled with markers. After the experiment, RGC morphology was analyzed using confocal microscopy. **B)** Transgenic mice that express the calcium indicator protein TN-L15 under the control of the thy-1 promoter were used. Flat-mounted retinae were imaged using two photon laser scanning microscopy. RGC types that express TN-L15 were identified using immunohistochemical markers.

Results: A) Light responses and their underlying synaptic currents were recorded in RGCs using scotopic and mesopic stimuli. The size of the receptive field centre matched the diameter of the dendritic tree of the RGC. Compared to centre responses, surround responses were smaller in amplitude and of opposite polarity. Using mesopic stimuli, in most RGCs excitatory synaptic currents faithfully reflected flicker frequencies up to 12.5 Hz. **B)** TN-L15 was expressed in the majority of RGCs. In all TN-L15-positive RGCs, increases in the intracellular calcium concentration were observed upon depolarization with high extracellular potassium or after stimulation with the glutamate receptor agonist AMPA, while only a fraction of cells responded to the glutamate receptor agonist Kainat.

Conclusions: Transgenic mouse lines that express genetically encoded calcium sensor proteins will be helpful to study calcium signals and synaptic input in RGCs *in vitro.* Electrophysiologically, synaptic currents that underlie RGC light responses can be analyzed in detail. Our approaches will not only enable us to identify and classify RGC types on the basis of their light responses, but also to analyze signal processing in the retina in depth using pharmacological and transgenic approaches.

CANDIDATE GENE APPROACH IN CONE AND CONE-ROD DYSTROPHIES

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Purpose: Cone and cone-rod dystrophies are a clinically and genetically heterogeneous group of retinal degenerations characterized by decreased visual acuity, color vision defects, photophobia and decreased sensitivity in the central visual field. In cone-rod dystrophies patients later also experience progressive loss in peripheral vision and night blindness. Several genes have been reported to be associated with these disorders, and it has been shown that mutations in the *ABCA4* gene are responsible for a high percentage (30-60%) of autosomal recessively inherited cone-rod dystrophies. In contrast, no comprehensive study has been presented for the autosomal dominant forms. Only few reports exist describing mutations in the genes for *RDS, CRX, GUCA1A and GUCY2D*. Therefore, we screened 28 independent patients with a diagnosis of cone- and cone-rod dystrophies and an autosomal dominant mode of inheritance for mutations in these genes.

Methods: All coding exons of *RDS, CRX, GUCA1A and GUCY2D* were screened by PCR amplification out of genomic DNA with primers derived from adjacent intronic sequences followed by direct sequencing. Segregation of mutations with the disease within the respective families was confirmed by PCR/RFLP or direct sequencing upon availability of parents, siblings and offsprings.

Results: We found three mutations in the *RDS* gene, one new 1bp-deletion in the *CRX* gene, and four mutations in *GUCA1A*. In *GUCY2D* we observed two common mutations to be causative for cone- and cone-rod dystrophies in our patient collection.

Conclusions: Mutations in the genes *RDS, CRX, GUCA1A* and *GUCY2D* are frequently associated with autosomal dominant forms of cone and cone-rod dystrophies. Together mutations in these four genes account for almost 60% of all analysed patients.

INTRACELLULAR FUNCTION OF BESTROPHIN-1 ENCODED BY VITELLIFORM MACULAR DYSTROPHY GENE VMD2

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Introduction: Vitelliform macular dystrophy *(VMD2)* also known as Best macular dystrophy (BMD) is an autosomal dominant disorder characterized by egg-yolk macular lesions of retina, decrease slow 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. *VMD2* gene encodes bestrophin-1 which is expressed predominantly in RPE cells. Bestrophin-1 function has been proposed as chloride channel or to have regulatory function on voltage dependent Ca^{2+} channels. In this study, we investigated the expression, subcellular localization and intracellular function of endogenous and heterologously expressed bestrophin-1.

Methods: Immunocytochemistry: 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.
Western blot: Protein analysis of endogenous bestrophin-1 from freshly isolated RPE cells of adult human donor eyes, or heterologously expressed in HEK293 or CHO-K1 cells, were performed with protein extracts from different cell compartments.

Results: The subcellular distribution of endogenous bestrophin-1 on primary human RPE cells revealed cytoplasmic expression pattern. Detection of heterogously expressed bestrophin-1 revealed a comparable pattern in cytoplasmic compartment using bestrophin-GFP fusion construct. The fluorescence signal in cells were seen perinuclear. Cytosolic localization of endogenous bestrophin-1 freshly isolated from human RPE cells, and also heterogously expressed was confirmed by western blot analysis and support data obtained from immunocytochemistry. Here, bestrophin-1 is expressed in a different subcellular fraction containing cell organelles

Conclusion: 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.

THE AXON GUIDANCE MOLECULE NETRIN-4 IS EXPRESSED BY RETINAL (GLIAL) MÜLLER CELLS AND IS INVOLVED IN RETINAL ANGIOGENESIS

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The balance between pro- and antiangiogenic modulators is considered important for maintaining an antiangiogenic milieu in the healthy retina. Netrin-4 is an axonal guidance molecule and has also been reported to promote angiogenesis. The aim of this study was to explore whether netrin-4 is expressed by retinal (glial) Müller cells and whether it promotes angiogenesis-related activities of retinal endothelial cells.

Expression of netrin-4 was examined using reverse transcription (RT)-PCR, Western Blotting, and immunofluorescence analysis of retinal cells and tissue. In the presence of netrin-4, migration and proliferation of bovine microvascular retinal endothelial cells as well as phosphorylation of the mitogen-activated protein (MAP) kinases, ERK-1/-2, were analyzed.

Cultured Müller glial cells were found to express netrin-4. Hypoxic conditions resulted in increased mRNA levels of netrin-4. Retinal endothelial cells demonstrated increased migration, proliferation, and ERK-1/2 phosphorylation following stimulation through netrin-4 in a dose-dependent manner.

These results suggest that retinal glial (Müller) cells are able to modulate netrin-4 levels which may contribute to the balance between positive and negative modulators of angiogenesis in the retina. This balance could be shifted in hypoxic conditions when the Müller cells upregulate netrin-4. Netrin-4 might act in concert with other proangiogenic factors to stimulate angiogenesis by increasing ERK-1/2-mediated proliferation as well as migration of retinal endothelial cells.

EGR1 AND PU.1 REGULATORY NETWORKS CONTROL RETINAL MICROGLIA ACTIVATION IN THE MOUSE MODEL FOR X-LINKED JUVENILE RETINOSCHSIS

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Purpose: Genetic and biochemical evidence suggests that innate immunity plays a pivotal role in retinal degenerative disorders. Our previous work in the mouse model for X-linked juvenile retinoschisis (RS1h$^{-/Y}$) has identified microglia activation preceding photoreceptor apoptosis and retinal degeneration. In this study, our aim was to further characterize underlying early signaling events, transcriptional markers, and effector mechanisms in primary microglia and model cell lines.

Methods: Early postnatal retinae and *ex vivo* microglia cells from retinoschisin-deficient and wild type mice were subjected to DNA-microarray and realtime qRT-PCR analysis. Cells were functionally characterized and lineage markers and molecular activation patterns were examined. RAW264.7 and BV-2 cells were used as model system to study TLR4-dependent activation of microglia-specific genes and to characterize TLR4-responsive Egr1 promoter elements. Chromatin Immunoprecipitation (ChIP) was performed to identify *in vivo* Egr1 target genes in activated microglia and reporter gene assays were used to analyze microglia-specific promoters.

Results: After isolation, purification and culture, phase contrast micrographs and latex bead phagocytosis assays demonstrate a highly activated state of retinoschisin-deficient microglia cells compared to controls. Starting from the induced transcripts in retinal samples of RS1h$^{(-/Y)}$ mice, a transcriptional network of TLR4-activated and Egr1-dependent genes composed of Casp11, Lyzs, Clec7a, Fcer1g, Ccl6 as well as a group of highly expressed and Egr1-independent constitutive markers (Tyrobp, Cd68, and S100A6) could be specifically allocated to the microglia population in the diseased retina. Furthermore, LPS-stimulation of RAW264.7 and BV-2 cells mimicks endogenous microglia activation, up-regulates the TLR4-dependent gene cluster and targets a specific region in the proximal Egr1 promoter. We could also demonstrate that the strong expression of the negative regulator Tyrobp in activated microglia is critically dependent on the myeloid transcription factor PU.1, which binds and activates the proximal Tyrobp promoter.

Conclusions: TLR4 signaling and up-regulation of Egr1 are early events during microglia activation in Rs1h-deficiency leading to transcriptional induction of pro-inflammatory and activation-induced apoptosis pathways. The expression of potentially microglia-deactivating genes such as Tyrobp is Egr1-independent and requires the myeloid transcription factor PU.1. In conclusion, our studies on gene regulatory networks in activated microglia cells point to novel disease pathways and targets for therapeutic options in neurodegenerative retinal disease.

MÜLLER CELL-CONDITIONED MEDIUM PROMOTES THE FORMATION OF IPL-LIKE STRUCTURES DURING *IN VITRO* REGENERATION OF THE MAMMALIAN RETINA

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Purpose: Retinospheres represent a model system, which reflects the histotypic structure and developmental properties of the *in vivo* retina. Moreover, retinospheres can be easily manipulated pharmacologically and genetically. For this reasons, they are important tools for therapeutic screening and further understanding of retinal development. Although dissociated cells of the embryonic chicken can be transferred to histotypic retinospheres, a comparable mammalian system is not yet developed. Dissociated retinal cells of neonatal rats, cultured under conditions of constant rotation, give rise to three-dimensional retinospheres, but do not organise themselves in retinotypic stratification. The aim of this work is to improve the reorganisation process to achieve nuclear and plexiform layers similar to the *in vivo* retina.

Methods: Retinae from neonatal rats were dissociated and the cells were cultured under constant rotation. Retinospheres growing under these conditions were treated with Müller cell-conditioned medium (MC-CM). After several days the influence of the MC-CM on proliferation, apoptosis and morphological improvements was analysed with immunohistochemical stainings.

Results: Retinospheres treated with MC-CM showed a less homogeneous but significantly increased size. MC-CM led to an increase in proliferating cells and the period of proliferation was prolonged. Furthermore, the amount of bipolar cells was increased and the rise in the bipolar cell population was accompanied by a consolidated inner plexiform layer.

Conclusions: Retinospheres, derived from rat retinal cells, cultured under the influence of MC-CM, which represents a mixture of growth factors, could be kept in a proliferative state for a longer period. Thereby, the system is able to organise histotypically before the cells reach a completely differentiated state. The formation of an enhanced inner plexiform layer indicates an improved organisation. Further studies will show if MC-CM is also influencing other cell types.

VITAMIN C PROTECTS LIGHT DAMAGED HUMAN RETINAL PIGMENT EPITHELIAL CELLS (ARPE-19) FROM APOPTOSIS VIA THE INDUCTION OF HEME OXYGENASE-1 (HO-1)

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Introduction: Photochemical damage to retinal pigment epithelial (RPE) cells and photoreceptors is involved in the pathogenesis of age-related macular degeneration (AMD). Numerous studies have suggested that photochemical damage includes oxidative events by which retinal cells die by/via apoptosis in response to photodynamic stress. Transcriptional activation of the HO-1 gene was observed after light damage which is considered as an adaptive response to oxidative and cellular stress. HO-1 can provide cytoprotection against oxidative injury and cellular stress. Because ascorbic acid (AA) is known to prevent or delay retinal degenerative processes we investigated the effects of AA on photochemically damaged retinal pigment epithelial cells especially in respect of affecting HO-1 gene expression.

Methods: Differentiated ARPE-19 cells were incubated for 8h with 56µM AA. Uptake of AA was measured using HPLC analysis. To investigate the protective role of vitamin C against photochemical damage, we treated differentiated ARPE-19 cells with the photodynamic active substance Merocyanin 540C (MC540) for 4 h. Cells were illuminated with a light intensity of 50 mW/cm^2 and a light dose of $4J/cm^2$. HO-1 mRNA expression was measured by microarray analysis and real-time-PCR. The expression of HO-1 protein and PARP cleavage (apoptosis marker) was determined by western blot analysis 24h after irradiation. Futhermore, cell viability was assessed by LDH-release and MTT assay.

Results: Using microarray analysis, we could show that illuminated differentiated ARPE-19 cells significantly induced mRNA expression of protein phosphatase like MKP-1 at 8 fold versus control. These results were confirmed by protein expression using western blot anlysis. AA alone did not affect HO-1 expression and cell viability. Photochemical damage resulted in an increased number of dead cells undergoing apoptosis (PARP cleavage) and HO-1 expression was induced. But in the presence of AA, ARPE-19 cells were damaged less by photochemical stress. This ameliorated cell viability correlated significantly with a higher upregulation of HO-1 protein expression and down regulation of PARP cleavage. AA protected irradiated cells against cell death and apoptosis.

Discussion: The protective effect of AA against light damage might be attributed to its role as an antioxidant or to the induction of HO-1 or may be the result of both effects. In the next series of experiments, we will investigate the role of HO-1 in maintaining cell viability by inhibiting HO-1 activity by ZnPPIX. Research on the signal transduction pathway of HO-1 in the prevention of light damage may lead to a better understanding of the role of vitamin C in the prevention of AMD.

CELLULAR UPTAKE OF VITAMIN C (ASCORBIC ACID) IN DIFFERENTIATED HUMAN RETINAL PIGMENT EPITHELIAL CELLS (ARPE-19) BY THE SODIUM-ASCORBATE COTRANSPORTER (SVCT2)

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Introduction: Vitamin C is an electron donor and is a potent water-soluble antioxidant in humans. Vitamin C is known to mediate a variety of enzymatic reactions, including collagen synthesis, the basis for the defect in scurvy. Vitamin C also protects tissues from oxidative damage by scavenging free radicals. Vitamin C can not be synthesized in the human organism and therefore, it must be included in the diet. Ascorbic acid (AA) and dehydroascorbic acid (DHAA, oxidized vitamin C) are dietary sources of vitamin C in humans. DHAA is transported predominantly by specific glucose transporters (for example GLUT1, GLUT3 and GLUT4). Whereas AA is transported mainly through sodium-dependent vitamin C transporters (SVCT1 and SVCT2 proteins) that are encoded by the genes SLC23A1 and SLC23A2, respectively. The aim of this study was to determine whether differentiated ARPE-19 cells express the SVCT mRNA gene and consecutively to determine the intracellular concentration of AA in a time- and dose-dependent manner in these cells.

Methods: ARPE-19 cells (ATCC CRL-2302), a not transformed human diploid RPE cell line, display many properties typical of RPE *in vivo* if cultured more than 8 weeks. Within this time, the expression of RLBP1 and RPE65 mRNA, two specific gene markers which are expressed as a function of RPE cell differentiation, were upregulated. Furthermore, we determined the expression of SLC23A2 (SVCT2) mRNA using real-time-PCR and uptake of AA was measured using HPLC analysis.

Results: Differentiated ARPE-19 cells were incubated with the physiologically relevant AA concentration of 56 µM in a time dependent manner (10 min to 24 h). Supplemented differentiated ARPE-19 cells showed maximal cellular uptake of AA after 4 h incubation time (1.7 ± 0.28 mM). However, longer incubation times decreased the intracellular concentration of AA significantly. Therefore, we determined the cellular uptake of AA in a dose- (56, 280, 560 µM) and time dependent manner (4, 6, 8 h). Intracellular vitamin C concentrations reached a plateau level after 8 h when the differentiated ARPE-19 cells were supplemented with AA concentrations above 280 µM.

Discussion: We showed that differentiated ARPE-19 cells significantly induced the sodium-ascorbate cotransporter (SVCT2) which is probably responsible for the cellular uptake of AA. The cellular uptake of AA in differentiated ARPE-19 cells was comparable with intracellular vitamin C concentrations in various cell types found *in vivo* studies.

CYTOPROTECTIVE ROLE OF MITOGEN-ACTIVATED PROTEIN KINASE PHOSPHATASE-1 (MKP-1) IN LIGHT DAMAGED HUMAN RETINAL PIGMENT EPITHELIAL CELLS (ARPE-19)

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Introduction: MAP kinase phosphatses (MKPs) are of dual specificity. MKP-1 e.g. dephosphorylates MAP kinases like JNK, p38, and ERK at Thr/Tyr residues, thereby contributing to a down-regulation of MAP kinase activity which is important to switch the cell from the "survival" to the apoptotic program. The aim of our study was to determine whether light damaged retinal pigment epithelial cells change their expression of MKP-1 and to what extend changes in the MAP kinase pathway are involved in the induction of apoptosis in these cells.

Methods: ARPE-19 cells were differentiated by keeping the cells for more than 8 weeks in culture. To investigate the cytoprotective role of MKP-1 concerning light damage, we treated differentiated ARPE-19 cells with the photodynamic active substance Merocyanin 540C (MC540) for 4 h. Thereafter, ARPE-19 cells were illuminated with a light intensity of 50mW/cm^2 and a light dose of 1- 4 J/cm^2 in absence and presence of 56 µM ascorbic acid (AA). After 3 h and 24 h, mRNA was extracted and used for microarray analysis and gene profiling of various stress genes, transcription factors and protein phosphatases. Furthermore, the expression and activity of MKP-1 and MAP kinase family was investigated by western blot analysis. 24 h after the induction of light damage, PARP cleavage (apoptosis marker) was determined by western blot analysis. In addition, cell viability was assessed by the LDH assay.

Results: Analysis of microarray data showed, that in light damaged ARPE-19 cells the expression of protein phosphatases 1 and 8 (MKP-1: 8- and MKP-5: 5-fold) was significantly induced. The results of the microarray analysis were verified by western blot analysis using a light dose of 1- 4 J/cm^2. Low light doses (1 and 2 J/cm^2) induced MKP-1 expression and inactivated JNK, but p38 and ERK were not altered upon treatment. High light doses (3 and 4 J/cm^2) decreased the expression of MKP-1 which resulted in an activation of SAPK/JNK. After 24 h recovery time the high light dose treatment induced apoptosis and increased cell death. Vitamin C (56 µM) inhibited the decrease of MKP-1 protein expression upon high light dose treatment and resulted in a smaller activation of JNK. This "inactivation" of the JNK pathway resulted in decreased PARP cleavage (apoptosis) and ameliorated cell viability.

Discussion: The MAPK phosphatase, MKP-1 is capable of inactivating the stress-induced protein kinase (JNK). Inactivation of the JNK pathway inhibits apoptosis and ameliorates cell viability. The cytoprotective properties of MKP-1 do not appear to be mediated by the p38 or the ERK pathway but rather by the JNK pathway in differentiated ARPE-19 cells. Our results are consistent with other *in vitro* studies, which have shown that MKP-1 provides cytoprotection against stress-induced apopotosis by inhibiting JNK activity.

RD1 PHOTORECEPTOR CELL SURVIVAL AS A FUNCTION OF CALPAIN INHIBITION PARADIGMS

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Purpose: The rd1 mouse demonstrates an inherited retinal degeneration which allows for studies of the molecular mechanisms underlying 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, there is so far limited knowledge regarding the roles of calpain activity during inherited retinal degeneration.
Previously, we have shown that calpain expression in rd1 mouse retina is unchanged when compared to the corresponding wt counterpart. Activity of calpain is, however, dramatically increased in rd1 photoreceptors and correlates with markers of cell death. This suggested a causal connection between excessive calpain activity and cell death and further prompted us to study the effect of calpain specific inhibitors on photoreceptor viability in an *in vitro* retinal explant culture system and on *in vivo* retinae.

Methods: Retinae from rd1 and wt mice were cultured via an organotypic explant system under various treatment paradigms testing for the effects of calpain inhibitors on the photoreceptors. Calpain inhibitor XI was administered starting from the third day of culturing for the chronic paradigm and in the last 16 hours of the cultures for the acute treatment paradigm. Calpastatin peptide, a highly specific inhibitor of calpains, was also utilized in a sustained, chronic situation.

Results: Here we show that very low concentrations of calpain inhibitor XI, when applied chronically, or high concentrations, when applied in an acute fashion, protect rd1 photoreceptors from mutation-induced cell death. Unexpectedly, moderate to high concentrations of calpain inhibitor XI induce selective photoreceptor cell death when employed in a chronic application paradigm. Differentially, calpastatin peptide appears to preserve photoreceptors at a moderate concentration under chronic application.

Conclusions: The data suggest that calpain may be essential for both the promotion and prevention of cell death. These ambiguous results may be due to calpain isoform specific effects and should be an important consideration as the use of calpain inhibitors to prevent or delay photoreceptor degeneration is further studied.

CELL PHYSIOLOGIC ANALYSES OF RPE CELLS OF VMD2 DEFICIENT MICE

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Purpose: Best's disease, an early onset macula dystrophy, is caused by mutations in the *VMD2* gene expressed in the retinal pigment epithelium (RPE). Aim of this study was to investigate the cell physiology in RPE cells of *Vmd2*-deficient and wild-type *(wt)* mice.

Methods: RPE cells were isolated from murine eyes under Ca^{2+}-free conditions and held over night prior measurement. Cl^- membrane currents were measured under K^+ free conditions using the patch-clamp technique. Intracellular free calcium concentration ($[Ca^{2+}]_i$) was monitored using the Fura-2 Ca^{2+} imaging method.

Results: Freshly isolated RPE cells showed a Ca^{2+}-sensitive Cl^- current, but no difference in membrane conductance between *wt* and *Vmd2* deficient mice. RPE primary culture and ARPE-19 cell line showed a similar pattern of ATP (100nM) induced $[Ca^{2+}]_i$ responses. An initial fast $[Ca^{2+}]_i$ increase was followed by a slight transient decrease below resting $[Ca^{2+}]_i$ before a sustained increase followed. Emptying intracellular Ca^{2+} stores by thapsigargin (1µM) reduced the initial $[Ca^{2+}]_i$ increase. RPE cells from *Vmd2* deficient mice showed a faster and higher Ca^{2+}-response compared to RPE cells from *wt* mice.

Conclusions: These results seem to support our theory, that bestrophin, the product of the *VMD2* gene, might be involved in the Ca^{2+} homoeostasis of RPE cells and that impaired Ca^{2+} homoeostasis might be important for further understanding of Best`s disease.

RREPORTGENERATOR, A VERSATILE TOOL FOR AUTOMATIC STATISTICAL ANALYSIS OF HIGH THROUGHPUT DATA IN VISION RESEARCH

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Purpose: Since high-throughput analysis methods, in particular genome wide transcription profiling and transfected cell arrays (TCA), have become part of standard testing routines in vision research there is an increasing need for automatic analysis methods. While the statistical platform "R" (http://www.r-project.org/) and the vast collection of additional modules on CRAN and BioConductor allow very powerful statistical analysis, it's particular command-line syntax renders the program inaccessible for non-statisticians.

Methods: Via it's graphical user-interface RReportGenerator launches "R" and runs the data-analysis following a predefined scenario. Without any further user interaction the analysis reports containing the results are generated using the R-package Sweave producing an intermediate .tex file that in turn gets transformed into the final .pdf report.

Results: Here we present RReportGenerator which provides a tool to make available the advantages of the statistical platform "R" for automatic statistical analysis in a highly user-friendly way under Microsoft Windows. RReportGenerator is designed for routine executing of a predefined scenario / analysis type for a given problem and can be easily operated by non-experienced users via it's graphical interface. Scenarios are written in the R and Latex language and allow following a path of multiple steps of data-treatment including flexible generation of graphs and potential identification of warnings. The graphical user interface allows the user to simply choose among predefined analysis-scenarios / analysis-types to be applied this to a given data-set. In turn a pdf-report with the analysis results, tables and figures is generated that can be accompanied by supplemental data-sets for export to other programs (e.g. Excel). An example of mouse retina transcription profiling illustrates suitable tasks of quality control and analysis for automated analysis.

Conclusion: RReportGenerator allows performing routine statistical analysis benefiting from the statistical environment of R via a graphical user-interface allowing the selection of predefined analysis scenarios / analysis-types without requiring any knowledge in the syntax and use of R.

FUNCTIONAL ANALYSIS OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN THE VMD2 PROMOTER REGION TOWARDS HETEROGENIC PHENOTYPES IN BEST VITELLIFORM MACULAR DYSTROPHY (BMD)

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Purpose: Mutations in the VMD2 gene are associated with BMD, an early-onset autosomal dominant retinal disorder. Egg-yolk-like deposits in the macular area and abnormalities in the electrooculogram (EOG) are hallmarks of the disease. Late stage manifestations include atrophic changes and subretinal neovascularisation, ultimately leading to impairment or loss of visual function. BMD is a highly variable disease with a striking intrafamilial heterogeneity of clinical phenotypes even in carriers harbouring an identical pathogenic mutation. The aim of this study is to clarify whether single nucleotide polymorphisms (SNPs) within the VMD2 promoter may exert influence on gene expression and thus may provide a basis to understand the variable expressivity of disease manifestations.

Methods: *In silico* analysis of the human VMD2 promoter was done with the ELDorado tool of the Genomatix software package (Genomatix Software GmbH, Munich, Germany). Comparative genomics was performed to predict promoter regions. In addition, the ECR Browser (Decode, California, USA) algorithms were applied to extract additive genomic regulatory elements (RE) based on the comparisons of multiple vertebrate genomes. Putative eye specific transcription factor (TF) binding sites were identified by the MatInspector tool within the Genomatix package. Additionally, SNPs were extracted from public databases and aligned to human VMD2 promoter sequences. Finally the influence of SNPs on TF binding within the predicted REs will be investigated by luciferase reporter assays and electrophoretic mobility shift assays (EMSA).

Results: By computational analysis, five regions with putative regulatory functions in upstream regions of the human VMD2 gene were predicted. These REs were further analysed for eye specific TF binding sites. Approximately 50 sites for TF binding were predicted. Aligning the SNPs to the sites of TF binding highlighted several sites with SNPs directly located within their core sequences. Subsequently, the REs were amplified from genomic DNA and cloned into the pGL4.10 luciferase reporter vector (Promega, Madison, USA). Together with a constitutively-expressed ß-Gal standard, the RE constructs will be expressed in ARPE19 cells. The luciferase activity will then be measured and normalized relative to the ß-Gal expression. This will facilitate the definition of the minimal promoter sequences of VMD2 and will provide a test system to evaluate the influence of SNPs on these activities.

Conclusion: We have identified five additional putative REs in the human VMD2 upstream region, one of which has previously been shown to reveal promoter activities. We have also identified several SNPs within putative TF binding sites of the core sequences. More detailed molecular analyses are ongoing to further determine an influence of these polymorphisms on VMD2 promoter activities.

VARIATION IN RETINAL NEUROTRANSMISSION BETWEEN MOUSE STRAINS: HOW CAN WE INVESTIGATE FURTHER?

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It has previously been shown that a QTL located within 10cM of a marker on proximal chromosome 19 controls variation in timing and amplitude traits of the light-adapted electroretinogram between two strains of inbred mouse commonly used in vision research, particularly in the generation of transgenics: C57BL/6JOlaHsd and 129S2/SvHsd (Harlan, UK). The observed strain-specific differences in the light-adapted ERG b-wave are likely to be caused by a gene or gene(s) involved in modulating retinal neurotransmission. At present, a backcrossing experiment is being performed so the QTL may be refined and the gene(s) involved in such variation identified. Studying phenotypic variation between strains of inbred mouse is a valuable resource for the identification of novel genes, modifying, for example, retinal function. In order to ascertain whether the gene(s) causing variation in the retinal neurotransmission phenotype also cause variation in neurotransmission throughout the CNS, experiments have been designed to compare *in vitro* hippocampal electrophysiology between the two strains of inbred mouse. This system is currently being optimised. An optomotor approach has also been proposed so that functional vision may be assessed. By analysing phenotypic variation in the hippocampus between strains of inbred mouse, we can determine whether those modulators affecting retinal neurotransmission are the same as those putatively varying hippocampal neurotransmission.

EXPRESSION OF SIALOADHESIN FOLLOWING ENGRAFTMENT OF RETINAL TISSUE TO *RD1* MICE

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Purpose: Activated microglia/macrophages constitute an important source of cytokines and are essential for effective removal of celular debris. On the other hand, the activation of these cells can lead also to the release of cytotoxic agents which can contribute to accelerate the progression of cell death. Microglial cells normally do not express sialoadhesin (Sn, CD169, or Siglec-1), a sialic acid binding receptor, but have been shown to do so when exposed to serum. The purpose of the present work was to examine the expression of Sn in the course of photoreceptor cell degeneration and following transplantation of retinal tissue.

Methods: The expression of Sn was analyzed by immunohistochemistry in retinas of *rd1* mice and of wild-type controls, postnatal days 2 to 46 (P2 – P46). For the transplantation studies, neonatal (P2) fragmented retinal tissue derived from transgenic mice expressing green fluorescent protein (GFP) was injected into the subretinal space of adult *rd1* mice and of wild-type controls. The expression of Sn was examined 21 days post-transplantation. Retinal pigment epithelium cell markers and the microglial marker, CD11b, were used to identify cells expressing Sn.

Results: In *rd1* mice, microglial cells appeared weakly labelled in the inner central retina at P11 and in large numbers also in the outer nuclear layer from P12 and onwards. Nevertheless, no Sn labelled cells were observed in the *rd1* mouse retinas in these regions at any of the ages examined. Following transplantation, however, a variable number of Sn-positive cells were detected within the graft as well as in the host retina. In the latter, most Sn-labelled cells were seen in the subretinal space and did not express GFP.

Conclusions: The present study shows that the significant activation of microglia/ macrophages in the early stages of degeneration in the *rd1* mouse is not accompanied by Sn expression. However, Sn-expressing cells are observed following transplantation. Considering that the grafted tissue derived from P2 donors and that the Sn-labelled cells did not express GFP, it appears that the majority of the cells expressing Sn are of host-origin. The occurrence of such cells could be of significance for the long-term survival of the grafts.

OLIGONUCLEOTIDE MICROARRAY BEASED EXPRESSION ANALYSIS OF THE CPFL1 MUTANT – A MOUSE MODEL OF CONE DYSTROPHIES

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Purpose: The *cpfl1* mutant *(cone photoreceptor function loss 1)* is a mouse model carrying mutations in the cone specific phosphodiesterase 6C *(pde6c)*. The phenotype is characterized by a loss of cone photoreceptor function and a progressive degeneration of the cones. To investigate the biological events leading to the loss of photoreceptors we performed microarray experiments in two age stages.

Methods: Retinas of *cpfl1* and wildtype mice at the age of 4 and 8 weeks were dissected. RNA was isolated and 3 samples each hybridized on Affymetrix MOE 430 2.0 microarrays. Chip analysis was performed using the Array Assist 4.0 software (Stratagene) selecting all transcripts with a minimum change in expression level of 1.5 fold with a p-value less than 0.05 (t-Test without multiple testing correction). Gene regulation networks were generated by the Ingenuity Pathways Analysis 3.1 software. To verify the data 11 transcripts were analyzed by qRT-PCR using the LC 480 system (Roche).

Results: 338 transcripts were differentially regulated in the retinas of the 4 week old mice and 223 in those of the 8 week old animals. There was an overlap of 30 % between the two experiments. A large number of genes encoding proteins involved in phototransduction were down regulated. Gene regulation networks revealed also misregulations of genes associated with cell death, proliferation and gene expression. All the transcripts chosen for Real-time validation could be verified.

Conclusions: The expression analysis of the *cpfl1* mutant highlighted a clear misregulation of components of the phototransduction cascade in accordance with the loss of visual function that characterizes the phenotype. The *cpfl1* mutant in general represents an appropriate animal model for the investigation of cone dystrophies in humans.

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GENE EXPRESSION PROFILING IN NORRIN DEFICIENT MICE

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Purpose: To investigate the molecular pathophysiology caused by a mutant Norrin *(Ndph)* gene, which in humans is associated with various forms of blindness, including Norrie disease (ND), exudative vitreoretinopathy (EVR), retinopathy of prematurity (ROP) and Coat's disease. In ND, at least part of the patients show extraocular symptoms including mental retardation and deafness.

Methods: Global gene expression analysis was performed on retinae from a mouse model for Norrie disease and compared to gene expression data of wild type mice. These expression studies were performed at postnatal day seven (p7) using Affymetrix microarrays . Subsequently, array results were verified by quantitative real-time PCR analyses.

Results: Out of 33 genes that were differentially expressed (threshold 1.6 fold) on the microarray, nine have been analyzed by quantitative RT-PCR. Most showed similar or consistent results in comparison to the array data. Four of these nine targets could be shown to have a significant different transcript level compared to wild type.

Conclusion: The 4 identified genes – *Slc38a5, ApoD, Agtrl and Plvap* – may play a role in the pathogenesis of Norrie disease and allelic traits. All of them could be associated with blood vessel development – a process that is affected in the retina of Norrin knockout mice and human patients. Future experiments will reveal whether or not Norrin directly regulates the transcription of angiogenic factors in the retina and additional tissues including brain and ear. Also, it will be interesting to study the expression levels of these genes in the brain of Norrin knockout mice, as so far no blood vessel abnormalities have been reported there.

HYDROXYL RADICAL INDUCED CA^{2+} RESPONSE IN THE HUMAN RETINAL PIGMENT EPITHELIUM

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In the retinal pigment epithelium (RPE) cells Ca^{2+} is involved in many important cellular tasks. Thus a disturbance of the Ca^{2+}-homeostasis in RPE cells may lead to serious functional impairments resulting in cell death. We showed that application of OH^- radicals resulted in a biphasic $[Ca^{2+}]_i$ increase. The initial, transient peak increased in a radical concentration-dependant manner. This Ca^{2+} increase could neither be inhibited by blocking the L-type Ca^{2+}-channel nor the reverse modus of the sodium/calcium exchanger NCX1. Thapsigargin decreased the first Ca^{2+} transient suggesting an involvement of intracellular Ca^{2+}-stores. A second, continuous $[Ca^{2+}]_i$ increase was seen after terminating the OH^- application in 33% of the experiments using 1.5x $[OH^-]$ and in 100% of the experiments using 2x $[OH^-]$ and was accompanied by a decreased cell survival. The application of Thapsigargin elevated the incidence of the 2nd Ca^{2+} increase in the 1.5x experiments to 75%. By using calpastatin, a specific blocker of calpain, the second Ca^{2+} increase could be abolished in 90% of the experiments using 2x $[OH^-]$. Calpain is known to cleave NCX in a $[Ca^{2+}]_I$ dependant manner preventing proper Na/Ca exchange. We conclude that extracellular OH^- can lead to a [radical] dependant Ca^{2+}-activation and Ca^{2+}-overload. By blocking calpain and thus rescuing NCX function the Ca^{2+} overload and subsequent cell death are abolished.

NON-SYNDROMIC AUTOSOMAL RECESSIVE CONE-ROD DYSTROPHY AND ENDOCOCHLEAR HEARING IMPAIRMENT IN A CONSANGUINEOUS FAMILY CARRYING A NOVEL SPLICE SITE MUTATION IN THE MERTK GENE AND A TRUNCATING MUTATION OF THE DFNB59 GENE

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Purpose: To describe the phenotype of a consanguineous family from Morocco segregating autosomal recessive cone-rod dystrophy (CORD) and congenital progressive hearing loss (DFBN).

Methods: All family members underwent detailed clinical investigation including best-corrected visual acuity, slit lamp examination, stereoscopic funduscopy and digital fundus photography, Goldmann kinetic visual fields, electroretinography, fundus autofluorescence imaging (cSLO, HRA-2, Heidelberg engineering, Heidelberg, Germany), third-generation optical coherence tomography (StratusOCT, Zeiss, Dublin, California, USA) and high-resolution OCT (SOCT, Optopol, Torun, Poland).

Results: The disease loci were mapped to 2q13-q14.1 for CORD and to chromosome 2q31.1-q32.1 for DFNB, respectively. CORD was shown to be due to homozygosity for a novel splice site mutation in the *MERTK* gene, c.2189+1G>T, and DFBN due to homozygosity for a 1-bp-insertion in exon 2 (c.113_114insT) of the *DFNB59* gene. Detailed clinical investigation of the six siblings revealed combined severe CORD and DFBN in two of them, while there is isolated CORD in three and non-syndromic DFBN in one of them. The siblings affected by CORD exhibited a very similar phenotype, which was least severe in the youngest sibling (II:1) and most advanced in the oldest (II:6). Best-corrected visual acuity was 20/50 and 20/60 in the right and left eye of II:1, and 20/100 and 20/400 in the right and left eye of II:6, respectively. Kinetic perimetry was relatively well preserved for Goldmann III4e, but showed variable concentric constriction for I4e. Electroretinography showed detectable signals in II:1, but the amplitudes were markedly reduced and the photopic responses were delayed. In all other affected sib-

lings, scotopic and photopic ERG responses were undistinguishable from noise. Fundus autofluorescence imaging revealed spotted increased autofluorescence at the posterior pole in II:1 and, to a lesser degree, in II:3. In these two individuals, there was a small central spot of decreased autofluorescence surrounded by an increased autofluorescence. The older siblings showed patches of decreased autofluorescence. These patches were surrounded by focally increased autofluorescence. High-resolution OCT revealed reduced central retinal thickness, disrupted photoreceptor layer, granular appearance of the RPE layer and a highly reflective irregular nerve fiber layer. Audiological investigation indicated hair cell and vestibular dysfunction. Auditory neuropathy was excluded.

Conclusions: Although MERTK is generally considered an RP gene, these data confirm recent reports that extended the phenotypic spectrum to severe CORD (McHenry et al. IOVS 2004;45:1456-1463; Tschernutter et al. BJO 2006;90:718-723). The phenotypic severity of the CORD in the siblings correlated well with age. The fundus autofluorescence and high-resolution OCT findings may indicate that in early stages of the disease reduced RPE phagocytosis results in multifocal autofluorescent photoreceptor debris accumulating between the outer retina and the RPE (as in II:1). Increasing photoreceptor dysfunction would result in lower metabolic load on the RPE and thus a new balance between RPE phagocytic activity and the metabolic demand. This might lead to a normalization of the extramacular fundus autofluorescence (as seen in II:4, II:5, and II:6). Ultimately, photoreceptor and RPE cell death would occur as observed in the central macula of the affected individuals.

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DEFECTIVE COMPLEMENT CONTROL OF FACTOR H (Y402H) AND FHL-1 IN AGE RELATED MACULAR DEGENERATION

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Purpose: The common variant in the human complement Factor H gene (CFH), with Tyr402His, is linked to age-related macular degeneration (AMD), a prevalent disorder leading to visual impairment and irreversible blindness in elderly patients. 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.

Methodes: 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. In addition we recombantly expressed FHL-1, the alternative splice product of CFH, with the risk H402 and the nonrisk Y402 residues. Functional tests were performed to compare these Factor H and FHL-1 subtypes.

Results: Here we show that the risk variant CFH 402His displays reduced binding to C reactive protein (CRP), heparin and retinal pigment epithelial cells. This reduced binding can cause inefficient complement regulation at the cell surface, particularly when CRP is recruited to injured sites and tissue. In addition, we identify the factor H like protein 1 (FHL-1), as an additional protein that includes the variant residue 402 and thus confers risk for AMD. FHL-1 is expressed in the eye and the FHL-1 402His risk variant shows similar reduced cell binding and likely reduced complement regulatory functions on the cell surface.

Conclusion: CFH and FHL-1 may act in concert in the eye and the reduced surface binding may result in inappropriate local complement control, which in turn can lead to inflammation, disturbance of local physiological homeostasis and progression to cell damage. As a consequence, these processes may lead to AMD pathogenesis.

RETINAL FUNCTION FOLLOWING APPLICATION OF NEURAL STEM CELLS INTO A RHODOPSIN KNOCK-IN MOUSE MODEL OF RETINAL DYSTROPHY

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Purpose: Mutations of the rhodopsin gene cause approximately 10% of all inherited human retinal dystrophies. In our study we used a rhodopsin knock-in mouse model leading to retinal degeneration to test the effects of intraretinal transplantations of neural stem cells (NSCs) on retinal function. In a first step we were interested in the safety of the transplantation procedure in dependence of the age of the recipient animals at the time of cell injection.

Methods: NSCs were isolated from the striatum of embryonic EGFP-transgenic mice. Cells from the fifth passage were intraretinally injected into 10 days old (9 knock-in and 9 wild-type; (10-days-old group)) and 30 days old (8 knock-in and 8 wild-type; (30-days-old group)) mice. The contralateral eyes of each animal received vehicle injections, and served as controls. Mice were examined with 2,5 and 4,5 months of age by full-field electroretinogram (ERG) recordings. Here, we present scotopic and photopic ERGs recorded from anaesthetized mice.

Results: Four out of 16 mice that received injections at 30 days of age developed phtisis bulbi in either a cell- or a sham-injected eye. Interestingly, this was not observed in any of the 18 mice that were manipulated at 10 days of age. Some mice from both, the 10-days-old group and the 30-days-old group developed transparency defects, however, in these mice ERG responses could be recorded, although the effect of the transparency defect on the responses remains unknown. While wild-type mice from the 10-days-old group showed lower ERG responses in the cell- than in the vehicle-injected eyes, the opposite was true for knock-in mice. In the 30-days-old group, in comparison, both wild-type and mutant mice showed slightly lower ERG responses in cell-injected eyes when compared to vehicle-injected eyes. However, none of these differences reached statistical significance.

Conclusion: Intraretinal injections may lead to opacities of the transparent media of the mouse eye which might prevent behavioural testing of the visual abilities of treated animals. ERG recordings allow the functional characterisation of experimental animals that develop such secondary complications. Altogether, we did not observe any significant beneficial effect of intraretinal neural stem cell transplantations on retinal function in rhodopsin knock-in mice. However, in the 10-days-old group, there was a slight increase in the ERG response in cell-injected eyes when compared to vehicle-injected eyes which deserves further investigations.

A NEW CONCEPT TO COMBAT AN ORPHAN DISEASE

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Purpose: Batten disease is the most common neurodegenerative disorder of childhood and is a group of at least ten lysosomal storage disorders. Children suffering from the juvenile form usually start to have visual deficits at school age leading to blindness in a few years. This is paralleled by a relentless mental and physical decline and all these children will die at the age of 20 to 30 years. This autosomal recessively inherited disease should be of great importance to the ophthalmologists, who are usually the first to see the affected children. However, all orphan diseases suffer from delayed and/or false diagnoses and the aim of the NCL-foundation is to change this in Batten disease (neuronal ceroid lipofuscinosis). Our strategy could be an example for other rare diseases with methods that are applicable to different medical groups focussing initially on ophthalmologists.

Methods: The NCL-foundation sets incentives to initiate scientific publications and to have medical training sessions and symposia at congresses. Additionally, it publishes its own articles and aims to distribute scientific information nationwide. It initiates new cooperations with (non-)profit organizations with the goal of cooperative funding of promising research projects, hereby extending the scientific ncl-network.

Results:
1. Initiation of scientific publications: "The Role of the Ophthalmologist in the Management of Juvenile Neuronal Ceroid Lipofuscinosis" (Rüther et al., Klin. Monatsbl. Augenheilkd. 2006; 223: 542-544).
2. Distribution of information: Mailing of this publication to all German eye-clinics (123) asking them to integrate this article into their journal clubs and training sessions (9 positive feedbacks so far).
3. Publishing of own articles: "Die NCL-Stiftung stellt sich vor" (Stehr, Retina aktuell 4/2006; 102: 34).
4. Initiating medical training sessions and symposia at existing scientific congresses: DOG is in progress.
5. Extension of the scientific ncl-network: organization of five national ncl-congresses with growing international participation, as well as direct contact and visits to researchers.
6. Start collaborations with (non-)profit organizations to initiate research: Seed funding of a scientific project at the Charité together with the Ernst-und-Elfriede-Griebel-Förderungs- und Unterstützungsstiftung. The topic was "functional and morphological analysis of the retina of the CLN3-knock in mouse model" (Rüther).

Conclusions: The NCL-Foundation aims to set the basis for the accurate and early diagnosis of Batten disease. This will have an important impact on affected families and the efficiency of future therapies. The foundation is focusing upon developing a gene therapy, which will first be targeted to the eye.

CHARACTERIZATION OF A NEW TRANSGENIC RAT WITH RETINAL VASOREGRESSION

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Aim: Our previous study has demonstrated that retinal overexpression of a mutant gene associated with cilia function leads to retina degeneration. This study characterizes this new transgenic rat (TGR) in relation to neuron degeneration and vascular regression in the retina.

Methods: The study was carried out in TGR and SD rats from 1 to 7 months of age. Neuronal degeneration was analyzed using quantitation of retinal cell numbers and layer thickness. Vascular regression was determined in retinal digest preparations. The function of the retina was evaluated by the electroretinogram (ERG).

Results: The cell numbers and the thickness of the outer nuclear layer (ONL) represent the level of retinal degeneration. The cell numbers and the thickness representing neuronal degeneration were significantly reduced in SD rats from 1 up to 7 months. In comparison to SD rats, TGR rats showed decreased cell number (-31%) and thickness (-24%) of the ONL at the first month. The reduction progressed over time. However, SD and TGR retinae showed identical responses in the ERG at the first month. No response in the ERG was observed in 3-month TGR retinae. TGR retinae did not differ from SD retinae in formation of acellular capillaries at the first month. At the second month the formation of acellular capillaries increased significantly in the TGR retinae (+100%). Similar to the cell number and the thickness of the ONL, the segments of acellular capillaries increased significantly in TGR rats over all period.

Conclusion: Transgenic TGR rats have the primary neuron degeneration at first month, the secondary vasoregression and impaired retinal function at the following months of living.

RETINAL NEUROPROTECTION BY HYPOXIC PRECONDITIONING: IDENTIFICATION OF POSSIBLE NEUROPROTECTIVE TARGETS BY ANALYZING THE HYPOXIC TRANSCRIPTOME

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Purpose: Preservation of visual cells by neuroprotective treatments is a possible strategy to rescue vision in patients. In an animal model of induced retinal degeneration, hypoxic preconditioning stabilizes transcription factor HIF-1a and protects photoreceptors from apoptosis. To identify factors, which might contribute to retinal neuroprotection, 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 and 16h thereafter. Affymetrix gene chips were hybridized and normalized using GC-RMA, significantly regulated genes determined based on FDR determination and their implication in biological pathways analyzed by Ingenuity Pathway Analysis. Real-time PCR and Western Blotting were used to verify chip results and to investigate promising signaling pathways.

Results: Hypoxic pretreatment induced a gene expression pattern highly different from normoxic controls. Immediately after hypoxia 431 genes with various biological functions like transport, transcriptional regulation and apoptosis were differentially regulated. Pathway analyses revealed a strong impact of Cdnk1a, a protein central to cell cycle, antiapoptosis and proliferation pathways. Additionally, regulation of c-Myc target genes and interaction partners indicates the potential involvement of c-Myc pathways in neuroprotection. Reoxygenation caused gene expression to return quickly to a normoxic pattern.

Conclusion: Hypoxia induces predominantly pathways of cell cycle, proliferation and DNA repair. A central position seems to be occupied by Cdnk1a. Screening of these pathways revealed candidates which may be involved in neuroprotection by hypoxic preconditioning. Their role in preventing photoreceptor apoptosis is currently being analyzed.

Keywords: neuroprotection, hypoxic preconditioning, retinal apoptosis

HIGH RESOLUTION GENE EXPRESSION ANALYSIS IN THE HUMAN RETINA

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Purpose: Retinitis pigmentosa (RP) is the most common form of hereditary retinal degeneration, with a prevalence of about 1:4000. To date, little information is available on the expression patterns in the human retina of RP genes at the cellular level and the current knowledge is inferred mostly through the analysis of the mouse. To overcome this lack of information, we decided to generate an expression atlas of the 34 known genes responsible for RP in both human and murine eyes.

Methods: We retrieved appropriate templates for 34 genes responsible for Retinitis pigmentosa and we used them to perform RNA *in situ hybridization* (ISH) studies on human and murine adult eye cryosections.

Results: The majority of the analyzed RP genes showed similar expression patterns between human and mouse adult retina. However, the expression patterns of a significant subset of human RP genes significantly differ from their mouse orthologs. The most interesting differences were observed in the expression patterns of genes known to be involved in the visual cycle process. LRAT, RPE65, RLBP1 and RGR genes show significant differences in the expression between human and mice. Interestingly, some of these genes do not have a uniform expression pattern throughout the human retina. Precisely, higher levels of expression were observed in the central part of the retina, as compared with the peripheral part. We have confirmed that in the human retina these genes are expressed in cones both at RNA and protein level.

Conclusions: We generated a comprehensive expression atlas of genes involved in RP and carried out a detailed comparative analysis of gene expression in adult retina between human and mouse. This atlas will be a valuable tool to acquire novel hints on the putative function of some RP genes in the human retina and to translate research performed in the murine models of RP to humans.

DEVELOPMENT OF A PAN-EUROPEAN DATABASE TO PROVIDE PHENOTYPIC DATA OF PATIENTS WITH RETINAL DISEASES

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Objective: Building a database that provides information on the phenotype of hereditary and age related retinal diseases. Providing easy access for clinicians to a uniform phenotyping system and to foster research cooperation between clinicians and basic scientists.

Method: EVI-Genoret is a joint project of clinical- and basic scientists to study hereditary and age related retinal diseases. Clinicians contribute to the database by providing patient data detailing their clinical phenotype. These data may also be accessed by partners from genetic, proteomic, developmental or therapeutical components. The database has been developed in respect to: 1. to provide easy access for members and future associates of the consortium 2. to serve as a local database for patient management 3. to serve as a standardized phenotyping system in clinical routine 4. to be an integrative part of the EVI-Genoret Consortium Database.

Results: A SQL database has been developed that uses a Javascript interface allowing data entry and retrieval via the Internet. This ensures accessibility from different personal computers, irrespective of the operating system. No special software must be installed and no duplicate data is stored locally. Thus we can provide easy access to the database and a system of standardization of the phenotyping data entry for the EVI-Genoret Consortium. New members or associates of the consortium can easily join the database without adapting their local computers. This clinical database will be accessed by the consortium via the IBM WebSphere Information Integrator and will contribute to the relational EVI-Genoret Consortium Database which integrates genetic, functional genomics and standardisation data linked to human and animal model retinal disease. Thus the database itself is easily integrated without storing duplicate data.

Conclusion: This Javascript based Interface to the database can be effective in storing and retrieving clinical data where several centres are involved and local data storage must be avoided because of legal restrictions, technical restraints or financial aspects. EVI-Genoret is the first European project to build such a database to foster integrated research on hereditary and age related retinal diseases.

Supported by EVI-Genoret

BASAL CALCIUM ENTRY IN RETINAL PIGMENT EPITHELIAL CELLS

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Background: The retinal pigment epithelium (RPE) is involved in a variety of processes that help to maintain normal retinal function. Many of these tasks depend on intracellular free calcium ($[Ca^{2+}]_i$), for example photoreceptor phagocytosis, growth factor secretion, transepithelial transport mechanisms and cell differentiation. All these processes need extra- or intracellular signals to initiate an increase in $[Ca^{2+}]_i$. While there is some evidence that voltage-gated Ca^{2+} channels and purinergic receptors are involved in these Ca^{2+} fluxes nothing is known about the basal Ca^{2+} entry under resting conditions. The purpose of this study was to identify the molecular identity of Ca^{2+} channels involved in basal Ca^{2+} entry in RPE cells.

Methods: Intracellular Ca^{2+} concentration measurements were performed with ARPE-19 cells using FURA-2 as fluorescent Ca^{2+} indicator. The data obtained with the RPE cell line were confirmed with human RPE primary cultures. Additionally, RT PCR experiments were made with mRNA extracted from freshly isolated RPE cells and from ARPE-19 cells with oligonucleotides specific for different Ca^{2+} channels.

Results: ARPE-19 cells had an resting $[Ca^{2+}]_i$ of 106.4 ± 16.1 nM. This resting concentration was not changed by the application of the voltage-gated Ca^{2+} channel blocker nifedipin (10 µM). Gadolinium (100 µM); lanthanum (100 µM) and nickel (2 mM) reduced the $[Ca^{2+}]_i$ by 73, 76 and 98%, respectively. Additionally, the blocker of store-operated Ca^{2+} entry 2-aminoethoxydiphenyl borate (75 µM) reduced $[Ca^{2+}]_i$ by 69%. As these blockers are known to block Ca^{2+} conducting channels of the TRPC family we performed RT PCR experiments and found TRPC1 and TRPC4 to be expressed in ARPE-19 cells. In freshly isolated RPE cells TRPC7 was expressed in addition to TRPC1 and TRPC4.

Conclusions: Our data show that the basal entry of Ca^{2+} in RPE cells is mainly driven by TRPC1 and TRPC4 channels. Their basal open probability provides the basal $[Ca^{2+}]_i$ needed for the function of diverse Ca^{2+}-dependent processes in RPE cells.

KCNV2 MUTATIONS ARE COMMON IN PATIENTS WITH CONE DYSTROPHY AND SUPERNORMAL ROD RESPONSE

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Purpose: Cone Dystrophy with Supernormal Rod Response (CDSRR) is a retinal dystrophy which is characterized by specific alterations of ERG responses that features reduced and delayed cone responses, a reduction and marked delay of rod b-waves at low light intensities but an elevated rod b-wave amplitude at higher light intensities. There are controversial reports about the genetic basis of CDSRR: While Piri and coworkers have reported a mutation in *PDE6H* in a single family, recently Wu and colleagues identified mutations in the *KCNV2* gene in several patients and families. We have undertaken an independent study to investigate the *PDE6H* and *KCNV2* genes in a cohort of Danish and German patients with CDSSR.

Methods: 16 patients from 13 families underwent detailed ophthalmological examination including psychophysical (visual acuity testing, perimetry and color vision testing), electrophysiological (Ganzfeld-ERGs), and imaging technologies (funduscopy, OCT, FAF). The coding sequences and flanking intron/UTR sequences of *PDE6H* and *KCNV2* were screened for mutations by means of DHPLC and direct DNA sequencing of PCR-amplified genomic DNA.

Results: No mutations were detected in the *PDE6H* gene. In contrast, we were able to identify putatively pathogenic *KCNV2* mutations – either in homozygous or compound heterozygous state – in all of the patients in our study. Nine of the 10 identified mutations were novel including 3 missense and 6 protein truncating mutations. The mutations segregate perfectly following an autosomal recessive mode of inheritance in all available family members except for one family in which the unaffected father supposed to be heterozygous did not carry the mutation.

Conclusions: In our study we found that the phenotype of cone dystrophy with supernormal rod response is strictly associated with mutations in *KCNV2* suggesting that CDSSR is a genetically homogeneous condition.

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DIFFERENTIAL EXPRESSION OF METALLOTHIONEIN (MT I/II) IN ANIMAL MODELS OF PHOTORECEPTOR DEGENERATION

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Purpose: Metallothioneins (MT) are cysteine-rich proteins that are important for protection against metal toxicity and for zinc absorption and homeostasis. In addition, some isoforms have been shown to have redox capacities. Neuroinflammation, oxidative stress, and several other insults have been shown to promote up-regulation of metallothioneins. The purpose of the presented work was to assess the temporal expression pattern of metallothioneins I and II (MT I/II) in different mouse/rat models of *Retinitis Pigmentosa*.

Methods: Immunohistochemical staining of MT I/II was performed on retinal tissue of *rd1* mice, postnatal days 2 to 28 (P2 – P28), *rds* mice (P2 – P30), Royal College of Surgeon rats (RCS, P2 – P42), and wild type controls. Co-stainings against the retinal pigment epithelium (RPE) and Müller glial cell marker, cellular retinal binding protein (CRALBP), and against the microglial marker, CD11b, were carried out to identify MT I/II-expressing cells.

Results: In wild type rats and mice of all examined ages, only the RPE showed MT I/II expression. In *rd1* mice, microglial cells were weakly labelled in the inner central retina at P11 and also in the outer nuclear layer from P12 and onwards. Discrete Müller cell labelling was observed in the central retina at P12, expanding to the peripheral retina with increasing age. In *rds* mouse retinas, Müller cells in the centre were not stained positive until P16, but at P18 the expression already extended throughout the whole retina. In RCS rat retinas, MT I/II expression could also be observed mainly in Müller cells, but only from P30 and onwards.

Conclusions: An early up-regulation of MT I/II is observed in the mouse models in retinal glial cells, which appears to correlate with the progression of photoreceptor degeneration. In RCS rats, however, the expression of MT I/II appears to be involved with late secondary events rather than with the photoreceptor cell loss itself.

COMPREHENSIVE MUTATIONAL ANALYSIS OF STARGARDT DISEASE PATIENTS – IDENTIFICATION OF 24 NOVEL MUTATIONS IN THE ABCA4 GENE

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Mutations in the ABCA4 gene are known to cause various forms of macular dystrophy including Stargardt disease, autosomal recessive cone-rod-dystrophy (CRD) and inverse Retinitis pigmentosa. Due to the size and complexity of the ABCA4 gene, routine diagnostic services frequently rely on a chip-based analysis (ABCR Chip, Asper, Estonia) that searches for the presence/absence of 496 known ABCA4 variants. With this analysis, frequently only one pathogenic mutation can be detected leaving some uncertainty about the second mutational change.

In the course of routine genetic diagnosis we identified 62 patients with only a single pathogenic mutation which were clinically diagnosed with Stargardt disease (36), autosomal recessive CRD (11), Stargardt-like AMD (8) or other forms of macular dystrophy (7). To further asses the usefulness of the ABCR Chip analysis for diagnostic purposes, we have now directly sequenced all 50 coding exons of the ABCA4 gene in the 62 patients.

So far, we identified a second genetic alteration in 27 of the 62 patients. Of these, 24 variations were novel. Interestingly, we found four mutations which should be covered by the ABCR Chip. The 24 novel mutations consist of eleven missense-mutations and two nonsense mutations that are caused by single nucleotide changes. Six alterations are located in the splice site consensus sequence, thus probably leading to an altered splicing behaviour. Three small deletions that should result in a frame shift, one in-frame duplication and one in–frame combined insertion/deletion were detected.

In contrast to our expectations, after evaluation of over 80% of the ABCA4 sequences the second mutation could not be identified in 35 of the 62 (56%) patients analyzed. This may be due to the occurrence of a frequent type of mutation in ABCA4 not detectable by the PCR technology. For example, large genomic rearrangements including large deletions or inversions would not be detected by this method. In addition, intronic variations or mutations that are located in the promoter region would have been missed. This study will now facilitate to expand the repertoire of mutational analysis especially to those patients with an as yet unidentified second mutation.

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