PRO RETINA FOUNDATION

5th PRO RETINA
Research-Colloquium Potsdam

CONFERENCE REPORT

Retinal Degeneration

Focus on Therapy

An Interdisciplinary Dialogue

April 17th/18th, 2009

Potsdam, Seehotel am Templiner See

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FOR PREVENTION BLINDNESS

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PRO RETINA DEUTSCHLAND E. V. & THE FOUNDATION FOR PREVENTION BLINDNESS

WHO WE ARE

The patient-organisation, "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 spread throughout Germany.
At present (2008), PRO RETINA Deutschland e.V. counts more than 6,200 members. The Board, the Counsellors, the leaders of the regional groups and all active members are working on a non-profit basis, but they are supported by a fulltime working staff at our office which is located in Aachen (www.pro-retina.de).

WHAT WE DO IN RESEARCH

The jewel of all this work is the PRO RETINA-Foundation for Prevention Blindness, which was founded in 1996.
From the early beginning we have created a stable network with researchers and ophthalmologists for joined information and advice. We support research projects with direct financial funding – since the "Foundation for Prevention of Blindness" was established in 1996, more than 1.5 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.

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 of 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.

PROGRAMME

Friday, April 17th 2009

13:00-13:05 Franz Badura (PRO RETINA Research Foundation, research division):
Welcome and opening remarks

13:05-13:15 Claus Gehrig (Chairman, PRO RETINA Deutschland e.V.):
"From the patients view"

13:15-13:30 Eberhart Zrenner (Chairman, Scientific Medical Advisory Board):
"Retinal Degeneration – Focus on Therapy"

13:30-15:35 **Session 1: Molecular therapy in retinal degeneration – present and future**

Chairman: Prof. Bernhard Weber

1. Scott Robbie, London: *"Update on clinical trial of gene therapy for RPE65"*
2. Peter Humphries, Dublin: *"Gene therapy of dominant RP"*
3. John Neidhardt, Zürich: *"Gene therapy to rescue splice defects causing retinal degeneration"*
4. Uwe Wolfrum, Mainz: *"Molecular genetic strategies for the treatment of Usher syndrome in the retina"*
5. Paul Lingor, Göttingen: *"AAV-based gene therapy for de- and regeneration in the retina"*

15:35-16:15 Coffee break and scientific chitchat

16:15-18:20 **Session 2: Cell-based therapy approaches in retinal degeneration**

Chairman: Prof. Peter Humphries

1. Marius Ader, Dresden: *"Retinal cell differentiation into mature photoreceptors"*
2. Mike Karl, Seattle: *"Identification of neuronal precursor cells in the retina"*
3. Amanda Barber, London: *"Development of improved protocols for photo-receptor cell transplantation"*
4. Raymond D. Lund, Oregon: *"Photoreceptor progenitor cells in the treatment of retinal degeneration"*
5. Konrad Kauper, Neurotec, Rhode Island: *"Experience with the CNTF clinical trial"*

18:30 Dinner

19:30 "Swingin' Poster Session and Get Together"

PROGRAMME

Saturday, April 18th 2009

8:30-10:10 **Session 3: Addressing microglia in retinal degeneration and therapy**

Chairman: Prof. Olaf Strauß

1. Solon Thanos, Münster: *"Microglia in retinal degeneration"*
2. Florian Sennlaub, Paris INSERM: *"Chemokine receptor CX3CR1 in AMD"*
3. Jonathan Cooper, King's College London: *"Novel roles for glia in NCL pathogenesis"*
4. Thomas Langmann, Regensburg: *"Modulation of microglia activity by dietary lipids"*

10:10-11:00 Coffee break and scientific chitchat

11:00-12:15 **Session 4: Clinical trials – Planning, Implementation and Experience**

Chairman: Prof. Klaus Rüther

1. Holger Lüdtke, Tübingen: *"Study design and Randomization in clinical trials"*
2. Barbara Wilhelm, Tübingen: *"Performance of clinical trials"*
3. Paulus deJong, Amsterdam: *"Read-out and interpretation of clinical trials in retinal degeneration"*

12:20-13:00 **Poster Awards and Short Presentations**

13:00 Lunch and end of meeting

PRO RETINA
POTSDAM-MEETING

SPEAKER ABSTRACTS

Effect of gene therapy on visual function in Leber's congenital amaurosis

James W.B. Bainbridge, Ph.D., F.R.C.Ophth.[1,2], Alexander J. Smith, Ph.D.[3], Susie S. Barker, Ph.D.[3], Scott Robbie, M.R.C.Ophth.[1], Robert Henderson, M.R.C.Ophth.[1], Kamaljit Balaggan, M.R.C.Ophth.[1], Ananth Viswanathan, M.D., F.R.C.Ophth[1,2], Graham E. Holder, Ph.D.[1], Andrew Stockman, Ph.D.[3], Nick Tyler, Ph.D.[6], Simon Petersen-Jones, Ph.D.[7], Shomi S. Bhattacharya, Ph.D.[3], Adrian J. Thrasher, Ph.D., M.R.C.P., F.R.C.P.[8], Fred W. Fitzke, Ph.D.[3], Barrie J. Carter, Ph.D.[5], Gary S. Rubin, Ph.D.[2,3], Anthony T. Moore, F.R.C.Ophth.[1,2], and Robin R. Ali, Ph.D[2,3,8]

[1]UCL Institute of Ophthalmology and Moorfields Eye Hospital, London, United Kingdom; [2]NIHR Faculty, Biomedical Research Centre for Ophthalmology, Moorfields Eye Hospital and UCL Institute of Ophthalmology, London, United Kingdom; [3]UCL Institute of Ophthalmology, London, United Kingdom; [4]Moorfields Eye Hospital, London, United Kingdom; [5]Targeted Genetics Corporation, Seattle, Washington; [6]Department of Civil and Environmental Engineering, University College London; [7]Michigan State University, East Lansing; [8]Institute of Child Health, University College London

Purpose: A number of previous studies have demonstrated that recombinant adeno-associated virus (rAAV) vector-mediated gene replacement therapy improves retinal function and visual behaviour in animal models of retinal degeneration caused by defects in the gene encoding *RPE65*. The purpose of this study is to determine whether gene therapy for retinal dystrophy caused by *RPE65* mutations is safe and effective in humans.

Methods: In a phase I/II dose-escalation trial, we have administered by subretinal injection a rAAV-2/2 vector expressing human *RPE65* cDNA under the control of a human *RPE65* promoter in 4 human subjects with early onset severe retinal dystrophy caused by mutations in *RPE65*. We have examined systemic vector dissemination and immune responses, assessed the effect of vector administration on visual function using a range of psychophysical techniques, and performed detailed electrophysiology and retinal imaging studies.

Results: There have been no complications associated with the surgical delivery of vector in the subjects enrolled to date. We have detected no systemic dissemination of vector genome and no evidence of immune responses to rAAV vector capsid or *RPE65* proteins. We have found no evidence of any significant adverse effect on retinal function. There has been no clinically significant change in visual acuity or in peripheral visual fields on Goldmann perimetry in any of the four patients. We have detected no change in retinal responses on electroretinography. One patient has had significant improvement in visual function on microperimetry and on dark-adapted perimetry. This patient has also showed improvement in a subjective test of visual mobility.

Conclusions: The outcomes in the first subjects to date suggest that subretinal administration of rAAV vector is safe in humans. These findings provide support for further clinical studies of this experimental approach in other patients with mutant *RPE65*.

Gene therapy for dominant RP

Pete Humphries, Smurfit Institute of Genetics, Trinity College Dublin, Ireland.

Gene therapies have now shown efficacy in at least twelve animal models, including murine models of Stargardt macular dystrophy, retinoschisis, Leber congenital amaurosis with mutations in the RPE65, RPGRIP and AIPL1 genes, achromatopsia, rd10, Usher syndrome type 1B, adRP with mutations in the rhodopsin and IMPDH1 genes, as well as in the RCS rat (with null mutations in the Mertk gene) and the Briard dog (a naturally occurring model of LCA based on null mutations within the RPE65 gene). In addition, initial findings from phase 1 clinical trials in humans with LCA have also been reported. Some forms of autosomal dominant retinopathy may, arguably, represent more attractive initial therapeutic targets than others, owing to features associated with their molecular pathologies. The RP10 form of adRP is a severe disease caused by mutations within the IMPDH1 gene, evidence indicating that protein mis-folding underlies disease pathology. However, mice with a targeted disruption of this gene display only a very mild retinopathy, probably induced by limiting supplies of GTP to photoreceptors. (IMPDH1 is one of two rate-limiting enzymes of *de novo* guanine nucleotide biosynthesis and is the predominant enzyme expressed in photoreceptors). RNAi-mediated co-suppression both of wild type and mutant IMPDH1 transcripts could drastically improve disease pathology by converting the dominant condition into a mild recessive equivalent and we will provide data validating this concept in mice. We show, in addition, that systemic administration of GTP following RNAi-mediated reversible opening of the blood-retina barrier to this compound, improves visual function in IMPDH1-/- mice.

Gene therapy to rescue splice defects causing retinal degeneration

John Neidhardt (PhD), Division of Medical Molecular Genetics and Gene Diagnostics, Institute of Medical Genetics, University of Zurich, Switzerland

Purpose: Mutations in genes associated with retinal degeneration cause visual handicap or blindness. Irrespective of the disease gene, up to 20 % of the cases are caused by mutations which affect splicing of the transcript and lead to exon skipping, intron retention, or usage of a cryptic splice site. We have developed and evaluated a gene therapeutic approach to correct splice donor site mutations.

Methods: Novel mutations were identified using homozygosity mapping and direct sequencing. RT-PCR methods were applied to characterize splice patterns. Minigenes were cloned to establish splice assays by chemical co-transfection with U1snRNA expression constructs in COS7 cells or retinal explants. Lentiviral transduction was used to correct abnormal splicing in patient-derived cell lines.

Results: We identified and characterized two novel mutations affecting splice donor sites in rhodopsin and the Bardet-Biedl syndrome gene 1, respectively. Both mutations lead to aberrantly spliced transcripts. To maintain normal splicing, splice donor sites need to be recognized by complementary base pairing of the splice factor U1snRNP (U1) with the pre-mRNA. To overcome the deleterious effect of the mutations, we adapted U1 to perfectly match the mutated splice donor site and found that exon skipping can be rescued in up to 90% of the affected transcripts. These results were established using minigene splice assays. Treatments of patient-derived cell lines with adapted U1 demonstrated the rescue of endogenously expressed mutant transcripts. The unaffected control transcript showed no side-effects from the U1 therapy.

Conclusion: Our results demonstrate the feasibility and high efficiency of U1-mediated therapeutic interventions to treat splice donor site mutations. These findings have implications on the development of gene therapeutic protocols for various other diseases caused by similar mutations.

Molecular genetic strategies for the treatment of Usher syndrome in the retina

Uwe Wolfrum
Institute of Zoology, Cell & Matrix Biology, Johannes Gutenberg-University of Mainz, Germany

Purpose: The human Usher syndrome (USH) is the most frequent cause of inherited combined deaf-blindness. It is genetically heterogeneous, assigned to three clinical types. The most severe form is USH1, characterized by profound prelingual hearing loss, vestibular areflexia, and prepubertal onset of *retinitis pigmentosa.* So far no treatment for the ophthalmic component of USH exists. Here we evaluate different molecular genetic strategies for retinal USH therapy, namely gene addition, gene repair and read-through of nonsense mutations.
Here we focus on the *USH1C* gene which encodes the scaffold protein harmonin. Harmonin is expressed in retinal photoreceptors in form of numerous splice variants and act as a key scaffold of the protein interactome related to USH. For our analyses we assess the *USH1C* p.R31X mutation present in a German family.

Methods: Harmonina1 splice variant addition was tested in harmonin knock-out mice using recombinant Adeno-associated virus (rAAV) (serotype 5). Gene repair of the *USH1C* p.R31X mutation was assessed by homologous recombination mediated by specific zinc-finger nucleases (ZFN) so far *in vitro.* Read-through efficiency of the *USH1C* p.R31X mutation by aminoglycosides and PTC124 was tested *in vitro,* in cell cultures and in retinal explants. Toxicity of aminoglycosides/PTC124 was analyzed by TUNEL assays in combination with molecular markers in retinal sections.

Results: Subretinal injected rAAV transduced harmonina1 in the retina of harmonin knock-out mice. Preliminary data in our gene repair strategy indicated that the designed ZFN are expressed in nuclei and cleave the target DNA. The read-through of the p.R31X mutation is mediated by all tested aminoglycosides and PTC124 in cultured cells but also in the retina restoring the scaffold function of harmonin. Müller glia cells are the primary cellular targets of the obvious toxicity of commercial aminoglycosides used in ophthalmic applications. This toxicity is drastically reduced for modified aminoglycosides and for PTC124.

Conclusion: Although rAAV mediated gene delivery is straight forward the numerous splice variants makes *USH1C* a difficult target for gene addition approaches. Gene repair by homologous recombination mediated by ZFN is the most elegant but also the most challenged approach. Drastically reduced toxicity combined with their read-through efficiency reveals the high potential of modified aminoglycosides and PTC124 as new therapeutic agents for *USH1C* and other genetic conditions.

Supports: FAUN-Stiftung, Foundation Fighting Blindness (FFB); DFG (GRK1044/1), ProRetina

AAV-based gene therapy for de- and regeneration in the retina.

Paul Lingor

Department of Neurology, University Medicine Göttingen, Germany and
DFG-Research Center for Molecular Physiology of the Brain

Purpose: Lesion-induced apoptotic cell death and axonal degeneration as well as an insufficient regeneration potential contribute to dysfunction and disease progression in numerous CNS disorders and retinal pathology. Successful restoration will thus depend on the simultaneous targeting of all above mentioned processes. We have therefore evaluated the therapeutic potential of small molecule drugs and viral vectors in models of retinal ganglion cell apoptosis and regeneration in vitro and in vivo.

Methods: Primary retinal ganglion cell cultures and immortalized RGC-5 cells were used for the evaluation of cell death, neurite growth and regeneration. To counteract apoptotic cell death and increase the regenerative response small molecule kinase inhibitors, growth factors, siRNAs and adeno-associated viral vectors overexpressing target genes or shRNA were employed. In vitro experiments were followed by in vivo studies, where we performed optic nerve transection and crush experiments.

Results: Apoptotic cell death could be attenuated by injection of siRNA into the lesioned optic nerve, which resulted in a downregulation of apoptosis mediators. We could further demonstrate that small molecule inhibitors of ROCK as well as shRNA-mediated inhibition of ROCK increase survival and regeneration in retinal ganglion cells in vitro and in vivo. AAV-mediated expression of the bcl-2-binding protein BAG1 was able to both attenuate apoptosis and increase the regenerative response, which was attributed to its interaction with antiapoptotic proteins of the bcl-2 family as well as an inhibition of ROCK-activity via Raf-1 kinase. In an attempt to more precisely dissect the inhibitory signalling cascade, we have now generated AAV vectors expressing shRNAs targeting RhoA, ROCK and LIMK.

Conclusion: Strategies addressing simultaneously degenerative cell death and regenerative response are promising for restoration of retinal pathology. AAV vectors have proven to be useful research tools mediating robust and cell-type specific transgene expression for overexpression or downregulation of target proteins, which merit their evaluation as a therapeutic alternative in retinal disorders.

Retinal cell differentiation into mature photoreceptors after transplantation

Marius Ader[1], Wasi Oriyakhel[2], Jochen Haas[1], Dominic Eberle[1], Gila Jung[2], Gisbert Richard[2], Pete Humphries[3], Jane Farrar[3], Udo Bartsch[2]

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Purpose: Impairment of vision and blindness due to loss of photoreceptors are one of the most prevalent causes of disability in industrialized countries. The adult mammalian retina lacks efficient endogenous repair mechanisms and thus is unable to regenerate photoreceptors lost due to injury or inherited diseases. Cell-based strategies might represent a possible treatment option to replace lost photoreceptors in retinopathies.

Methods: Three cell populations were used for transplantation experiments into the retina of rodents: neural stem cells, retinal stem cells, or primary retinal cells isolated at the peak of rod photoreceptor generation, i.e. during the first postnatal week of mice. All donor cell populations were isolated from EGFP-transgenic mice to allow their detection in the host tissue. Cells were grafted into the vitreous and/or sub-retinal space of adult mice and their integration and differentiation potential was analysed.

Results: Neural- and retinal stem cells integrated preferentially into inner retinal layers (e.g. retinal ganglion cell layer, inner plexiform layer, or inner nuclear layer) and survived for prolonged time periods. Although stem cells adapted neuronal and glial phenotypes, differentiation into mature photoreceptors was not detected. In contrast, integration into the outer nuclear layer and differentiation into mature rod photoreceptors was observed when primary retinal cells were used for grafting. Furthermore, primary retinal cells isolated at postnatal day 4 showed a significant increase in their capacity to integrate and to differentiate into mature photoreceptors when compared with cells isolated at postnatal day 0 or 1.

Conclusion: The data implicates that cells committed to the photoreceptor lineage rather than stem/progenitor cells have the greatest potential for integration into host retinas and photoreceptor differentiation. Thus, young photoreceptors represent prime candidates for the development of cell replacement therapies for diseases characterized by photoreceptor loss.

Identification of neuronal precursor cells in the retina

Mike O. Karl

Introduction: Retinal regeneration is well established in non-mammalian vertebrates. After retinal damage in fish this regeneration is complete; in chicken it is limited to inner retinal neurons. Whether a similar process might occur in mammals is not conclusive. I examined Müller glia as a potential cell source for neuronal regeneration in adult mice.

Methods: To address this question in vivo I damaged adult mouse retina by intraocular injection of neurotoxins or exposure to bright light. Two days later I subsequently injected growths factors. Several growths factors were tested and animals were sacrificed at various time points.

Results: Although Müller glia migrate after NMDA neurotoxic injury, they do not proliferate. However, if any one of a number of growth factors is injected after the NMDA-induced damage, the Müller glia re-enter the cell cycle. Subsequent application of EGF, FGFa, Shh, Wnt3a or a GSK 3α/β inhibitor, but not TGFß2, induce proliferation in vivo. The combination of FGF1 and insulin was most successful in stimulating regeneration in the mouse retina. NMDA damage, followed by FGF1/insulin injections in a line of mice in which green fluorescent protein (GFP) is expressed from the GAD67-promoter revealed that some of the progeny of Müller glia (BrdU+) differentiate into GAD67-expressing neurons (amacrine, ganglion, or horizontal cells). Exposure to bright light leads to photoreceptor damage and subsequent injection of growth factors does not increase the number of regenerated GAD67-GFP neurons compared to growth factor injections alone. None of the treatments led to regeneration of mGluR6-GFP labeled bipolar cells.

Conclusions: Thus, Müller glia can be induced to proliferate, dedifferentiate and regenerate retinal neurons, most likely amacrine cells, in the mouse retina. Loss of retinal interneurons, but not photoreceptors increases the number of regenerating interneurons significantly. Although more limited than chicken or fish retina, the adult mouse retina has the potential to regenerate inner retinal neurons after damage.

Development of improved protocols for photoreceptor cell transplantation

AC Barber, E West, RE MacLaren, RR Ali, RA Pearson.
Department of Genetics, UCL Institute of Ophthalmology, London, UK

Purpose: Photoreceptor cell transplantation provides a novel approach to restore sight in retinal diseases that involve photoreceptor loss. Photoreceptor transplantation can successfully restore the pupillary reflex in models of retinal degeneration. However, greater numbers of integrated cells are required to restore visual acuity. Integration may be improved by targeting physical barriers within the host environment. The outer limiting membrane (OLM) is compromised of adherens junctions between Müller cells and photoreceptors and forms a barrier separating the interphotoreceptor matrix from the outer nuclear layer (ONL). Here, we assessed whether integration of transplanted photoreceptors can be improved by chemical, molecular or genetic manipulation of the OLM.

Methods: Transient disruption of the OLM was induced using a chemical or molecular approach. In the first instance a glial toxin, dl-alpha-aminoadipic acid (AAA) was administered intravitreally prior to subretinal transplants of dissociated early post-natal retinal cells. For the molecular disruption of the OLM siRNA targeting ZO-1, a component of the OLM, was administered subretinally prior to retinal cell transplants. The number of integrated photoreceptors in the ONL was counted 3 weeks post injection and compared to the control injected contralateral eye. To assess genetic manipulations of the OLM the $Crb1^{rd8/rd8}$ mouse model was used. This model has a defect in Crb-1; a protein involved in the formation of the OLM adherens junctions. These mice display a progressive retinal degeneration accompanied by fragmentation of the OLM. $Crb1^{rd8/rd8}$ mice received subretinal injections of donor retinal cells. Again, the number of integrated photoreceptors was assessed and compared to wildtype controls.

Results: Both the pharmacological and molecular approaches resulted in reversible disruption of the OLM and did not result in significant long term damage to the host retina. Moreover, when combined with cell transplantation, both treatments resulted in a significant increase in the number of integrated photoreceptors compared to control injected eyes. In comparison to wildtype controls, transplantation into the $Crb1^{rd8/rd8}$ mouse also showed significant improved integration.

Conclusion: The OLM is an important natural barrier that impedes integration of transplanted cells and disruption of the OLM should be considered in future protocols to improve photoreceptor transplantation. This study also highlights that it is possible to temporarily disrupt the OLM with no significant long term damage to the host retina.

Limiting the degeneration of photoreceptors with forebrain-derived progenitor cells.

Ray Lund, PhD, Casey Eye Institute, OHSU, Oregon, USA

We have explored the potential for slowing progress of photoreceptor loss in Royal College of Surgeons rats, in which photoreceptors die as a result of a retinal pigment epithelium (RPE) defect, by transplanting human-derived forebrain progenitor cells into the subretinal space at an age before degeneration has advanced. The donor cells form a sheet between the host RPE and the photoreceptors, to which they become closely related. Some cells also enter the retina where they show neuron-like features, but they do not take on retinal cell morphological or antigenic characteristics. Cells in both locations can survive for more than 200 days. Substantial photoreceptor rescue is achieved for these prolonged periods. Most important, we tested two visual functions – acuity, and luminance threshold responses across the visual field - and found substantial rescue of both functions over time. Transient rescue of full field ERG was also achieved.

To test whether these cells might also be effective in another human disease model, we did similar transplants in a mouse model of Usher 2A. (Liu et al., PNAS 104, 4413, 2007). We found that although no overt morphological changes were seen until later ages in untreated mice, deterioration of acuity and contrast sensitivity functions was seen as early as 70 days of age with continued deterioration over time. Cell transplantation at P70 resulted in restoration of behavioral measures to wild type non-dystrophic mouse levels and this was sustained for at least 6 weeks.

As a step towards translation to clinic, we introduced cells to the subretinal space of normal non-human primate eyes, to ensure that cells can be delivered to a human-like eye without negative effects. Such transplants distributed in the subretinal space and survived for at least 6 weeks, even without immunosuppression: furthermore there was no deterioration in multifocal ERG responses from preoperative levels, when measured at various times post-transplantation.

In summary, the forebrain-derived progenitor cell appears to be a promising cell for application in retinal degenerative diseases involving photoreceptor dysfunction, not only caused by RPE disorder but by an intrinsic photoreceptor mutation.

Encapsulated cell technology and the protection of photoreceptors in retinal degenerative diseases

K. Kauper, Neurotech Pharmaceuticals, Lincoln RI

Ophthalmic disorders represent a rapidly growing disease area that is associated with the aging population. Their sight is threatened by age related macular degeneration, diabetic retinopathy, glaucoma, or retinitis pigmentosa (RP). Few effective treatments for these disorders are available to date, in part due to lack of effective mechanisms to deliver therapeutic molecules to the retina. The encapsulated cell technology (ECT) allows the controlled, continuous, and long-term administration of protein drugs in the eye, where therapeutic agents are needed, eliminating the clinical issues associated with chronic intraocular injections or potential systemic toxicity due to intravenous route of drug delivery.

Neurotech's lead product to date uses the ECT platform to deliver ciliary neurotrophic factor (CNTF) to the retina as a potential chronic treatment for RP and geographic atrophy (GA). Preclinical studies in the S334ter-3 rats and *rcd*1 canines have shown the effectiveness of CNTF to protect the photoreceptor cells from degeneration in these animal models. In follow-up investigations in over 200 human patients a significant body of data has been collected proving the safety of the ECT treatment modality in human application. Furthermore, clinical data from these human studies has corroborated the beneficial results seen in earlier animal models and suggests the beneficial role CNTF may have in the initial protection of photoreceptor cells and/or the prevention of further degeneration of the critical photoreceptor cells responsible for human vision.

This presentation will review Neurotech's encapsulated cell technology, pre-clinical and clinical trial results and discuss the positive role CNTF may have in providing a potential treatment option for those patients with diseases affecting the photoreceptors.

Microglia in retinal degeneration

Solon Thanos, Dept. Experimental Ophthalmology, University Eye Hospital Münster, Domagkstr. 15, 48149 Münster, Germany

Purpose: To review the role of microglial cells, members of the monocytic lineage, which represent the resident immunocompetent cells of the central nervous system including the retina. The retina shows particular peculiarities such as a double blood retinal barrier.

Methods: Microglial cells invade the retina in response to naturally occurring neuronal death during embryonic development and remodelling. Staining of microglial cells can beseessed with immunohistochemsitry, vital phagocatotic loading or in vivo imaging.

Results: Resident microglial cells are extremely sensitive to changes in their microenvironment arising from either traumatic or chronic neurodegeneration, inproper wiring, hereditary diseases or infection and become rapidly activated. In their activated state, the cells undergo drastic morphological changes, upregulate a variety of receptors and secrete soluble factors which contribute to recognition and phagocytotic clearence of dying or malfunctioning neurons. Microglial cells can migrate through the retinal tissue and contribute to local phagocytotic processes.

Conclusions: It is concluded that microglial cells are involved in experimentally induced or naturally occurring retinal neurodegenerations. Activation of microglial cells can result in devastating fate of neurons or induce retinal disease. Expanding on the mechanisms, we shall discuss on approaches to pharmacologically interfere with the microglial activation and neurophagy. The protagonistic role of microglial cells in the outcome of certain diseases help designing microglial targeted treatments with potential benefit for neuronal survival and regeneration in clinically relevant conditions.

Inflammatory chimiokine receptor deficiencies lead to different degrees of spontaneous subretinal microglial cell accumulation and photoreceptor degeneration

Florian Sennlaub, William Raoul, Constance Auvynet, Serge Camelot, Sophie Lavalette, Mathieu Rodero, Charles Feumi, Francine Behar Cohen, Sylvain Chemtob and Christoph Combadière

Retinal microglial cells (MCs) are physiologically located in close proximity to the retinal vessels in the inner retina. They accumulate subretinally in aging, light induced injury, and retinal degeneration models. Similarly, activated MC accumulation in the photoreceptor cell layer has been reported in age-related macular degeneration (AMD), the leading cause of irreversible blindness. We have previously shown that retinal MC mobility and distribution is controlled by the inflamatory chimiokine receptor (iCR) CX3CR1. Here we show that populations of human and murine retinal MCs express the iCR CCR1, CCR2 and CCR5 additionally to CX3CR1. Significant progressif age dependent subretinal MC accumulation was observed as early as 3m in CCR1-/- and CCR5-/-, from 6 months in CX3CR1-/-, but only at 18m in WT and CCR2-/- mice. A significant number of subretnal CCR1-/-, CCR5-/- MCs and to a lesser extent CX3CR1-/- MCs were binucleated and appeared bloated by intracellular autofluorescent lipid accumulation, giving a "drusen-like" appearance in fundoscopy. Subretinal MC accumulation was associate with significant spontaneous photoreceptor degeneration in CX3CR1-/- and CCR1-/- and to a much lesser extent in CCR2-/- and CCR5-/- mice at 18m. These results show that individual inflammatory CR deficiencies lead to different degrees of spontaneous subretinal MC accumulation, "drusen-like" appearance and neurotoxicity. These findings reveal a novel pathogenic process with important implications for the development of new therapies for retinal degenerative diseases such as AMD.

Novel roles for glia in NCL pathogenesis

Dr Jonathan D Cooper, Pediatric Storage Disorders Laboratory, Department of Neuroscience, Institute of Psychiatry, King's College London.

Purpose: The neuronal ceroid lipofuscinoses (NCLs, Batten disease) are a group of at least ten fatal inherited lysosomal storage disorders that affect mostly children. Each form of NCL is caused by a mutation in a different gene, resulting in different ages of onset depending on which gene is mutated. Clinically these disorders all display visual failure leading to blindness, progressively worsening seizures and relentless declines in cognitive and motor abilities ending in an early death.
Pathologically these disorders also display retinal degeneration and a profound loss of neurons within the brain. However, this cell loss does not occur globally, but displays remarkable selectivity, especially early in pathogenesis. Unusually, this loss of neurons in the NCLs is preceded by localized glial activation, which serves as an accurate predictor of where neuron loss subsequently occurs. This raises the possibility that glia are important players in NCL pathogenesis, which we have been investigating both *in vivo* and *in vitro*.

Methods: Immunohistochemical staining was performed for markers of astrocytes, microglia, neuronal markers (including Nissl staining), presynaptic proteins and other inflammatory markers. Unbiased stereological analysis of these markers was performed in different NCL mouse models, using the thalamocortical system as a series of well-defined pathways that are affected in these disorders. Pure microglial or astrocyte cultures and neuron co-cultures derived from NCL mouse models also provides a powerful model system to investigate the role of neuron-glial interactions in pathogenesis.

Results: Although similar pathological events occur in each subtype, the sequence in which they happen during disease progression differs markedly between forms of NCL. There is a complex relationship between astrocytes, neurons and microglia, with our recent data pointing towards novel roles for astrocytes at the synapse. In Juvenile NCL (JNCL), an early glial activation precedes neuron loss by many months, but appears to be attenuated with incomplete morphological transformation of both astrocytes and microglia. Primary microglial cultures from JNCL mice also display an incomplete morphological response to LPS stimulation, and a distinctive cytokine expression profile as detected via antibody arrays.

Conclusions: Taken together our data provide the first evidence for altered glial neurobiology in these disorders and raise the possibility that altered microglial function contributes directly to the outcome and progression of disease.

Modulation of retinal microglia by dietary lipids

Stefanie Ebert [1], Karin Weigelt [1], Yana Walczak [1], Wolfgang Drobnik [2], Richard Mauerer [2], David A. Hume [3], Bernhard H.F. Weber [1], and Thomas Langmann [1]

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[2] Synlab Medical Care Service, Weiden, Germany
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Purpose: Microgliosis, disturbed lipid metabolism, and photoreceptor degeneration are common phenomena in retinal dystrophies. The aim of this study was to identify a potential link between these events using a prototypic model of inherited retinal degeneration, the retinoschisin-deficient ($Rs1h^{-/Y}$) mouse.

Methods: To visualize microglia, $Rs1h^{-/Y}$ animals were crossed with transgenic MacGreen mice, which express EGFP under the control of the macrophage-specific *csf1r* promoter. Microarray analysis and qRT-PCR were performed for molecular profiling of microglia. A DHA supplementation study was carried out with $Rs1h^{-/Y}$ mice and lipid profiles of the retina, brain, and plasma were determined by gas-chromatography mass-spectrometry. Retinal histology, photoreceptor apoptosis, and microglia markers were analyzed.

Results: Lipid droplet-containing activated microglia were detected in retinal sections of early postnatal $Rs1h^{-/Y}$ / MacGreen mice before the onset of overt neuronal cell death. Microarray analysis of isolated microglia revealed induction of transcripts involved in lipid droplet formation and eicosanoid synthesis. Analysis of retinal phospholipids revealed a significant decrease in docosahexaenoic acid (DHA) levels in $Rs1h^{-/Y}$ mice. A dietary intervention study was performed to establish a potential link between microglia activation, reduced DHA levels, and neurodegeneration. Female $Rs1h^{-/-}$ mice and their $Rs1h^{-/Y}$ litter were either subjected to a diet enriched with 2% DHA, or a standard chow. Supplementation with DHA enhanced photoreceptor survival and converted lipid-bloated microglia to a quiescent phenotype with reduced pro-inflammatory gene expression. As a consequence, retinal DHA levels may control the activity of microglia and thereby may affect the progression and extent of retinal degeneration.

Conclusions: We conclude that dietary DHA supplementation could be a treatment option for inherited retinal degeneration that restores both microglial homeostasis and neuronal viability.

Study design and randomization in clinical trials

Holger Lüdtke, Datinf GmbH, Tübingen

Introduction: The success of a clinical study will depend critically upon the effort you apply in its initial planning. A poor design may result in a dramatic decrease of power of the study and, in most cases, cannot be corrected afterwards. There is no "best" study design in general. The design has to be adapted to the question and external circumstances e.g. ethical considerations. In randomized controlled trials (RCT), randomization plays an essential role to be protected against selection bias of known and unknown factors, especially in trials with a small number of subjects.

Content: Clinical studies can be divided into different categories, descriptive, observational and experimental trials. These are tools for different purposes as generating hypotheses, investigating the relationship between cause and disease or to demonstrate the efficacy of a therapy. Depending on the purpose the study designs are different: simple case reports, case-control and cohort studies, or randomized controlled trials. Beside the selection of the primary endpoint, important details of the RCT are different methods of blinding, possible control groups or crossover and randomization.
Regarding a difference between two groups this can be due to a real effect associated with the two groups, to chance or to a systematic, not controlled difference between the groups. The aim of the randomization is to obviate the latter one. There are different types of randomizations, simple randomization, permuted block randomization, stratified randomization, covariate-adaptive randomization or outcome-adaptive randomization. Randomization also includes the allocation concealment, from sealed envelopes to central randomization via internet.

Summary: The presentation will focus on different study designs and randomization techniques and discuss its advantages and disadvantages.

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Special aspects of clinical trials in ophthalmology

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Introduction: What is the meaning of abbreviations like EC, CRO, SIV, ISF or IC? How can an investigator be a sponsor? To be effective as an investigator and study centre one should be familiar to terms and procedures in the world of clinical trials. What is related to the planning and performance of a clinical trial in ophthalmology? How can the quality of study procedures be optimized? These and many more questions arise related to clinical trials.

Content: After a general part dealing with the legal framework of clinical trials and the principles of good clinical practice special aspects of ophthalmological clinical trials are explained by several recent examples. All necessary steps in trial preparation and performance are described including aspects often underestimated or forgotten. Dealing with visually impaired or even blind patients implies special additional demands in clinical trials. On the other hand there are high technical demands and the need of certifications asked for in high performance study procedures and examinations. The increasing administrative and procedural complexity of clinical trials is an increasing challenge to cope with alongside of clinical routine work. The Centre of Ophthalmology at the University of Tübingen provides a special unit where medical and technical stuff is dealing exclusively with clinical trials, the STZ *eyetrial*. STZ *eyetrial* is a certified member of the European Vision Institute's sites of excellence for clinical trials (EVI.CT.SE). STZ focuses on trials with innovative pharmaceuticals therapeutics as well as medical devices like retinal implants. STZ promotes clinical trial performance at the Centre of Ophthalmology, supports physicians involved in clinical trials and guarantees high quality standards in sponsor-initiated as well as investigator-initiated projects.

Conclusion: Quality aspects have to be dealt with growing intensity in future ophthalmological trials demanding increasing professionalism. Specialised teams in ophthalmological centres may be one possible solution to face this challenge and, in parallel, to satisfy the needs of participating patients. Intensive training in trial matters for study teams is necessary to cope with quality demands and time lines in trials serving research for the benefit of visually impaired patients.

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The long road to data interpretation in clinical trials

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Background: Pharmaceutical companies are in desperate need of good ideas for developing new drugs and the EC is starting a large programme for cooperation between investigators and these companies.

Purpose: To describe to medical researchers some pitfalls in the road towards setting up and completing a clinical trial.

Methods: descriptive lecture on personal and hear-say experiences in clinical trials

Results: The paramount steps for a researcher to start a study are:

1. Analyze her/ his own motives for developing a new therapy
2. Provide a sound theoretical basis and proof of principle
3. Be sure disease under study is well described
4. Look for reliable pharmaceutical partners
5. Develop an excellent study design with experienced persons in pharmaceutical partner
6. Make clear agreements on the rights and obligations of all partners
7. Describe what to do with unexpected results
8. Insist on your independent analysis of the raw and final clinical data
9. Be very specific on the publication policies
10. Carefully examine feasibility of the study, e.g. amount of visits of the patients, impact of the tests.
11. Scrutinize inclusion and exclusion criteria
12. Divide mentally the number of promised available patients in a certain time span by half or three in order to get a real guestimate.
13. Develop good case report forms (CRFs) and adapt them after pilot study
14. Insert properly qualified personnel in the study team
15. Make flowsheet with all necessary visits on it.
16. Make good monitor plan and build in resilience when schedule is too tight

Financial disclosures: none.

PRO RETINA
POTSDAM-MEETING

POSTER ABSTRACTS

Mechanism of photoreceptor cell death in P23H-1 and S334ter-3 mutant rhodopsin transgenic rats

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Purpose: To examined the expression and activity of Calpain and PARP during photoreceptor cell death in transgenic rats with P23H and S334ter rhodopsin mutations.

Methods: Retinas of P23H-1, S334ter-3 and wild type Sprague Dawley (SD/CD) rats at different developmental ages (PN0 – PN30) were examined by conventional histological techniques, TUNEL staining, immunohistochemistry and immunoblotting using specific antibodies for PAR and PARP. Monitoring of calpain or PARP activities at the cellular level was investigated using enzymatic in situ assays (Paquet-Durand et al., 2006; 2007) on unfixed cryosections.

Results: In S334ter-3 rats many photoreceptors and few cells in the inner retina were positive for TUNEL assay during the whole development. In P23H-1 rats only some photoreceptors were positive for TUNEL assay however, positive cells remains detectable as late in the development as PN30. In addition, calpain activity was considerably increased in photoreceptor cells in the P23H-1 and S334ter-3 retinas and absent on CD rats. We observed PARP activity only in a subset of cell bodies in P23H-1 and S334ter-3 photoreceptors, but never on the CD retinas. Contradictory to previous studies made in rd1 mouse, PARP immunostaining was found to be increased in both transgenic models when compared to CD rats. PAR immunostaining showed numerous positive cells in the outer retina of both transgenic rats, nevertheless, their amount was higher in the S334ter-3. PAR positive cells were not detected in the CD retina.

Conclusions: These results coincide with previous studies using other animal models for retinitis pigmentosa, indicate that activation of calpains and PARP could be a general mechanism involved in photoreceptor cell death, which elevates the possibility of using calpain and/or PARP inhibitors as therapeutic agents to prevent or delay photoreceptor degeneration.

Identification and characterization of interacting proteins to the USH1G protein SANS

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Purpose: The human Usher syndrome (USH) is the most common form of inherited combined deaf-blindness. It is genetically heterogeneous and can be clinically divided into 3 subtypes (USH1-3). Mutations in USH proteins lead to profound inner ear defects and degeneration of retina. The 10 so far identified USH genes encode proteins of diverse protein families. Former studies revealed that all known USH1 and USH2 molecules are integrated in protein networks. Here we investigated the role of the USH1G protein SANS (scaffold protein containing ankyrin repeats and SAM domain) in these USH networks.

Methods: Screening bovine retina library by yeast-2-hybrid (Y2H) system using different domains of SANS as baits. GST-pull downs for validation. Analyzing subcellular localization of putative interacting proteins via immunofluorescence and immunoelectron microscopy.

Results: Y2H screens with SANS´s C-terminal SAMPBM (sterile alpha motif, PDZ-binding domain) identified three different PDZ proteins, including the MAGUK protein MAGI2 as putative interactors of SANS. GST-pull down assays confirmed direct interaction between PDZ5 of MAGI2 and SANS C-terminus. Indirect immunofluorescence showed partial co-localization of both proteins in the retina. ImmunoEM demonstrates association of MAGI2 with transport vesicles in photoreceptor cells. Y2H screens with SANS´s central domain identified 24 additional proteins as putative SANS binding partners. Here we further analyzed the binding of SANS to Alsin which is related to the ALS neurodegenerative disease and associated with vesicular trafficking.

Conclusions: Direct binding of SANS to vesicle associated MAGI2 and subcellular distribution of the complex confirm SANS´s participation in inner segment transport. Furthermore, this interaction indicates a molecular link of USH protein networks to establishment of planar cell polarity which is closely associated with MAGUK protein function. Binding of SANS to Alsin provides first evidence for connection of the interactome related to the USH disease with molecules associated with ALS. Interaction with both proteins give therefore hint for a role of SANS in a transport network necessary for maintenance and function of photoreceptor cells. Defects of complex partners may lead to dysfunction of the entire network causing photoreceptor degeneration as seen in USH patients.

Supports: DFG GRK 1044/2; FAUN; Pro Retina Deutschland; Forschung contra Blindheit

Comparative analysis of GCAP1 mutants involved in hereditary cone dystrophies

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Purpose: Guanylate cyclase-activating protein 1 (GCAP1) operates as Ca^{2+}-sensor in rods and cones of the vertebrate retina and regulates the target enzyme, the photoreceptor guanylate cyclase (GC), in a Ca^{2+}-dependent manner. This GCAP1/GC-system plays a prominent role in phototransduction and light adaptation. So far several mutations in the GCAP1 gene were discovered in patients suffering from retinal diseases leading to a dysfunction in Ca^{2+}-sensor properties. Impairment of GCAP1 function includes the constitutive activation of photoreceptor GC at physiological Ca^{2+}-concentration and destabilization of the three-dimensional GCAP1 structure. The aim of this study was to test a set of recently described GCAP1 mutants (Kitiratschky et al; 2009) for alteration of their molecular properties.

Methods: Wildtype GCAP1 and the mutants E89K, D100E, L151F and G159V were heterologously expressed and purified. Protein samples were analysed for GC-activating properties and in particular for the impairment of protein folding by limited proteolysis and tryptophane fluorescence spectroscopy. Monomer-dimer equilibria were investigated by high performance liquid chromatography.

Results: All tested GCAP1 mutants displayed a shift in their Ca^{2+}-sensitivities leading to constitutive activators of GC at physiological Ca^{2+}-concentration (Kitiratschky et al., 2009). However, all tested mutants fold into a more compact structure when Ca^{2+} is bound, which is not different from the wild-type. The presence of Ca^{2+} did further trigger the dimerization of the mutants D100E, L151F and G159V, where the most pronounced dimerization was observed for the L151F mutant.

Conclusion: In a comparative analysis of all known GCAP1 mutations that are related to cone dystrophies we discuss three main cellular routes of photoreceptor dysfunction. Mutations can affect the Ca^{2+}sensitive activation of GC and/ or induce a destabilization of the GCAP1 structure. We here show that a distorted monomer-dimer equlibrium could result from certain point mutations. GCAP1 dimers are known to be inactive. Furthermore, GCAP1 dimers could act as seeds causing the formation of cellular aggregates, a typical phenomenon observed in the pathogenesis of many diseases.

Kitiratschky, V. B., Behnen, P., Kellner, U., Heckenlively, J. R., Zrenner, E., Jägle, H., Kohl, S., Wissinger, B., Koch, K.-W. (2009) Mutations in the GUCA1A gene involved in hereditary cone dystrophies impair calcium-mediated regulation of guanylate cyclase. Human Mutation –submitted-

Supported by Pro Retina Deutschland e.V.(Pro-Re/Prom-Stip.Koch/Behnen.1)

Comparison of primary retinal cells versus expanded retinal stem cells in their ability to generate photoreceptors in a cell density – dependent manner.

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Purpose: One of the treatment strategies proposed for retinal degenerations is to use cell transplantation to replace photoreceptor cells that are lost due to disease or injury. For clinical use it is important that donor cells are easily available in sufficient quantity. Here an in vitro differentiation assay was performed on primary cells isolated from retinas of newborn mice as well as expanded retinal stem cells to investigate their capacity to form photoreceptor cells.

Methods: Retinal cells were isolated from wild-type mice at postnatal day 0. Some cells were seeded on coverslips at various densities (5 – 8 x 10^5 cells/well) and subjected to differentiation in 1% fetal calf serum (FCS). Some cells were expanded in DMEM/F12 medium supplemented with B27 and EGF/FGF2 to generate retinal stem cell cultures. After second passage retinal stem cells were also subjected to differentiation using 1% FCS. Morphology was progressively compared. After 8 days cells were fixed in 4% paraformaldehyde (PFA) and analyzed via immunocytochemistry using photoreceptor-specific markers. Recoverin- (early photoreceptor marker) and rhodopsin (late rod marker)-positive cells were quantified and results compared between both primary and expanded cells in corresponding densities.

Results: Expanded retinal stem cells following serum treatment generate a homogenous population of big, flat cells. Regardless of the cell density, expression of recoverin as well as rhodopsin was not observed. On the other hand primary retinal cells are highly heterogenous, generate cells of neuronal as well as non-neuronal morphology. The proportion of recoverin-positive cells appeared to remain at the same level independently of the cell density. However, the relative amount of rhodopsin-positive cells increased with higher cell densities.

Conclusion: High cell density determines maturation of young photoreceptors derived from primary retinal cells. However, after expansion and passaging these cells loose ability to adopt a photoreceptor fate. Thus, optimal culture conditions for the propagation of retinal cells that retain the potential to generate photoreceptor cells have to be established to make these cells applicable for cell-based strategies following photoreceptor loss.

Gene expression analysis of GDNF-induced Mueller Glial- derived transcripts: Potential candidates for neuroprotective proteins

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Purpose: Retinal Mueller Glial cells (RMG) are the major type of glia in the mammalian retina. Glial-Cell-Line-Derived Neurotrophic Factor (GDNF) is a powerful neuroprotective factor for photoreceptors in the retina which acts via RMG cells (Hauck et al., 2006). Upon stimulation with GDNF, RMG cells secretion neuroprotective factors, which enhance survival of rods, cones and other retinal neurons. Unfortunately, those GDNF-induced neuroprotective factors have not been identified to date. The purpose of this work is to identify GDNF-induced neuroprotective factors from RMG.

Methods: Explanted retinas from hGFAP: eGFP mice were directly stimulated with GDNF for 24h before RNA isolation. Additionally, after 24h incubation with or without GDNF, RMG were isolated from the retinas with FACS sorting and used for RNA isolation. From all experiments expression profiling of mRNAs was performed with Illumina microarrays. Results were confirmed by real-time PCR and selected candidate genes were further validated in a photoreceptor survival bioassay.

Results: A total of 46 genes were found regulated (ratios ≥ 2 or ≤ 0.5) in response to GDNF stimulation from total mouse retina and from FACS sorted RMG cells. Among these genes, seven transcripts were both, increased upon treatment and coding for a secreted protein and were thus included in the confirmation experiments. One of these candidates (osteopontin) was obtained as purified protein and tested for survival promoting activity on primary photoreceptors *in vitro*. We found that osteopontin enhances survival of photoreceptors in a concentration-dependent manner. Additionally, we could confirm expression of osteopontin in RMG *in situ*.

Conclusions: Gene expression analysis showed that the stimulation of explanted retinas and primary RMG cells in culture with GDNF specifically changes gene expression profiles. Among GDNF-induced transcripts are novel candidate factors for neuroprotective activity. We are currently testing a set of prioritized candidates for their therapeutic properties towards future clinical application.

Integrins alpha 5/alpha v and protein tyrosinphosphatase 1 (PTP1) in retinal pigment epithelium – key players in fibrotic and angiogenic processes?

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Background: Integrins play an essential role in physiological processes of the retina and the underlying retinal epithelium (RPE). They provide cell adhesion, regulatory functions in photoreceptor phagocytosis and cell proliferation and migration. As tyrosine phosphorylation via tyrosine phosphatases as PTP1 and tyrosine kinases is important in integrin-mediated signaling processes, it also may play a role in RPE under physiological and pathological conditions (e.g. fibrosis and angiogenesis at the posterior pole). Aim of the study was an immunohistochemical analysis of the RPE using different antibodies.

Methods: Immunohistochemical analysis (indirect immunofluorescence) using cryosections of different tissues (porcine, bovine, rat, human) and cultured retinal pigment epithelium cells (ARPE19) using integrin alpha 5, integrin alpha V and PTP1 antibodies, as well as antibodies against ß-catenin and bestrophin-1 for double staining.

Results: Integrin alpha 5 and alpha v were found *in situ* in human and bovine retinal tissue; integrin alpha 5 was mostly located apically. In human choroidea, we found few integrin alpha 5/ PTP1 positive cells. Porcine RPE was integrin alpha 5 and alpha V negative. *In vitro* confluent ARPE19 cells were integrin alpha 5 and alpha V positive at the basal and apical cell membrane after fixation and staining of crysections; they showed only weak positivity for PTP1. In the rat eye, we found weak reactivity for integrin alpha 5, integrin alpha V and PTP1 at the RPE level.

Conclusion and Perspective: Integrin alpha 5 and alpha V are of functional and structural importance in the RPE. The role of PTP – especially on integrin mediated signaling – has to be determined. Functional assays are underway to analyze their interactions, which might be putative targets of therapeutical intervention.

Luteolin as a potent antagonist of neurotoxic microglia activation

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Purpose: Neurodegenerative diseases including retinal dystrophies are associated with a loss of limiting control mechanisms of innate immunity. Sustained microglia activation may lead to increased apoptosis and retinal tissue damage. The aim of this study was to analyze whether the flavonoid Luteolin has modulatory effects on microglia gene expression and function.

Methods: DNA-microarray analysis and real-time qRT-PCRs were performed with Luteolin-treated non-activated and LPS-activated BV-2 microglia-like cells. NO production of BV-2 cells and primary microglia cells was determined by Griess reaction. Apoptosis of 661W photoreceptor cells treated with microglia conditioned media was quantified by measuring caspase 3 and caspase 7 activities.

Results: Luteolin caused a dose-dependent inhibition of LPS-mediated pro-inflammatory and adhesion molecule gene expression. Moreover, Luteolin induced the transcription of several genes involved in cellular oxidative stress response. Neurotoxic NO release was strongly decreased in Luteolin-treated microglia and reduced apoptosis was detected in 661W photoreceptor cells cultured in the presence of conditioned media from Luteolin-treated microglia.

Conclusions: Luteolin antagonizes the release of toxic radicals from activated microglia and thereby excerts neuroprotective effects. Luteolin is a promising candidate for *in vivo* studies investigating a potential therapeutic effect in retinal degeneration.

A cell for a cell – therapeuthical replacement approaches for the treatment of retinopathies

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Aim: Retinitis pigmentosa (RP) as one of the most common forms of inherited retinal degeneration is, beside age-related macular degeneration, diabetic retinopathy, or cone-rod dystrophy, characterized by a significant progressive loss of photoreceptor cells. Currently, there is no effective treatment available. We are working on a cell based approach for the treatment of these kinds of retinopathies by replacing degenerated photoreceptors with photoreceptor precursor cells which may then integrate into the outer nuclear layer (ONL) of the retina and differentiate into mature photoreceptor cells. If these precursor cells were able to integrate and differentiate in a significant number it might be possible to rescue the loss of photoreceptor cells and improve visual function.

Methods: We used wildtype mice and a mouse model of retinal degeneration (p347s) which is characterized by a significant loss of photoreceptor cells within a time window from 24 days to 4 months after birth. Subretinal injections were performed with precursor cells extracted from postnatal day 4 (P4) retinas of transgenic rhoGFP and actinGFP mice. Further analysis was done by immunohistochemical staining of 30µm Vibratome sections.

Results: We observed that injected cells were able to integrate into the ONL with a success rate of approximately 0,01%. These rhoGFP-positive donor cells developed the morphology of mature photoreceptor cells and expressed photoreceptor specific markers (rhodopsin, recoverin). Another fraction of the transplanted cells (approx.: 0,1%) which were not able to integrate are found in the subretinal space between the outer segment and retinal pigmented epithelium. This heterogeneous population of rhoGFP-positive cells which do not show a morphology like a mature photoreceptor is characterized by cells expressing photoreceptor specific markers like rhodopsin and recoverin and cells not expressing these markers.

Conclusion: We could show that transplanted P4 retinal precursor cells were able to integrate into the ONL of wildtype and p347s mice. Nevertheless the observed numbers are currently too low for an effective treatment of retinopathies. Furthermore the functionality of the integrated cells remains unproven. Further studies which will characterize the different fractions of transplanted cells may provide more information about the nature of these cells and their potential to integrate and form new photoreceptor cells.

Modulation of retinal microglia by dietary lipids

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Purpose: Microgliosis, disturbed lipid metabolism, and photoreceptor degeneration are common phenomena in retinal dystrophies. The aim of this study was to identify a potential link between these events using a prototypic model of inherited retinal degeneration, the retinoschisin-deficient ($Rs1h^{-/Y}$) mouse.

Methods: To visualize microglia, $Rs1h^{-/Y}$ animals were crossed with transgenic MacGreen mice, which express EGFP under the control of the macrophage-specific *csf1r* promoter. Microarray analysis and qRT-PCR were performed for molecular profiling of microglia. A DHA supplementation study was carried out with $Rs1h^{-/Y}$ mice and lipid profiles of the retina, brain, and plasma were determined by gas-chromatography mass-spectrometry. Retinal histology, photoreceptor apoptosis, and microglia markers were analyzed.

Results: Lipid droplet-containing activated microglia were detected in retinal sections of early postnatal $Rs1h^{-/Y}$ / MacGreen mice before the onset of overt neuronal cell death. Microarray analysis of isolated microglia revealed induction of transcripts involved in lipid droplet formation and eicosanoid synthesis. Analysis of retinal phospholipids revealed a significant decrease in docosahexaenoic acid (DHA) levels in $Rs1h^{-/Y}$ mice. A dietary intervention study was performed to establish a potential link between microglia activation, reduced DHA levels, and neurodegeneration. Female $Rs1h^{-/-}$ mice and their $Rs1h^{-/Y}$ litter were either subjected to a diet enriched with 2% DHA, or a standard chow. Supplementation with DHA enhanced photoreceptor survival and converted lipid-bloated microglia to a quiescent phenotype with reduced pro-inflammatory gene expression. As a consequence, retinal DHA levels may control the activity of microglia and thereby may affect the progression and extent of retinal degeneration.

Conclusions: We conclude that dietary DHA supplementation could be a treatment option for inherited retinal degeneration that restores both microglial homeostasis and neuronal viability.

TMEM16B, a novel protein with calcium-dependent chloride channel activity, interacts with PSD95 at the photoreceptor synapse

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Purpose: Molecular compartmentalisation of the presynaptic plasma membrane forms the basis for transmission of light induced electrical signals to second order neurons at the highly specialized photoreceptor ribbon synapse. The precise organization of synaptic components is achieved by scaffolding proteins. The aim of this study is to analyze the interaction between TMEM16B, a novel transmembrane protein with calcium-dependent chloride channel activity and photoreceptor presynaptic proteins.

Methods: Antibodies against mouse TMEM16B and photoreceptor proteins were used for immunohistochemistry and Western blotting. Yeast two-hybrid and GST-pulldown assays were applied to determine protein-protein interaction.

Results: TMEM16B co-localizes with scaffolding proteins PSD95, VELI3 and MPP4 at mouse photoreceptor synaptic membranes. The intracellular C-terminus of TMEM16B contains a consensus PDZ class I binding motif that is capable and required to interact with PDZ domains of PSD95.

Conclusion: Our results suggest that TMEM16B is a novel component of a presynaptic protein complex recruited to specialized plasma membrane domains of photoreceptor terminals to evoke Ca^{2+}-dependent Cl^- currents that regulate synaptic output.

The Crumbs complex in retinal development and degeneration: Using animal paradigms to study the molecular basis of human retinal dystrophies

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Two forms of retinal dystrophies, autosomal recessive retinitis pigmentosa (RP) and autosomal Leber congenital amaurosis (LCA) are associated with mutations in the *Crumbs homologue 1 (CRB1)* gene. The retinas of LCA patients with mutations in CRB1 lack the distinctive layered structure seen in healthy tissue, suggesting a failure to develop properly. *Crumbs* was initially identified in *Drosophila* as a key regulator of epithelial polarity by regulating formation of the adherens junction. Ensuing work identified Crumbs to be part of a protein complex, the core components of which are the transmembrane protein Crumbs and the scaffolding proteins Stardust, *D*PATJ and *D*Lin-7. Defects in individual components lead to disintegration of embryonic epithelia, morphogenetic defects in photoreceptor cells and light-dependent retinal degeneration.

Despite a basic understanding of Crumbs complex functions in various tissues, its molecular functions remain unclear. We use *Drosophila* and *Danio rerio* (zebrafish) as genetically tractable model organisms to study the molecular and cell biological functions of the Crumbs complex. Current work in *Drosophila* is aimed at identifying additional components of the Crumbs complex using genetic and biochemical methods. We are also using live imaging and immunohistochemistry to further characterise the Crumbs mutant phenotype.

The zebrafish genome encodes five *crumbs* genes, a single *stardust* gene and three *lin7* genes. Mutations in one of the *crumbs* orthologues, *oko meduzy*, and in the *stardust* orthologue *nagie oko* affect the polarized structure of the neuroepithelium. Loss of *crb2b* function affects the differentiation of apical characteristics in photoreceptors. In order to understand the cell biological function of the Crumbs protein complex in polarity of zebrafish photoreceptor cells, we plan to induce mutations in *crb2b* and analyse these mutants by immunohistochemistry and in vivo imaging.

Given that the Crumbs complex displays a high degree of structural and functional conservation between *Drosophila,* zebrafish and mammals, our results will contribute to the understanding of retinal development and degeneration.

Differentiation of in vitro expandable retinal stem/progenitor cells

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Purpose: Vision impairment is a widespread debilitating condition affecting approximately 2% of the young and 12% of the elderly population in the European Union. Degeneration of retinal ganglion cells or photoreceptors represents the cause for retinopathies in the majority of affected patients. Currently, no effective treatments exist. For these conditions the transplantation of cells to replace degenerating retinal neurons might be a possible therapeutic option. For this, culture conditions have to be identified to expand cells in a multipotent and proliferative state and maintain their potential to differentiate into specific retinal cell-types like ganglion cells or photoreceptors. The *Notch* gene and the signalling through the Notch pathway plays a critical role during neural development in the vertebrate retina by regulating cell fate and proliferation. Here we investigated the influence of Notch signalling on the differentiation capacity of retinal stem/progenitor cells.

Methods: Retinal stem/progenitor cells were isolated from rhodopsin-green fluorescent protein (rhoGFP) transgenic mice at postnatal day 0/1 and cultured in defined medium containing epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2). Subpopulations of cells proliferated under these conditions and were expandable over several passages. We established a two step differentiation protocol: cells were first cultured for 5 days with FGF-2 but without EGF followed by 5 days in medium without growth factors. The γ-secretase inhibitor DAPT, a small molecule that can block Notch signalling in retinal stem/progenitors cells, was added for defined time periods to the retinal stem/progenitor cells.

Results: During expansion the majority of cells expressed the proliferation marker Ki67 and specific markers for undifferentiated neural cells like nestin or pax6. Following differentiation cells were negative for Ki67 and nestin but showed reactivity for neuronal (ß-tubulin-III) or glial (GFAP) markers. Addition of DAPT significantly increased the number of cells that expressed the neuronal marker ß-tubulin-III.

Conclusion: Cells isolated from the developmental mouse retina are expandable in vitro and have the potential to differentiate into neuronal and glial cell types, thus fulfilling the minimal requirements for a tissue-specific stem cell. Inhibition of the Notch-pathway significantly increases the differentiation of retinal stem/progenitor cells along the neuronal linage. Following characterization of generated neuronal subtypes these cells may be useful for transplantation experiments to test their potential to replace degenerated retinal neurons in retinopathies.

Reduced expression of spliceosomal proteins elicits eye defects in zebrafish: An animal model for retinits pigmentosa

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Purpose: Retintis pigmentosa (RP) is characterized by a progressive photoreceptor degeneration leading to visual impairment and blindness. Besides mutations in retina specific genes, also mutations in the essential splice factors Prp3, Prp8 and Prp31 are associated with RP. To gain insight into the mechanisms by which mutations in these ubiquitously expressed proteins lead to the tissue specific phenotype observed in RP, a zebrafish model for reduced Prp31 activity was established. As mis-spliced pre-mRNAs are expected to undergo rapid degradation, gene expression profiling was used to identify such transcripts. Furthermore, a similar approach was used to validate Prp4 as an RP-linked splice factor.

Methods: Rescue experiments were carried out to confirm that the reduced expression of Prp31 is a valid model for the haploinsufficiency found in RP patients. Defects in visual processing were assessed by testing Prp31 deficient larvae for the presence of the optokinetic nystagmus (OKN) and retinal integrity was evaluated by immunohistochemistry. In order to identify transcripts affected by a reduced level of Prp31, a microarray analysis was performed. The impact of the RP-linked Prp4 missense mutation was investigated by *in vitro* splicing assays, overexpression studies and in the zebrafish model.

Results: Mutant Prp4 did affect neither splicing *in vitro* nor zebrafish development. Rather, RP-linked mutations in both Prp4 and Prp31 led to a loss of functional protein. Mimicking this haploinsufficiency by morpholino-mediated knockdown resulted in a reduced OKN and rod-photoreceptor specific defects. Interestingly, gene expression profiling of Prp31 deficient larvae revealed a significant down-regulation of several retina-specific and even RP-linked transcripts.

Conclusions: An RP-like phenotype can be induced in zebrafish larvae by reducing the levels of either Prp31 or Prp4. The identification of RP-linked transcripts affected by Prp31 deficiency is consistent with the idea of the RP-phenotype as a consequence of the mis-splicing of a subset of mRNAs important for photoreceptors.

Regulatory interaction between voltage-dependent calcium channels and Rab-GTPase in RPE

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Abstract: Secretion is triggered by activation of voltage-dependent calcium channels (VDCCs). Small GTPases known as Rab proteins play important roles in targeting and docking of secretory vesicles to the plasma membrane. Since VDCCs can interact with other proteins of the fusion machinery including SNARE complex, it could be possible that they interact with Rab proteins as well. However functional modulations of ion channels by Rab proteins remain less understood. The aim of this study is to investigate the direct interaction between Rab27a and Cav1.3 channels in isolated environment, and to observe the effect of endogenously expressed Rab27a on Cav1.3 channels. For this purpose, whole-cell patch clamp experiments were conducted in heterologous expression system in Chinese hamster ovary (CHO) cells as well as with endogenous Cav1.3 channels and Rab27a in a human retinal pigment epithelial (RPE) cell line, ARPE-19. Furthermore immunocytochemistry was performed to observe localization of the proteins in isolated system. In heterologous expression system, the voltage-dependence of Cav1.3 channels in presence of Rab27a was shifted toward negative value. In addition the activation time constant of Cav1.3 channels was smaller in each membrane potential. These findings suggest cells which express Rab27a have fast activation of Cav1.3 channels and larger amount of calcium influx when depolarized. Endogenous Cav1.3 channels in ARPE-19 cells, on the other hand, showed opposite results. The voltage-dependence of Cav1.3 channels was shifted toward positive value and the activation was slower with Rab27a overexpression. Immunocytochemistry showed co-localization of Cav1.3 channels and Rab27a on the plasma membrane in heterologous expression system. We conclude that Rab27a can modulate functions of Cav1.3 channels and here the first evidences of a functional modulation of ion channels by Rab protein have been shown. The differences between observations in CHO and ARPE-19 cells will help to find additional regulators of this signaling cascade.

Gene therapy studies for USH3: Characterization of the knockout mouse retina and search for an efficient AAV vector

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Purpose: Usher syndrome type 3 (USH3) is an autosomal recessive disorder characterized by retinitis pigmentosa and progressive sensorineural deafness. USH3 is caused by mutations in the *CLRN1* gene. The recently generated *C1rn1* knockout (ko) mice have no histological or functional retinal abnormalities when studied by conventional methods, i.e. ERG, whereas they do have progressive hearing loss. The aims of this study were (1) to perform ERG on isolated *Clrn1*-ko-retina in order to detect possible subtle ERG abnormalities and (2) to search for suitable AAV vectors for delivery of wt *Clrn1* into the *Clrn1*-ko-cochlea.

Methods: Photoreceptor responses were measured with aspartate-isolated ERG from P80 wt and *Clrn1*-ko mouse isolated retinas. Transduction efficiencies of recombinant AAV2/1, 2/2, 2/5 and 2/8 vectors encoding GFP marker or HA-tagged *CLRN1* were studied *in vitro* in HEK-293 cells and mouse cochlear tissue cultures. AAV2/1 and 2/2 encoding GFP were also studied *in vivo* in wt mice by microinjection of vector constructs to cochlea.

Results: Comparison of ERGs between wt and *Clrn1*-ko mouse revealed no significant differences. Viral transduction experiments showed that AAV2/1 and 2/2 transduce HEK-293 cells more efficiently than AAV2/5 and 2/8. Transduction efficiency of self-complementary AAV2/2 was significantly higher than that of conventional AAV2/2. AAV2/1 and 2/2-mediated GFP expression was present in the organ of Corti of wt mouse 6 days after cochlear injection.

Conclusions: Vector-based gene therapy is proven to be a suitable method for treating retinal dystrophies. We found no retinal phenotype in the *Clrn1*-ko mouse. Therefore we plan to test *Clrn1*-replacement in the *Clrn1*-ko mouse cochlea. Positive results may indicate that *CLRN1* replacement in USH3 patients' retina could be therapeutic.

Identification of novel interaction partners of USH1G protein SANS by Tandem Affinity Purification

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Purpose: The human Usher syndrome (USH) is the most common form of combined deaf-blindness and clinically divided into 3 types (USH1-3) which are genetically heterogeneous: 10 identified USH genes encode proteins of diverse protein families. All USH1 and USH2 proteins are organized in protein networks within the USH protein interactome. Here, we focus on the scaffold protein SANS (scaffold protein containing ankyrin repeats and SAM domain) (USH1G).
Previous analyses revealed direct binding partners to SANS indicating a crucial role of SANS in integrating USH networks in photoreceptor cells. Since little is known about the composition of the entire protein complexes, we apply Tandem Affinity Purification (TAP) strategies to analyse the function and integration of complex compounds via direct physical binding and indirectly via bridging molecules. The novel combination of the two small tags StrepII and FLAG (SF-TAP) allows a fast but gentle *in situ* isolation of protein complexes in their cellular context without digestion.

Methods: N- or C-terminal tagged SF-N-SANS or SF-C-SANS were expressed in HEK293T cells. After SF TAP, proteins of the recovered complex were separated by SDS-PAGE, silver stained and determined by MALDI-TOF-TOF, or directly analysed by LC-MSMS. Phosphorylation and dephosphorylation of SF N SANS was tested *in vitro* with casein kinase 2 (CK2) and serine/threonin phosphatase 2A (PP2A).

Results: LC-MS/MS data of SANS-TAP-tag analyses revealed numerous potential complex partners which belong to different protein families, e.g. cytoskeleton, scaffold proteins, and signal proteins. The data confirmed the association of SANS with microtubule cytoskeleton and the interaction with USH1C protein harmonin. Furthermore, we identified several novel proteins, e.g. PP2A. Assessment of phosphorylation and dephosphorylation assays revealed that serine/threonin sites of SANS are phosphorylated by CK2 and dephosphorylated by PP2A.

Conclusion: The SF-TAP procedure is a powerful tool for the *in situ* decipherment of protein complexe compositions and enables identification of potential SANS complex partners under physiological conditions. The obtained data indicate regulation of SANS´s scaffold function by phosphorylation. Our findings enlighten the function of USH protein networks and thereby pathophysiological mechanisms causing senso neuronal degenerations in human USH.

Supports: DFG-GRK 1044/2, FAUN Stiftung, Pro Retina Deutschland; Forschung contra Blindheit-Initiative Usher Syndrom

Connective tissue growth factor induces changes in the actin cytoskeleton of human trabecular meshwork cells

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Purpose: A pathological increase in intraocular pressure (IOP) is the major risk factor for glaucoma, which is characterized by a loss of axons in the optic nerve head. The increase in IOP is due to an enhanced resistance in the outflow pathways of aqueous humor in the anterior eye. There is considerable evidence that the acto-myosin system in the (HTM) plays an important role in modulating trabecular outflow resistance. The information on endogenous factors that modulate contractility and architecture of the HTM actin cytoskeleton is incomplete. Connective tissue growth factor (CTGF) is expressed at high amounts in HTM cells in situ. Recently, we showed that CTGF is a potent inducer of extracellular matrix in HTM cells. In this study, we analyzed, if the changes in HTM biology induced by CTGF do also affect their actin cytoskeleton.

Methods: HTM cells were treated with CTGF at different concentrations (2.5-100 ng/ml). Changes in the expression and distribution of cytoskeletal proteins were examined by real-time RT-PCR, western blotting and immunohistochemistry. Cells of an immortalized HTM cell line (HTM5) were stable transfected with a pSilencer(siCTGF)-Vector. The actin cytoskeleton of HTM5-siCTGF cells was compared to that of control HTM5 cells under normal conditions and after heat-shock.

Results: CTGF treatment of HTM cells caused an increase of α-smooth-muscle actin, α-actinin, αB-crystallin and their mRNAs. The knock-down of CTGF in HTM5-siCTGF cells resulted in an increase in the number of focal contacts as compared to untreated HTM5 cells. Stress applied as heat shock to HTM5 cells substantially induced the expression of α-actinin, of the small heat shock protein αB-crystallin that is known to bind to actin, and caused a marked increase in the number of actin stress fibers. Similar effects were completely absent in HTM5-siCTGF cells.

Conclusion: CTGF is a potent mediator of the actin cytoskeleton in HTM cells. Together with the inducing effects on extracellular matrix synthesis in HTM cells, CTGF might lead to an increased stiffness of HTM cells. An increase in HTM stiffness might contribute to an increase in HTM outflow resistance and POAG.

Supported by DFG Forschergruppe FOR 1075

The pathogenic factor of hepatic retinopathy, glutamine, induces osmotic swelling of retinal glial (Müller) cells

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Retinal observations on patients, who died on chronic liver failures, showed cytoplasmatic swelling and gliotic alterations of Müller cells, which was called hepatic retinopathy (HR). Müller cells play a crucial role in the ion and water homoestasis of the retina; this function seems to be disturbed in HR. Chronic liver diseases are associated with an increase in the blood ammonium level. Müller cells are capable to detoxify ammonia, which reaches the retina from the vessels, through the glia-specific enzyme glutamine synthtase (GS). GS catalyses the formation of glutamine from ammonia and glutamate. In HR, glutamine is suggested to be elevated in the extracellular space of the retina due to the high activity of GS. The aim of the present study was to investigate (i) whether extracellular glutamine alters the volume regulation of Müller cells, (ii) whether a glutamine-induced Müller cell swelling can be inhibited pharmacologically, and (iii) whether glutamine disturbs the mitochondrial integrity of Müller cells.

Freshly dissected retinal slices and isolated Müller cells were used to investigate the size of Müller cell bodies in the absence and presence of glutamine. To mimic pathological alterations of the osmotic gradients in the retinal tissue, a hypotonic solution (60% of control osmolarity) was applied. Normally, hypotonic exposure does not alter the size of Müller cell bodies. However, administration of glutamine (5 mM) in the hypotonic solution resulted in a swelling of Müller cell bodies. The swelling of Müller cells was significantly reduced by administration of inhibitors of oxidative/nitrosative stress as well as by substances which inhibit the transfer of glutamine into mitochondria and its conversion inside. Isolated Müller cells were stained with the cationic dye JC-1, which allows to record the alterations of the mitochondrial membrane potential. Administration of glutamine (5 mM) in the hypotonic solution caused a significant greater decrease in the mitochondrial membrane potential than administration of the hypotonic solution alone.

The data suggest that increased glutamine levels as occurring in HR may induce the formation of oxidative/nitrosative stress in the mitochondria of Müller cells. A disturbance of the cellular energy level may result in cytotoxic edema of Müller cells.

Key words: glutamine, retinal edema, mitochondria

The novel Activated Microglia WAP Domain Protein, AMWAP, is induced during microglia activation and acts as a negative regulator of proinflammatory response

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Purpose: Activated microglia contribute to neurodegenerative processes. Via DNA-microarray analysis, we have previously identified high transcript levels of the novel gene AMWAP in activated retinal microglia. As other Whey Acidic Domain proteins play a role in the homeostatic regulation of inflammation, our aim was to functionally characterize this novel protein with a special emphasis on inflammatory processes.

Methods: A recombinant AMWAP-GFP fusion protein was over-expressed in BV-2 microglia and RAW264.7 macrophages, visualized by fluorescence microscopy, and inflammatory marker transcripts were detected by qRT-PCR. A luciferase reporter gene assay was used to determine the influence of AMWAP on chemokine promoter activity. Trypsin-protection assays and scratch-migration tests were performed to study the influence of AMWAP on cell adhesion and migration.

Results: Heterologous expression of an AMWAP-GFP fusion-protein in BV-2 cells and RAW264.7 cells decreased transcript levels of the chemokines Ccl2 and Ccl6, as well as the cytokines IL-6 and IL-1,. AMWAP expression also repressed Ccl2 promoter activity, indicating a direct regulatory role. Trypsin-protection assays showed forced cellular adhesion in AMWAP-over-expressing cells, suggesting a serine-protease inhibitory potential. Wound-scratch migration assays also demonstrated a lower migratory potential of AMWAP-enriched microglia.

Conclusion: Our data indicate that AMWAP may act as a counter-regulatory peptide dampening the inflammatory response and inhibiting the migration of microglia, two intitial immune reactions during early retinal degeneration.

Morphological characterization of the retinal degeneration in two rat models for Retinitis Pigmentosa: P23H-1 and S334ter-3 rhodopsin mutations

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Purpose: Retinitis pigmentosa (RP) is approximately 10%, inherited by autosomal dominant (AD) pattern. Human homologous rat models for ADRP offer the possibility to study the underlying degenerative mechanisms of this disease and are valuable tools to test potential therapeutic treatments. The goal of this study was to investigate structural changes in the cellular organisation of the retinae of transgenic rats with P23H and S334ter rhodopsin mutations.

Methods: Retinae from heterozygous P23H-1, S334ter-3 and wild type Sprague Dawley (SD/CD) rats were collected at various postnatal ages (PN0 - PN30) and were examined by conventional histological techniques.

Results: Basic histological examination revealed a rapid and continuous outer nuclear layer (ONL) degeneration in S334ter-3 and comparatively slower degeneration in P23H1 transgenic rats. S334ter-3 retina does not reach their whole postnatal development. **Rhodopsin** immunostaining showed no fully developed outer segments of photoreceptors present at any postnatal stage. There are no remaining rods at PN 30. In the P23H-1 mutants, however, photoreceptor outer segments developed fully before beginning to degenerate. **M-Opsin** staining showed that most of the photoreceptors left in the ONL are cones at PN20 in S334ter-3; however, outer segments did not development normally. In P23H-1 the cones were observed to be correctly aligned at the outer border of the retina and the antibody expression was visible on cell body and outer segments. Expression of **calretinin** in cells in the inner nuclear layer and in the ganglion cell layers of mutant rats showed no difference than CD retinae. In both mutant models, staining of rod bipolar and amacrine cells by **PKCα** was similar to CD controls but the staining intensity of their axons within the inner plexiform layer was decreased at PN30. In spite of reduction in number of photoreceptors the pattern of bipolar cells expressing **recoverin** was preserved in both mutants. Muller cells in transgenic retinae showed up-regulation of **GFAP**, an indicator of reactive gliosis.

Conclusions: We conclude that the development of the inner retina is not altered at least until PN30 in both the transgenic models. Due to rapid outer retinal degeneration, S334ter-3 is a good model for short term experiments. Meanwhile, P23H-1 is an appropriate model for clinical experimental purposes.

DIRECT INTERACTION BETWEEN ARMS2 (LOC387715) AND FIBULIN-6 (HEMICENTIN-1)

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Purpose: Age-related macular degeneration (AMD) is strongly associated with polymorphisms on chromosomal region 10q26. Recent data suggest a causal relationship between the lack of ARMS2 synthesis and the development of AMD. The present study was undertaken to gain an understanding of the genuine (patho)physiological role of the poorly characterized ARMS2 protein.

Methods: Yeast two-hybrid screen was performed using ARMS2 as bait and a human placental cDNA library as prey, in order to identify interacting partners of ARMS2. Co-precipitation and pull-down assays were employed to validate these interactions. Rat monoclonal antibodies to ARMS2 were raised against two peptides corresponding to the predicted amino acid sequence (NP_001093137) and used in immunohistochemical analyses.

Results: Fifty-four clones isolated form quadruple dropout plates were deemed potential binding partners of ARMS2. Sequence analyses of the prey plasmids showed that the majority code for extracellular proteins; many of them are known constituents of the extracellular matrix. Our in vitro experiments also confirmed the secretion of ARMS2, and the specificity of these interactions. Additionally, ARMS2-specific immunohistochemical staining of human tissues was confined to cell-sparse regions mostly comprised of matrix proteins.

Conclusions: These results demonstrate a new role for ARMS2, as constituent of the extracellular matrix. Besides, the data obtained from these experiments enabled us to propose a protein interaction map, which links ARMS2 to proteins already implicated in AMD and other macular dystrophies.

Support: RETNET (MRTN-CT-2003-504003 to M.U.); EVI-GENORET (LSHG-CT-2005-512036 to M.U.); INTERACTION PROTEOME (LSHG-CT-2003-505520 to M.U.); and Deutsche Forschungsgemeinschaft (SFB 571 A5 to C.D.)

Persistent expression of polysialic acid in the outer nuclear layer of the zebrafish retina

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PURPOSE: Polysialic acid (polySia) is a post-translational modification of the neural cell adhesion molecule NCAM, which in the vertebrate brain is dynamically regulated during development and crucially involved in developmental and adult neurogenesis. As fish grow throughout life their retinas also increase in size by persistently generating new neurons from stem- and progenitor-cells located at the retinal margin as well as in the central part of the retina. However a possible contribution of polySia to these processes has not yet been addressed.

METHODS: PolySia as well as NCAM expression was examined by immunohistochemistry. Additionally polySia was colocalised with the Müller glia specific protein CRALBP. Dividing cells in the adult retina were marked by BrdU. For controls, polySia was enzymatically cleaved by endoneuraminidase N.

RESULTS: NCAM expression was already present as early as 2.3 days post fertilization (dpf) on cell somata and fibers in the entire retina. In contrast to NCAM polySia immunoreactivity could be detected first at 4.3 dpf in the nascent outer nuclear layer (ONL) of the retina, primarily in the ventral position. Later on in the adult zebrafish, polySia expression of the ONL extended to the entire retina. By analysing polySia expression retinas of the transgenic zebrafish line XopsEGFP-N1, expressing EGFP in rod photoreceptors or by colocalisation with the Müller glia-specific protein CRALBP, the location of polySia expression could be disclosed on radial Müller glia processes in the ONL and on processes forming a small band below horizontal cells. Short-time BrdU administration revealed proliferating neuroblasts in the retinal margin as well as rod-precursors in the ONL. Colocalization with polySia demonstrated that both were devoid of polySia expression.

CONCLUSION: PolySia-negative rod-precursors face a polySia-NCAM-positive microenvironment within the ONL presented by radial Müller glia. In accordance with polySia functions described for other neurogenic systems, this pattern indicates that polySia provides environmental cues that might be relevant for the generation of new rods.

Induction of Early Growth Response-1 Mediates Microglia Activation In Vitro But Is Dispensable In Vivo

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Purpose: We have previously identified activation of microglia and induction of the early growth response gene 1 (Egr1) in the retina of retinoschisin-deficient ($Rs1h^{-/Y}$) mice. We hypothesized that microglial expression of the transcription factor Egr1 and the downstream signaling events might support retinal microgliosis.

Methods and Results: Egr1 transcript levels were determined in RNAs isolated from early postnatal retinas and primary microglia from $Rs1h^{-/Y}$ mice and wild-type controls. Egr1 mRNA expression was strongly induced in retinoschisin-deficient retinas as well as in *ex vivo* isolated microglia. Increased microglial Egr1 protein expression was concordantly detected in retinal sections of $Rs1h^{-/Y}$ mice using immunohistochemistry. Prominent activation-dependent Egr1 mRNA and protein expression was also confirmed in murine BV-2 microglia.
Using binding site prediction and chromatin immunoprecipitation, we identified that the Egr1 promoter itself and the microglial marker genes Clec7a and Caspase11 are direct transcriptional targets of Egr1.
Over-expression of Egr1 in BV-2 cells by adenoviral infection promoted Clec7a and Caspase11 mRNA synthesis, whereas expression of the Egr1 repressor NAB2 blocked the transcription of these genes.
To analyze whether Egr1 was absolutely required for microglial marker expression *in vivo,* transcript levels were quantified in $Rs1h^{-/Y}$ / $Egr1^{-/-}$ retinas. No significant differences in activation marker expression could be measured in retinal tissue from $Rs1h^{-/Y}$ / $Egr1^{-/-}$ mice compared to $Rs1h^{-/Y}$ mice, suggesting that lack of Egr1 does not impair transcription of microglia genes *in vivo*.

Conclusions: Our findings suggest that increased Egr1 expression is present in activated retinal microglia and contributes to their activation. However, up-regulation of Egr1 is not absolutely required for retinal microglia activation *in vivo*.

Frequency of the Complement Factor H Related Genes Within a German AMD cohort

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PURPOSE: Age-related macular degeneration (AMD) is the most common cause of blindness in the Western World with approximately 4.5 Mio affected individuals in Germany. Recent studies show that several complement genes are associated with AMD and increase or decrease the individual risk for this complex retinal disease. Deletion of an 84 kb chromosomal fragment on human chromosome 1 that includes the two "Complement Factor-H-Related" genes CFHR1 and CFHR3, reduces the risk for AMD.

METHODS: Here, we determined the allelic frequency of CFHR1, CFHR2, CFHR3 and CFHR5 in a German cohort of 110 AMD patients and 67 unaffected age-matched controls. The copy numbers of the four CFHR genes were analyzed by multiplex ligation dependent probe amplification. The corresponding expression levels of CFHR1, CFHR2 and CFHR3 were further analyzed on protein level by Western Blotting. The protein data could confirm the genetic data.

RESULTS: The AMD group showed a significant lower deletion frequency of CFHR1 and CFHR3 compared to the age-matched control group. The genes for CFHR2 and CFHR5 were 100 % biallelic. In addition did we identify six individuals (four AMD patients and two Controls) with different copy numbers of CFHR1 and CFHR3.

CONCLUSION: This suggests a new breakpoint within the complement factor H gene cluster, which includes CFHR1 and excludes CFHR3. The CFHR1 deletion may therefore be stronger associated with AMD development than CFHR3 deletion.

AMD associated Y402H polymorphism of complement Factor H affects complement control on the surface of necrotic ARPE cells

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PURPOSE: Age-related macular degeneration (AMD) is the most common cause of blindness in the Western World and affects about 50 million people worldwide. The complement system is involved in the pathogenesis of this retinal disease and several polymorphisms in the Factor H gene are associated with AMD. The most prominent is the Y402H variation within the Factor H protein, which increases the risk for AMD about 50%.

METHODS: Here we analyzed the role of the Y402H polymorphism of Factor H and FHL1 for binding to necrotic retinal pigment epithelial cells (ARPE). Factor H was purified from plasma of genotyped AMD patients, and the corresponding FHL1 variants were generated by in-vitro mutagenesis and recombinantly expressed.

RESULTS: The four resulting protein variants Factor H_{Y402}, Factor H_{H402}, $FHL1_{Y402}$ and $FHL1_{H402}$ were compared for binding to living and necrotic ARPE cells, as well as to ligands which are involved in the inflammatory process. Both variants of Factor H and FHL1 bound with similar intensity to necrotic ARPE cells but the H402 risk variants of both regulators show reduced binding to the monomeric form of the C-reactive protein (mCRP). This results in a reduced mCRP-mediated recruitment of the complement regulators to necrotic ARPE cells. Immunofluorescence microscopy further revealed that the recruitment occurred at the side of severe damage. In addition, mCRP enhance cofactor activity of Factor H and FHL1, both in fluid phase and on the surface of necrotic cells. This activity was again reduced for both H402 risk variants.

CONCLUSION: The diminished recruitment of the complement regulators Factor H and FHL1 to the surface of necrotic ARPE cells may reduce local complement control within the sensitive structures of the eye. This results in inflammation and injury of retinal cells. The subsequent accumulation of cellular immune deposits at the Bruchs membrane may lead to Drusen formation and AMD progression.

Autocrine cell volume regulation of Müller cells: Involvement of voltage-gated Calcium and Sodium channels

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Müller cells, the principal glial cells of the retina, dehydrate the retinal tissue in dependence on the neuronal activity and clear the extracellular space from excess of water. Hypoosmotic conditions in the presence of inflammatory mediators or under oxidative stress, and during blockade or down-regulation of potassium channels, induce swelling of Müller glial cells.

In cell swelling experiments using acutely isolated retinal Müller cells of the rat, we determined whether receptor agonists which evoke a calcium response in Müller cells may prevent the osmotic swelling of Müller cells. We found that VEGF, NPY, HB-EGF, ANP and EPO prevented the osmotic swelling of the cells. Activation of specific receptors evoked a calcium-dependent, exocytotic release of glutamate from retinal glial cells, and subsequent stimulation of glial group I/II metabotropic glutamate receptors. Activation of glutamate receptors evoked an autocrine swelling-inhibitory purinergic signalling cascade that was calcium-independent. In further experiments we investigated the involvement of voltage-gated sodium and calcium channels in the autocrine regulation of Müller cell volume. We found that the inhibitory effect of the agonists on the osmotic swelling of retinal glial cells is prevented in the presence of antagonists of voltage-gated sodium (tetrodotoxin) and T-type calcium channels (kurtoxin). In contrast, the swelling-inhibitory effect of glutamate remained unaffected in the presence of the blockers. The data suggest that voltage-gated sodium and calcium channels are implicated in the release of glutamate from retinal glial cells which mediates the autocrine regulation of cellular volume.

Keywords: glia; retina; VEGF; glutamate; calcium channel; sodium channel

Identification of novel genetic defects in outbred cone-rod dystrophy patients using homozygosity mapping

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Purpose: To identify the causative gene-defects in patients with autosomal recessive cone-rod dystrophy (arCRD).

Methods: Homozygosity mapping was performed with high-density SNP microarrays to identify new disease-causing genes and mutations. DNA samples of 27 arCRD patients from 11 non-consanguineous families and 42 isolated CRD-patients mostly from the Netherlands and Germany were analyzed for homozygous regions.

Results: Initial analysis of the homozygosity data revealed the causative gene mutation in four families, including one known mutation in *ABCA4,* two novel mutations in genes known to cause a retinal dystrophy other than CRD *(CABP4, PROM1)* and one novel mutation in the newly identified gene *EYS*. Affected siblings shared one to three significant homozygous regions varying in size from 2 to 28 Mb. In two families, the causative mutations were found in the largest overlapping homozygous region, spanning 9 and 28 Mb, and in two families a mutation was identified in the second largest region, encompassing 5 and 10 Mb. We reevaluated the phenotype in patients carrying mutations in *CABP4* and *PROM1*. In the sib pair with the *PROM1* mutation we confirmed the diagnosis of CRD. Interestingly, in the sib pair with the *CABP4* mutation, detailed characterization of the phenotype led to the description of a new phenotype, named congenital cone-rod synaptic disorder. Analysis of homozygous regions in the other families and isolated cases is ongoing.

Conclusions: Using homozygosity mapping in CRD patients from outbred populations, we unraveled the molecular cause in at least 4 of 11 families, in one of which mutations were identified in the novel gene *EYS*. Furthermore, we show that molecular knowledge of the disease may lead to a better phenotypic understanding.

The drusen-like phenotype in MCP-1/Ccl2 knockout mice is caused by an accelerated accumulation of swollen autofluorescent subretinal macrophages

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Purpose: Drusen, which can be defined clinically as yellowish white spots in the outer retina, are cardinal features of age-related macular degeneration (AMD). MCP-1/Ccl2 knockout (MCP-1) mice have been reported to develop drusen and phenotypic features similar to AMD including an increased susceptibility to choroidal neovascularisation (CNV). Here we investigate the nature of the drusen-like lesions *in vivo* and further evaluate the MCP-1 mouse as a model for AMD.

Methods: We examined eyes of 2-25 month old MCP-1 and C57Bl/6 mice *in vivo* by autofluorescence scanning laser ophthalmoscopy (AF-SLO), electroretinography, and measured the extent of laser- induced CNV by fluorescein fundus angiography. We also assessed retinal morphology using immunohistochemistry and quantitative histological and ultrastructural morphometry.

Results: The drusen-like lesions of MCP-1 mice comprise accelerated accumulation of swollen CD68-positive macrophages in the subretinal space that are apparent as autofluorescent foci on AF-SLO. These macrophages contain pigment granules and phagosomes with outer segment and lipofuscin inclusions that might account for their autofluorescence. We only observed age-related RPE damage, photoreceptor loss and sub-RPE deposits but, despite the accelerated accumulation of macrophages, we identified no spontaneous CNV development in senescent mice and found a reduced susceptibility to laser-induced CNV in MCP-1 mice.

Conclusion: These findings suggest that the lack of MCP-1 leads to a monocyte/macrophage trafficking defect during aging and to an impaired recruitment of these cells to sites of laser injury. Other, previously described features of MCP-1 mice that are similar to AMD may be the result of aging alone.

Mice have light-induced retinal damage at low light intensity.

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Light damage is an important and well establish model to study degenerative mechanism in the retina. So far this light damage assay has been used in high light intensity directly leading to photoreceptor damage. In order to study involvement of supportive tissues we examined light damage at the lower threshold levels. For this purpose mice were previously adapted to dark conditions followed by an exposure to a light intensity of 8000 lux for 30 minutes.
After 3 days tunnel assays revealed few signals of apoptosis and after 9 days histology showed a variety of different patterns in the retina of mice exposed to 8000 lux. Changes in retinal structure were detected close to the optic nerve close to temporal area. These changes comprise thickening of the outer nuclear layer whereas others regions show loss of nuclei in the outer nuclear layer and outer segment.
The complex pattern of retinal aberrations after threshold light damage suggests much more signaling pathways involved. Study of the complexity of these pathways will lead to the identification of new degenerative mechanisms.

Impact of PKA on photoreceptor degeneration in the rd1 mouse model

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PURPOSE: PKA, a cAMP dependent protein kinase, is important in activating the transcription factor CREB (cAMP response element-binding protein) which is crucially involved in neuronal survival. Previously it has been demonstrated that in the rd1 mouse, a model for retinal degeneration, retinal CREB mRNA is significantly downregulated (Azadi et al., 2006). Furthermore, PKA bears the potential to stabilize ICER (inducible cAMP early repressor) which is the endogenous inhibitor of CREB. To investigate this bivalent role of PKA and the impact of PKA on photoreceptor cell survival we specifically activated or inhibited PKA activity in retinal explants of the rd1 mouse.

METHODS: Retinas from rd1 mice at postnatal day 5 were cultured in an organotypic culture system for 6 days in total. After 2 days in vitro (DIV) retinal explants were treated for 4 days by adding PKA inhibitor (RP-8-CPT-cAMPS) or activator (SP-8-CPT-cAMPS) to the culture medium. Cell death after treatment was assessed using TUNEL assay and cell death rate was calculated as TUNEL positive cells / μm^2 of outer nuclear layer (ONL). Immunohistochemistry with antibodies specific for ICER and pCREB were performed.

RESULTS: After treating retinal explants of rd1 mice for 4 days with a PKA inhibitor we could not observe any significant differences in the number of dying cells within the ONL (3.7 control vs. 3.2 treated) as revealed by TUNEL staining. In an additional set of experiments we could demonstrate that 4 days treatment of retinal explants with a PKA activator showed no significant changes in the number of dying cells (3.4 control vs. 3.1 treated). ICER expression could be disclosed in some cells of the ONL in rd1 and wild type animals.

CONCLUSIONS: Increased PKA activity as well as PKA inhibition has no apparent impact on photoreceptor cell death in the rd1 mouse model, at least under the short term treatment conditions used here. Further investigations will be needed to demonstrate any potential role of PKA in fine tuning of CREB dependent transcription or on other parameters of the retinal degeneration in the rd1 mouse.

Systemic activation of the renin-angiotensin system causes inhibition of the intraocular renin-angiotensin system

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The renin-angiotensin system (RAAS) plays a role in the development of diabetic retinopathy, age-related macular degeneration, and intraocular angiogenesis. An active intraocular RAAS has recently been shown to exist in the human eye and it seems to be functionally separated from the systemic RAAS by the blood/retina barrier. To study a possible interaction of the systemic RAAS with the intraocular RAAS we investigated cells of the retinal pigment epithelium (RPE) which form a part of the blood/retina barrier. Freshly isolated RPE cells showed the expression of angiotensin-2 receptors 1A and 1B by means of RT-PCR and by western-blot. Immuno histochemical analysis of porcine retina revealed localization of angiotensin-2 receptors in the RPE basolateral membrane which faces the blood stream in the choroid. Application of angiotensin-2 to cultured porcine RPE resulted in a biphasic increase in intracellular free Ca^{2+}: after an initial transient rise followed a sustained elevation to lower levels than those of the initial peak. Using quantitative-PCR, RPE cells were found to express renin. Reduction of the plasma angiotensin-2 levels in mice by systemic administration of the angiotensin-converting enzyme inhibitor enalapril resulted not only in an up-regulation of the renin expression in the kidney but also in the RPE as well in the retina. In cryosections of the retina renin was only found in the RPE of enalapril treated mice by immune cytochemistry. In conclusion, the kidney influences the function of intraocular tissues by the systemic RAAS. RPE cells which form the blood/retina barrier are stimulated by angiotensin-2 in the plasma and reduce the renin secretion into the eye by Ca^{2+}-dependent regulation of renin expression.

Pericentrin, a centrosomal protein, identified at the basal-body complex in mammalian photoreceptor cells

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Purpose: Pericentrin – also known as kendrin - is one of the best-studied mammalian centrosomal proteins. Pericentrin has been demonstrated to mediate microtubule organization by centrosomal anchoring of γ-tubulin complexes that nucleate microtubules. It is characterized by coiled-coil domains throughout most of its structure and a PCM (peri-centrosomal-matrix) targeting motif called the PACT-domain near its C-terminus. The multiple coiled-coil domains of pericentrin mediate interactions between resident structural lattice proteins of the PCM and a number of regulatory and transient centrosomal proteins, including protein kinase A, cytoplasmic dynein and the γ-tubulin complex. The PACT domain targets pericentrin and other PCM proteins, such as the protein kinase A anchoring protein AKAP450, to the centrosome. Jurczyk *et al.* (2004) investigated the role of pericentrin in centrosome related structures, the primary cilia. They showed that pericentrin localizes to the base of primary and motile cilia and is involved in ciliary development and ciliary function in mammalian cells.

Methods: With immunocytochemistry, light microscopy and laser capture microdissection in combination with RT-PCR we examined the distribution of Pericentrin and the centrosomal interaction-partners in retinae of wildtype mice.

Results: In the vertebrate retina, photoreceptor cells are morphologically and functionally arranged in several compartments. The light sensitive photoreceptor outer segment is linked with an inner segment, which contains the typical energy producing and protein synthesizing components of an eukaryotic cell, via a modified non-motile cilium, termed the connecting cilium. Using the method of laser capture microdissection in combination with RT-PCR techniques, we were able to show the gene expression of all known pericentrin-isoforms in the mouse retina. Furthermore, we found with immunocytochemical and high-resolution light microscopical analyses that pericentrin and several interacting partners, known from the centrosome, are co-localized at the basal body and the centriole of the connecting cilium, where the protein-transport from the inner to the outer segment takes place.

Discussion: The presence of pericentrin at the connecting cilium, the site of transport regulation and interaction with transport molecules like IFTs (intraflagellar transport molecules) suggests a role of pericentrin in the ciliary transport in photoreceptor cells. Studying pericentrin function may help us to understand the regulation of protein transport in photoreceptor cells and provide new insights into human disorders related to defects in ciliary function.

Role of Ca^{2+}-channels in the regulation of phagocytosis by retinal pigment epithelium

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Phagocytosis of photoreceptor outer-segments through the retinal pigment epithelium (RPE) is a physiological important renewal process in the retina. Incomplete clearance of the photoreceptor outer-segment fragments cause photoreceptor degeneration and loss of vision. Recently new regulatory mechanisms of L-type Ca^{2+}-channels in this phagocytosis process have been described (Karl et al. 2008, Cell Signal). Aim of this study was to investigate the functional relevance of these features.

For in vivo measurement of retinal phagocytosis we labeled the outer segment protein rhodopsin in

paraffin embedded retinal cross-sections of mice and quantified rhodopsin-positiv phagosoms in the RPE at different times of the day. Analyzing the photoreceptor outer-segments clearance we detected a increased activity in the morning. 30 minutes after light onset a peak number of phagosomes of 12 ± 3 phagosomes per 100µm retina was detected compared with the afternoon, where we could survey about 4,5 ± 2 phagosomes.

In conclusion, phagocytosis shows a circadian regulation with a peak after onset of illumination in the morning. These investigations will provide a route towards a more detailed understanding of the functional role of Ca^{2+}-channel in the regulation of phagocytosis.

Norrin is an angiogenic factor that protects against vascular degeneration and induces retinal recapillarization in oxygen induced retinopathy

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Purpose: To analyze, if norrin, the secreted protein product of the Norrie disease gene *(NDP)*, has direct angiogenic and vasoprotective properties *in vitro* and *in vivo* in mice with oxygen induced retinopathy (OIR). $Ndp^{(y/-)}$ mutant mice that are deficient in norrin lack the deep capillary layers of the retina, a phenotype that is completely rescued in mixed βB1-norrin/$Ndp^{(y/-)}$ mice with transgenic overexpression of norrin (Ohlmann et al., *J. Neurosci.* 2005).

Methods: Human recombinant norrin was isolated and purified from conditioned cell culture media of 293-EBNA cells by chromatography. Human retinal vascular endothelial cells (HRMEC) were treated with recombinant norrin to analyze its effects on proliferation, viability, migration and tube formation. In addition, OIR was induced inβB1-norrin mice with transgenic ocular overexpression of norrin and in their wild-type littermates by exposure to 75% O_2 at postnatal day 7 (P7) for either 18 hours or 5 days. Following O_2 exposure, flat mounts of FITC-dextran-perfused retinae were isolated and areas with obliterated vessels were quantified by morphometry. For controls, Dickkopf (DKK)-1 was added to block WNT/β-catenin signaling, as norrin activates the classical WNT pathway by acting primarily via the frizzeled-4 receptor.

Results: Recombinant norrin significantly increased proliferation, viability, migration and tube formation in HRMEC. The effects could be blocked significantly by adding DKK-1. Following exposure to 75% O_2 for either 18 hours or 5 days, vasoobliterated areas were significantly smaller in βB1-norrin mice as compared to wild-type littermates. Moreover, after O_2 exposure for 5 days, recapillarization of vasoobliterated areas was markedly increased in βB1-norrin mice as compared to wild-type controls. Again, the effect on recapillarization was decreased significantly by adding DKK-1.

Conclusions: Norrin is an angiogenic factor for retinal microvascular endothelial cells with an important role for development, recapillarization and maintenance of retinal capillaries.

Supported by DFG Research Unit (Forschergruppe) FOR 1075

The USH 1 and 2 protein network at the photoreceptor ciliary complex

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Purpose: The human Usher syndrome (USH) is the most common form of combined deaf-blindness. USH is genetically heterogeneous with at least 12 chromosomal loci assigned to three clinical types, USH1-3. All identified USH type 1/2 proteins can be connected by the scaffold protein harmonin (USH1C) within an USH interactome. 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, immunohistochemistry and immunoelectron microscopy of mouse retinae, pharmacological BAPTA treatment; protein-protein interaction assays: co-transfection of cell lines, GST-pull down assays; pharmacological microtubule destabilization in cell and organotypic retina cultures.

Results: SANS co-localizes with USH2A, VLGR1b/GPR98 (USH2C), and the scaffold protein whirlin (USH2D) in the ciliary apparatus - particularly in the periciliary region of photoreceptor cells. Whirlin directly binds to SANS and to the other USH2 proteins via its PDZ-domains. The long ectodomains of both transmembrane proteins extend into the gap between the adjacent membranes of the connecting cilium and the apical inner segment. Analyses of Vlgr1/del7TM mice revealed the ectodomain of VLGR1b necessary for the formation of fibrous links projecting through this gap. Microtubule destabilization assays provide evidence for the association of SANS with the microtubule system.

Conclusions: In the ciliary specialization of photoreceptor cells, USH proteins are integrated into a protein network, associated with the microtubule cytoskeleton. The USH protein network is bridged by SANS to the microtubule transport machinery, whereas whirlin provides the cytoplasmic anchor for USH2A isoform b and VLGR1b. The molecular components integrated into the periciliary ridge complex may contribute to the handover of cargos between the inner segment and the transport through the connecting cilium. Defects of complex part-

ners may lead to dysfunction of the entire USH network and cause photoreceptor degeneration in USH patients.

Support: Forschung contra Blindheit - Initative Usher Syndrom; ProRetina Deutschland; FAUN-Stiftung; Deutsche Forschungsgemeinschaft GRK 1044/2

PKG inhibition protects photoreceptors in two mouse models for Retinitis Pigmentosa

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Purpose: Inherited photoreceptor degeneration in retinitis pigmentosa (RP) is a major cause of blindness in the developed world. Although causative genetic mutations have been indentified in many cases, the underlying neuronal degeneration mechanisms are largely unknown. Mutations affecting the levels of cyclic-guanosine-mono-phosphate (cGMP) – e.g. in the gene encoding for phospodeesterase 6 (PDE6) – are often associated with rapid photoreceptor cell death. Since cGMP is known to cause activation of cGMP-dependent protein kinase (PKG), we investigated the role of PKG in retinal degeneration mechanisms.

Methods: We employed specific antibodies for the detection of cGMP and PKG activity dependent products to determine the extent of cGMP induced PKG activation in wild-type (wt), rd1, and rd2 mice – the latter two being widely used human homologous animal models for retinal degeneration. We used organotypic retinal explant cultures derived from these animals, to test compounds inhibiting or activating PKG and/or PDE6 in vitro. To confirm in vitro observations, we employed three different in vivo application techniques (topical application, intravitreal injection, subtenon injection). For evaluation of treatment outcomes histological staining, immunofluorescence, immunoblot, and TUNEL techniques were used.

Results: We demonstrated for the first time a strong elevation of cGMP in situ, in both rd1 and rd2 photoreceptors. We found that activation of PKG hallmarks photoreceptor degeneration in rd1 and rd2 mice. When induced in wt retinae, PKG activity was both necessary and sufficient to trigger cGMP-mediated photoreceptor cell death. Target specific, pharmacological inhibition of PKG activity in both rd1 and rd2 retinae strongly reduced photoreceptor cell death in organotypic retinal explants and increased long-term photoreceptor survival. Likewise, inhibition of PKG in vivo, using three different application paradigms resulted in robust photoreceptor neuroprotection in the rd1 retina.

Conclusions: Our results suggest a critical role for PKG activity in cGMP-mediated photoreceptor degeneration mechanisms and highlight PKG as a novel target for the pharmacological intervention in RP. Since elevated cGMP levels were found in the rd2 retina and are known to occur also in other forms of inherited retinal degeneration, cGMP-PKG-induced cell death may be relevant for a number of genetically different forms of RP.

Acknowledgements

This work has been supported by grants from the EU (RETNET: MRTN-CT-2003-504003, EVI-GENORET: LSHG-CT-2005-512036), Crown Princess Margaretha's Foundation for Vision Research (KMA), Crafoord Foundation, Foundation Fighting Blindness (FFB), Deutsche Forschungsgemeinschaft (DFG: PA1751/1-1), Kerstan Foundation, Lund University Hospital Funds, and Segerfalk Foundation.

Interaction between Bestrophin-1 and ßsubunits of Voltage dependent calcium channels

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Best vitelliform macular dystrophy is an autosomal inherited macular degenenration that is caused by mutations in the BEST1 gene encoding for bestrophin-1. The diagnosis of Best's disease is usually based on a reduction of the light-peak amplitude in the patient's electrooculogram (EOG). Mice which lack the ß4-subunit of voltage dependent calcium channels (VDCC) show a reduced light-peak whereas mice lacking bestrophin-1 show larger light-peaks compared to wildtype mice. Thus also the alteration of VDCC activity could explain the patient's EOG.

The aim of this study was to investigate the interaction between bestrophin-1 and the ß1 or ß4 subunits of voltage dependent calcium channels. ß-subunits were tagged with c-Myc or 6xHis tag to facilitate their detection with antibodies. ß1 or ß4 constructs were heterologously expressed together with bestrophin-1 in HEK-293 cells and in a cell line of human retinal pigment epithelial cells, ARPE-19. Immunocytochemistry, immunoprecipitation and western blot were performed to investigate interaction between ß-subunits and bestrophin-1. With confocal microscopy we could show co-localisation of bestrophin-1 with ß1His or ß4myc on the plasma membrane.

The ß-subunits showed co-immunoprecipitation with bestrophin-1 in heterologous expression in HEK-293 cells. The molecular basis of this interaction is most likely an interaction between the SH3 domain of the ß-subunits and proline-rich motifs on bestrophin-1. Immunoprecipitation was thus performed with a bestrophin-1 that lacks four of the proline-rich motifs on the C-terminus (ΔCT-PxxP) and the ß-subunits. The lack of co-immunoprecipitation indicates the crucial role of the proline-rich sites for the interaction of ß-subunits and bestrophin-1. In summary, we showed that bestrophin-1 binds to L-type calcium channels to modulate VDCC activity in the light-peak.

High phenotypic variability within families in Best macular dystrophy

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Background: Best macular dystrophy (BMD) is associated with autosomal dominant mutations in the *BEST1* (formerly *VMD2*) gene. The clinical diagnosis can be difficult due to highly variable penetrance and expressivity. We present five families with known mutations and extremely variable fundus findings inter- and intrafamilial.

Methods: Retrospective analysis of the morphological and functional data. Mutational analysis in the *BEST1* gene was done by direct sequencing of the 10 coding exons of the gene.

Results: Five unrelated families, each with affecteds in two generations, were examined. *BEST1* gene mutations were identified in all reported family members. One son of family #523 presented with foveal subretinal hemorrhage in both eyes, a very rare complication of BMD. His brother had a vitelliform lesion only in the right eye whereas the left eye was almost normal. Their father had no fundus changes. The EOG was pathological in all three family members. The son in family #502 and the daughter in family #522 presented with typical lesions (pseudohypopyon, vitelliruptive, atrophic lesions). However, both mothers had only slight fundus changes, not resembling BMD, and only one of four eyes had visual disturbances. In family #282, two sisters had normal fundus and vision. Both sons of one sister had very asymmetrical fundus changes with unilateral choroidal neovascularisation. The son of the other sister had symmetrical vitelliform lesions. The EOG was reduced in four family members and not recorded in one mother. The father and his son of family #269 showed both fundus lesions, however, the lesions were extrafoveal located in the father. The EOG light rise was reduced in both men.

Conclusions: BMD is known as a monogenic macular dystrophy. However, the highly intrafamilial phenotypic variability indicates that additional factors may modify the *BEST1*-related consequences of the identified mutations. Environmental factors, genetic interactions or varying expression of *BEST1* may influence the phenotype.

The role of poly-ADP-glycohydrolase (PARG) in inherited retinal degeneration

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Purpose: Inherited retinal degenerations such as Retinitis Pigmetosa (RP) are initiated by gene defects in rods, cones or the retinal pigment epithelium (RPE) and often lead to an irreversible loss of visual function. The retinal degeneration (rd) 1 mouse is a well studied model for RP in which the gene encoding for the beta subunit of the rod photoreceptor cGMP phosphodisterase 6 (PDE6) is mutated and consequently non-functional. PDE6 dysfunction causes accumulation of cGMP which has been shown to be associated with excessive poly (ADP-ribose) polymerase (PARP) activation and oxidative stress in the rd1 mouse. The aim of this study was to investigate the role of the PARP antagonist poly-ADP-glycohydrolase (PARG) in retinal degeneration.

Methods: PARG knockout (KO) and wild-type (wt) mouse retinal explants were cultured with/without zaprinast, a selective inhibitor of cyclic GMP-specific phosphodiesterase, to simulate in vitro a situation comparable to the rd1 mouse model. Immunofluorescent detection of cGMP was used to confirm zaprinast effects; TUNEL staining, PAR immunohistochemistry, PARP activity assay were performed for analysis of dying cells and PARP activity, respectively.

Results: Immunofluorescence showed that in both wt and PARG KO cultured retinae zaprinast treatment induced an accumulation of cGMP in the outer nuclear layer. Detection of degenerating cells using the TUNEL assay showed a treatment induced increase in the number of positive cells in the outer nuclear layer (ONL) of wt. Compared with wt, zaprinast treated PARG KO retinae showed less TUNEL positive cells in the ONL but more such in the inner nuclear layer (INL). Detection of PARP activity in wt and rd1 in vivo retinae showed many positive cells in the rd1 ONL and very few positive cells in the wt ONL, confirming previous results. In contrast to this, PARG KO retinae showed a lot of PARP activity positive cells in the INL, but none in the ONL.

Conclusions: The activity of PARG may have important functions in the retinal photoreceptor cell death in the rd1 mouse and could be new targets for therapeutic developments. Moreover, an understanding of the roles of PARG in retinal degenerations could shed new light on the existence or absence of alternative pathways for degradation of poly-ADP-ribose-polymers.

HDAC activity is altered in rd1 mouse photoreceptors

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Purpose: The mechanisms behind retinal degeneration in Retinitis pigmentosa are still largely unknown, but appear to involve effects on gene transcription. Histone deacetylases (HDACs) and Histone acetyl transferases (HATs) are enzyme families which control the acetylation state of lysine residues in histones and other proteins. HATs add acetyl groups into the lysine residues, and HDACs are in charge of removing them. Acetylation of nucleosomal histones affects chromatin density and therefore gene expression. The aim of this study was to investigate the relevance of histone acetylation and deacetylation, focusing on the activity of HDACs, in rd1 mouse photoreceptor degeneration.

Methods: Immunofluorescence and Western Blot experiments were performed to study general acetylation in the photoreceptor cell layer of wt and rd1 retinae. Immunostainings for specific acetylated nucleosomal histones were also used. A newly adapted in situ activity assay was performed to study activity of HDACs in rd1 and wt retinae. Retinae from rd1 and wt animals were cultured in an organotypic explant system and treated with specific inhibitors and activators of HDACs. Cell death was assessed using TUNEL assay.

Results: The immunostaining against acetylated proteins in general, and histones in particular, revealed hypoacetylation in photoreceptor cell bodies. The HDAC activity assay allowed to efficiently resolve activation of HDACs at the cellular level, showing an enhanced positive signal in rd1 outer nuclear layer, when compared with wt. The application of HDAC class III (a.k.a. sirtuins) inhibitor (Nicotinamide) and activator (Resveratrol) did not significantly affect viability of degenerating photoreceptor cells.

Conclusions: The acetylation balance in the retina of rd1 mice seems to be altered. HDACs may be involved in the development and progression of photoreceptor degeneration in the rd1 mouse. Further research is needed to delineate the precise role of these enzymes in the degeneration process and to evaluate their potential for a treatment of photoreceptor cell death.

mRNA expression profiling of the *Cnga3* knockout mouse with focus on the dorsal-ventral gradient in retinal degeneration

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Purpose: The *Cnga3*$^{-/-}$ mouse is an animal model lacking the A subunit of the cone specific cyclic nucleotide gated channel. The phenotype is characterized by a loss of cone photoreceptor function and a progressive degeneration of the cones, particularly in the ventral part of the retina. To elucidate this dorsal ventral gradient in degeneration we performed whole genome expression analyses of dorsal and ventral areas of *Cnga3*$^{-/-}$ and wildtype retinas at the age of 4 weeks.

Methods: Expression analysis of *Cnga3*$^{-/-}$ and wildtype retinas was performed using Illumina MouseWG-6 v1.0 Expression BeadChips. Differential regulated transcripts with a minimum change in expression level of 1.5 fold (p-value $\leq$ 0.05) were obtained and gene regulation networks were generated by the Ingenuity Pathways Analysis software. To verify the data several transcripts were analyzed by qRT-PCR. The study was performed in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Visual Research.

Results: Physiological differences in expression patterns of the dorsal and ventral retina were analyzed in wildtype samples, showing 264 differently regulated transcripts. Differences between the wildtype and *Cnga3*$^{-/-}$ retinas appeared with 579 differently regulated transcripts in the dorsal areas and 608 in the ventral retina. In contrast to the wildtype, *Cnga3*$^{-/-}$ mice showed a differently regulation of 227 transcripts in the ventral retina. The results could be successfully verified by qRT PCR.

Conclusions: Expression analysis of different retinal areas showed clear differences in expression patterns of the dorsal and ventral regions. Analysis in *Cnga3*$^{-/-}$ retinas discovered clear differences in expression patterns. These results could help to elucidate the molecular events leading to the dorsal-ventral gradient of cone degeneration in *Cnga3*$^{-/-}$ mice.

Epithelial sodium channels (ENaCs) in the mouse retina

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Purpose: Glaucoma, the most frequent optic nerve disease, is characterized by an increased intraocular pressure (IOP) leading to ganglion cell death and hence to blindness. Searching for factors involved in the pathogenesis of glaucoma, a previous study showed an upregulation of the epithelial sodium channel subunit α (ENaC α). Here we examined the distribution of ENaCs in the mouse retina and their possible involvement in the pathogenesis of glaucoma.

Methods: Specific antibodies for the ENaC α, β and γ subunits were generated. With immunocytochemistry and light microscopy we examined the distribution of the ENaC subunits in retinae of C57BL/6 wildtype, DBA/2J, and DBA/2JRj mice at 2, 6 and 10 months of age.

Results: All three ENaC subunits are present in wildtype retina: ENaC α is diffusely distributed in both plexiform layers, the outer (OPL) and inner (IPL) plexiform layer. ENaC β is found in two distinct strata in the IPL and as punctate label in the OPL. ENaC γ is present as punctate staining in the photoreceptor layer, the OPL and the inner nuclear layer (INL). Additionally, somata are labeled in the INL and the ganglion cell layer. So far the comparison of ENaC subunit distribution and expression between wildtype, DBA/2J and DBA/2JRj retinae (age 2, 6 and 10 months) showed no obvious differences.

Discussion: Our newly generated anti-ENaC antibodies specifically detect all three ENaC subunits in the mouse retina. Unexpectedly and most surprisingly, the ENaC subunits seem to be preferentially localized in the synaptic layers of the retina. Their distribution patterns indicate that the ENaC subunits could form functional channels composed of the subunits α/β and α/γ. So far our results from immunocytochemistry – comparing ENaC subunit distribution in the three mouse lines at different postnatal ages – do not support a vital involvement of ENaCs in the pathogenesis of glaucoma. This study, however, is only the beginning of our effort to establish the role of ENaCs in retinal function and their possible involvement in the pathogenesis of glaucoma.

Supported by a grant SFB 539/A7 to J.H.B. and R.E.

CFH, ARMS2 and C3 confer risk for susceptibility but not for disease progression of geographic atrophy due to age-related macular degeneration

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Background: Age-related macular degeneration (AMD) is the most prevalent cause of blindness in industrialized societies. Variants in the complement factor H *(CFH)*, the complement component 3 *(C3)* and the age-related maculopathy susceptibility 2 *(ARMS2)* genes have been established to confer significant risks for disease susceptibility. Their role for disease progression and thus their significance for developing therapeutic intervention remain unknown.

Methods: We tested for an association between genetic variants in CFH, C3 and ARMS2 and disease progression of late atrophic AMD (geographic atrophy). Patients were selected from the multicenter FAM study cohort (n=619) and compared with 612 controls. Patients were investigated by fundus autofluorescence imaging. A quantitative phenotype of disease progression was computed based on longitudinal observations.

Results: In a subset of 99 cases with pure bilateral geographic atrophy, variants in *CFH* (Y402H), *ARMS2* (A69S) and *C3* (R102G) were strongly associated with disease ($P = 1.6 \times 10^{-9}$, $P = 2.6 \times 10^{-12}$, and 3.2×10^{-3}, respectively). Median progression rate of geographic atrophy over a mean follow-up of 3.0 years was 1.61 mm^2/year with high concordance between the two eyes. Despite sufficient power, no association between the progression rate and the genetic risk variants at the three loci was observed ($P>0.13$).

Conclusions: Variants at *CFH, C3,* and *ARMS2* confer high risks for geographic atrophy, but not for disease progression. As a consequence, therapeutic options specifically addressing the

three susceptibility factors may not be helpful to alleviate disease progression once late atrophic AMD has developed. Other so far unknown susceptibility factors may be involved.

Support: European Commission (EU FP6), Integrated Project "EVIGENORET" (LSHG-CT-2005-512036); German Research Foundation (DFG) Heisenberg Fellowship SCHO 734/2-1; DFG Priority Research Programme Age-related Macular Degeneration (SPP1088); DFG HO1926/1-3; DFG WE1259/18-1, the Ruth and Milton Steinbach Foundation New York and the Alcon Research Institute.

Norrin mediates neuroprotective effects on retinal ganglion cells via induction of neurotrophic factors in Müller cells

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Background: $Ndp^{(y/-)}$ mutant mice that are deficient in the Norrie disease gene *(Ndp)* show a continuous loss of retinal ganglion cells (RGC), a phenotype that is completely rescued in mixed βB1-norrin/$Ndp^{(y/-)}$ mice with transgenic overexpression of norrin, the secreted protein product of *Ndp* (Ohlmann et al., *J. Neurosci.* 2005). To analyze, if norrin has neuroprotective properties, we investigated its effects on RGC survival in mouse eyes following N-methyl-D-aspartate (NMDA) induced RGC damage.

Methods: Recombinant human norrin was isolated and purified from conditioned cell culture medium of HEK 293-EBNA cells. To induce RGC death, 3 µl NMDA [10 mM] were injected into the vitreous body of C57/Black6 mice while the fellow eye received 3 µl of a combination of NMDA [10 mM] and norrin [5 ng/µl]. To determine the degree of RGC damage, the number of axons in cross sections of the optic nerve and of RGC in meridional semithin sections of each eye was quantified. In addition, TUNEL labelling was performed on meridional sections, and the number of labelled nuclei was quantified. The expression of mRNA for several neurotrophic factors was investigated by quantitative real-time RT-PCR of treated retinae and of cultured retinal Müller cells after a 7 h incubation with norrin [40 ng/ml].

Results: Three weeks after injection of NMDA, the number of optic nerve axons was almost two-fold higher in eyes injected with combined NMDA/norrin (20,383 ± 3,101; mean ± SEM) as compared to eyes that received NMDA only (11,150 ± 1,013), an effect that was statistically significant ($p<0.05$). Similarly, perikarya of surviving RGC were significantly more numerous in norrin/NMDA injected eyes (3.6 ± 0.5 RGC per 100 µm) when compared to eyes injected with NMDA only (2.3 ± 0.2 RGC per 100 µm; $p < 0.025$). Comparable results were obtained by TUNEL labelling. By real-time RT-PCR, a significant increase in mRNA for bFGF, LEDGF, BDNF, CNTF and also GFAP was observed in eyes injected with combined NMDA/norrin as compared to retinae that received NMDA only. The results correlated with the up-regulation of the mRNA level of several neurotrophic factors in cultured Müller cells (bFGF, BDNF, PEDF and CNTF) after incubation with norrin.

Conclusion: Norrin has neuroprotective properties on retinal ganglion cells, which are partly mediated by the induction of neurotrophic factors in Müller cells.

Supported by DFG Research Unit (Forschergruppe) FOR 1075 and Pro Retina e.V.

Strategies to fight an orphan disease

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Purpose: Because of their rarity orphan diseases are often overlooked by the general public. Indeed, very few people recognize names such as "Retinits pigmentosa", "Stargardt's disease" or "Batten disease". Even the medical experts tend to misdiagnose these rare diseases. Different strategies could be used to increase medical awareness.
I will present one route focussing on ophthalmologists, which are usually the first doctors to encounter children with Neuronal Ceroid Lipofuscinosis (Batten disease). But most often they tend to misdiagnose it as Retinitis pigmentosa.

Methods: To raise the profile of an orphan disease called Neuronal Ceroid Lipofuscinosis (OMIM 204200) a corresponding article was initiated which was published in an ophthalmology journal (Klinisches Monatsblatt der Augenheilkunde). Extra prints of this report were sent to all German eye-clinics with the request to integrate it into their journal clubs and training sessions. The foundation asked for feedback via telephone and/or email if this article was discussed.

Results: As a result, 31 (26.5%) of the clinics organized an intern medical NCL-meeting (journal club). However, an equal amount of clinics gave a negative response (31 / 26.5%) and the majority (55 clinics, 47.0%) have not yet responded.

Conclusion: Early diagnosis will be essential for developing effective therapies. Additionally, a correct diagnosis is important to prevent false medication and to provide helpful family counselling. Further steps need to be taken to inform all doctors with the aim to raise the professional awareness of NCL to get more researchers involved and strengthen lobbying for this devastating disease.

Genetic etiology and clinical consequences of complete and incomplete achromatopsia

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Purpose: To investigate the genetic causes of complete and incomplete achromatopsia (ACHM), and to assess the association between disease-causing mutations, phenotype at diagnosis, and visual prognosis.

Methods: The ophthalmologic clinical data of probands with complete ACHM (N=47), incomplete ACHM (N=32) and their affected relatives (N=18), were registered from medical charts and updated by ophthalmologic examination. Mutations in the *CNGB3, CNGA3,* and *GNAT2* gene were analyzed by direct sequencing .

Results: Five *CNGB3* mutations were identified in 55/63 (87%) of patients; the most common mutation was p.T383IfsX13 (80%). We also detected a novel frameshift mutation p.G548VfsX35. *CNGA3* mutations were detected in 3/63 (5%) probands, and no mutations were found in the *GNAT2* gene. ACHM subtype, visual acuity, color vision, refractive error, and macular appearance were equally distributed among the *CNGB3* genotypes, but appeared to be slightly worse among *CNGA3* genotypes. Visual acuity deteriorated from infancy to adulthood in 12% of patients, leading to 20/200 in 61%, and even lower than 20/200 in 20% of patients.

Conclusion: In this well-defined cohort of ACHM patients, the disease appeared much more genetically homogeneous than previously described. The *CNGB3* gene was by far the most important causal gene, and T383IfsX13 the most frequent mutation. ACHM subtype did not associate with a distinct genetic etiology, nor were any other genotype-phenotype correlations apparent. The distinction between complete and incomplete subtypes of ACHM has no clinical value, and the assumption of a stationary nature is misleading.

Activation of microglia in the retina evoked by varied stimuli

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In the retina, resting microglia cells are located in the inner retinal layers. In virtually every retinal disease microglia are activated. In spite of first models for examination of microglial functions, the interaction of microglia in retinal diseases is not yet well understood. To optimize therapeutic interventions a better knowledge of microglia functionality and regulation is required. One aim of the present study was the characterisation of microglia activation after partial surgical vitrectomy in the rabbit eye. Furthermore, a viable model to characterize and influence microglial activation in the guinea pig was established.

During partial vitrectomy of the rabbit eye a part of the vitreous body was replaced by BSS or sodium hyaluronate. The retinae were fixed and stained with the microglia marker (GSA) and the proliferation marker *anti*-Ki67. The total number and the number of Ki67-positive microglia cells were significantly increased two days after partial vitrectomy compared to control. After one week the proliferation decreased strongly, only few (BSS) or no (sodium hyaluronate) cells were still positive for Ki67 in retinae from vitrectomized eyes. The number of microglia cells was similar to that in control retinae one week after treatment. Thus, the surgical procedure itself seems to induce solely a transient activation of microglial cells.

To further investigate and characterize microglial reaction, intravitreal application of LPS was used as activation stimulus. At varying time points after injection the retinae were fixed and stained with the microglia markers, GSA and Iba 1. Three days after the injection of LPS the number of microglia cells doubled whereas after seven days the number of cells was increased threefold. After 14 days the number of microglial cells decreased considerably. It can be concluded that the LPS-induced microglial activation is also a transient process. Furthermore we aimed at a selective manipulation of microglia using a specific immunotoxin. Intravitreal application of the immunotoxin induced a strong microglial reaction and atypical morphological alterations.

Both *in vivo*-models are suitable to investigate processes of microglia activation and proliferation and to study the influence of microglia cells on neuronal degeneration. The LPS-model offers a valuable possibility to characterize the effect of pharmacological interventions. The immunotoxin might be a useful tool for inducing artificial, dystrophic modifications in microglial functionality.

Effect of a heterozygous novel mutation (G846X) on the phenotype in CRB1-related Leber Congenital Amaurosis

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Purpose: to compare the phenotypes of two CRB1-associated LCA patients with the G846X/C948Y and C948Y/C948Y genotypes.

Patients and methods: 7-year-old male with searching eye movements with onset less than 1 year of age and 30-year-old male with micronystagmus and night-blindness since 2-3 years of age. Detailed clinical examinations included best corrected visual acuity testing, fundoscopy and photodocumentation, full-field ERG and ocular coherence tomography (OCT). Molecular genetic screening was performed by LCA-microarray and confirmed by direct sequencing.

Results: Patient 1 (younger, compound heterozygous) has a visual acuity of 0,04 (right eye) and 0,025 (left eye) with excentric fixation. Fundoscopy showed normal optic discs, normal vessels and an atrophic maculopathy of three disc diameter. OCT images revealed slightly modified foveal impression and a thickened neuroretina outside the central lesion.
Patient 2 (older, homozygous) has a best corrected visual acuity of 0,1 (right eye) and 0,2 (left eye). Optic discs are slightly decolorated, vessels are not attenuated. There is an unusual vitreoretinal surface irregularity with some focal thinnings of the central retina, without diffuse atrophic maculopathy. Full-field ERGs were extinguished in both patients.
Genetic examination revealed a novel G846X mutation as an allelic variant in a heterozygous form in Patient 1.

Conclusion: the novel nonsense mutation (G846X) may influence negatively the phenotype in CRB1-related LCA.

Expression profiling of microglia and macrophages using a novel lipidomic TaqMan Array

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Purpose: Lipidomics is the analysis of metabolism, transport, and localization of lipids. Substantial progress has been made to identify and quantify lipids by mass spectrometry. However, quantitative transcriptomics still remains to be integrated for a holistic view of lipidomics. Therefore, our aim was to use the TaqMan Array technology for the quantitative analysis of informative lipid-regulated genes. Of particular interest was to study dynamic gene expression in microglia and macrophages under conditions mimicking sterol overload, inflammation, and n3-fatty acid stimulation.

Methods: We have developed a Lipidomics TaqMan Array with 41 genes covering the four major ontologies sterol metabolism, fatty acid metabolism, lipid droplet, and transcription factors. Four reference genes for normalization and three inflammation markers were also included. Mouse primary microglia, BV-2 cells, and macrophages were stimulated with LXR/RXR agonists to mimic sterol loading, LPS/Ifng for pro-inflammatory stress, and docosahexaenoic acid (DHA) as anti-inflammatory lipid agonist. Data analysis was carried out with Applied Biosystems RQ Manager and Integromics StatMiner software.

Results: Lipidomic TaqMan Arrays showed that (a) stimulation of all three myeloid cell types with LXR and RXR agonists had an inductive effect on several genes of lipid metabolism, (b) the pro-inflammatory stimuli LPS and Ifng strongly repressed most lipid-related genes, with the exception of Cox2, and (c) co-incubation with DHA broadly attenuated the repressing effect of inflammation on lipid genes.

Conclusions: The Lipidomic TaqMan Array is an optimized tools for high-throughput quantitative transcript analysis of the most dynamic lipid genes. Its combination with the Real-time StatMiner further enables researchers to generate statistically evaluated high-quality data. This technology will be very useful for transcriptomics as part of the rapidly progressing field of lipidomics.

Compartmental mitochondrial energy status under physiological and pathophysiological conditions in the retina

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Purpose: The retinal degeneration 1 (*rd*1) mouse is a model for retinitis pigmentosa carrying a mutation in the rod photoreceptor-specific phosphodiesterase gene *(PDE6)* leading to increasing calcium level and oxidative stress in the rods. It is unknown how these mechanisms influence the spatio-temporal pattern of the degeneration process in the *rd*1 retina. In this respect, a special interest lies on the compartmental functionality of mitochondria as it is crucial for the maintenance of cell function and viability. Therefore we want to analyze the localization of physiological and pathophysiological mitochondrial activity in the outer retina of *rd*1 mice at different stages of degeneration.

Methods: The mitochondrial localization and functionality in *rd*1 was investigated by using the JC-1 and Mitotracker orange. Changes in mitochondrial transmembrane potential $\Delta\psi_m$ were assessed using JC-1, a lipophilic cation that accumulates in mitochondria. JC-1 shows green fluorescence at low concentrations, but inside mitochondria at concentrations above 0,1 µg/mL forms J-aggregates and produces red fluorescence. Intracellular distribution of the dyes was assessed by confocal microscopy (Nikon C1si).

Results: We used retinal slices (100µm in thickness) of *rd*1 and age matched wild type mice. At first we could show for wild type mice an intense labelling in the OPL (synaptic mitochondria) and the inner segments (IS) where most of the mitochondria are located. Secondly we identified numerous intensely JC-1 labeled puncta located in the ONL that could be explained as the fluorescence of juxtanuclear mitochondria in rods and cones. For the *rd*1 mice (P11 to P15) we could observe that JC-1 labelling was weaker and diffuse in the abovementioned layers. Furthermore, the JC-1 staining of the *rd*1 retina revealed the appearance of radial fibers in the ONL and IPL that could not be observed in the wild type. This observation coincides with the scenario of remodelling in the degenerating retina, with certain respect to Müller cell atrophy (also seen with Mitotracker orange).

Conclusion: The mitochondria in the outer nuclear layers of *rd*1 mice retina underwent a decrease of $\Delta\psi_m$ at the beginning of retinal degeneration (P11). To separate the mitochondrial activity in photoreceptors from that of Müller cells of *rd*1 retinae it has to be investigated whether the remaining photoreceptors and atrophied Müller cells contain mitochondria in the ONL and how functional they are.

Ca^{2+}-dependent Bk channels in human Rpe cells are coupled to voltage-dependent Ca^{2+} channels and not to Ryanodine Receptors

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Purpose: In different tissues the activation of BK channels has been shown to be coupled to voltage-gated Ca^{2+} channels and/or ryanodine receptors. As activation of BK channels leads to hyperpolarization of the cell they provide a negative feedback mechanism for Ca^{2+}-induced functions. Many cellular functions of the RPE are coupled to changes in $[Ca^{2+}]_i$. The aim of this study was to identify to which Ca^{2+} entry pathway the activation of BK channels is coupled in the RPE.

Methods: We used freshly isolated human RPE cells and the ARPE-19 cell line for molecular biological detection of BK channel α subunits. Patch-clamp measurements were used to characterize BK channels and Fura-2 to monitor changes in $[Ca^{2+}]_i$ in ARPE-19 cells.

Results: Freshly isolated human RPE cells and ARPE-19 cells have been shown to express BK channels. In ARPE-19 cells these channels have been shown to be functionally active. Application of iberiotoxin led to a block of outward currents by 28.15%. At +50 mV ARPE-19 cells had a BK channel-mediated current density of 2.42 pA/pF. Activation of ryanodine receptors by caffeine led to a significant increase in $[Ca^{2+}]_i$ by 34.16%. Nevertheless, caffeine-induced Ca^{2+} signals were not sufficient to activate BK channels. Instead, the activation of L-type Ca^{2+} channels by BayK 8644 caused a dramatic increase in BK channel activity and a shift of the reversal potential of the ARPE-19 cells by -22.6 mV.

Conclusions: We have shown here for the first time that human RPE expresses BK channels. These channels are activated in RPE cells by increases in $[Ca^{2+}]_i$ that are mediated by the opening of voltage gated L-type Ca^{2+} channels. As Ca^{2+} entering the RPE cells through these Ca^{2+} channels are known to be important for growth factor secretion and light-induced transepithelial transport, we speculate that BK channels coupled directly to these Ca^{2+} channels may provide a good tool for negative feedback control of the L-type Ca^{2+} channels.

Identification and mapping of large KCNV2 gene deletions in patients with Cone Dystrophy and Supernormal Rod Response

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Purpose: To investigate the genetic basis of Cone Dystrophy with Supernormal Rod Response (CDSRR) and the associated spectrum of KCNV2 gene mutations.

Methods: The *KCNV2* gene and flanking SNPs were analyzed by DNA sequencing. STR markers were genotyped by PAA gel separations. Gene dosage analyses were carried out by means of TaqMan probe and SYBR green based quantitative PCR (qPCR) assays and Nimblegen CGH array technology. Deletion junction fragments were obtained from Long Distance (LD) PCR amplifications and breakpoints covered by DNA sequencing.

Results: In a screen for KCNV2 mutations in patients with CDSRR and phenotypically similar retinal disorders we identified two unrelated subjects with a homozygous deletion covering ~ 9 kb of genomic DNA sequence including most parts of exon 1 and intron 1. Moreover we observed four independent patients with apparent homozygous point mutations that failed to segregate concordantly in the nuclear family. Using qPCR we could show a decrease in KCNV2 exon 1 gene dosage in all of those patients indicating the presence of heterozygous deletions. Applying loss of heterozygosity and segregation analysis of flanking SNP and microsatellite markers as well as further qPCR assays we could narrow the extent of the deletions in three subjects, that finally enabled amplification and sequencing of deletion junction fragments in two of the subjects. Those carried intragenic KCNV2 deletions of 11.7kb and 16.9kb, respectively. A further patient was investigated applying Nimblegen CGH arrays which allowed the identification of a ~ 80 kb whole gene deletion.

Conclusions: Based on our screening data we found large-sized deletions in 6 of 27 patients with KCNV2 mutations indicating that such genomic rearrangements have to be taken into consideration in the genetic diagnostics of CDSRR. Our data also demonstrate that a complete loss of KCNV2 is a common pathomechanism in CDSRR.

Identification of the receptor for Pigment Epithelium Derived Factor on retinal endothelial cells

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Introduction: Retinal neovascularization (RNV) is the primary cause of blindness in a wide range of ocular diseases such as proliferative diabetic retinopathy (PDR). Endothelial cells are one of the most important cell populations in RNV. Pigment epithelium-derived factor (PEDF) is the most potent natural antiangiogenic factor so far. It has been shown that the different activities of PEDF are mediated via binding to the cell surface. However, little is known about the identity of the receptor and molecular mechanism(s) by which PEDF functions to regulate endothelial cell behavior. In this study, we detected a potential receptor for PEDF using recombinant human PEDF (rhPEDF).

Methods: DNA encoding for human PEDF was amplified from human retinal pigment epithelium (RPE) cell mRNA and cloned into a plasmid that gives rise to the production of a PEDF fusion protein. Subsequently, rhPEDF was produced and purified from mammalian cell cultures. Expression of a putative PEDF receptor on bovine retinal endothelial cells (BRECs), RPE cells and human umbilical vein endothelial cells (HUVECs) was detected using rhPEDF-coated magnetic beads, by immunohistochemical staining and Western blotting. In particular, Western blots were used to assess the molecular weight of the PEDF-binding molecule.

Results: Purified rhPEDF bound to retinal cells and HUVECs. The putative PEDF receptor, with a molecular weight of about 160 kDa, was localized to the surface of BRECs, HUVECs and RPE cells.

Conclusions: Retinal cells express a ~160-kDa binding protein that may function as a receptor and probably accounts for PEDF-mediated effects.

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THIS MEETING IS FUNDED BY

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