PRO RETINA FOUNDATION

PRO RETINA POTSDAM-MEETING

10th PRO RETINA

RESEARCH-COLLOQUIUM POTSDAM

CONFERENCE REPORT

Retinal Degeneration

Diagnostic Advances, New Mechanistic Insights and Progress in Therapeutic Approaches

An Interdisciplinary Dialogue

March 27th/28th, 2015

Seminaris SeeHotel Potsdam

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HOPE2 HEREDITARY RETINAL DISORDERS From Patients Towards Therapies

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PRO RETINA DEUTSCHLAND E. V. & THE PRO RETINA-FOUNDATION FOR PREVENTION OF 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 60 regional groups, which are spread throughout Germany. At present (2015), PRO RETINA Deutschland e. V. counts more than 5,800 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 of 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 two 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, March 27th 2015

13:00–13:05 **Welcome remarks**

13:05–14:30 **Session 1** **Selected poster presentations**
Eight abstracts to be selected

14:30–15:10 **Keynote lecture**
Steven J. Fliesler, Buffalo
A decade of progress in retinal degeneration research: Cage-side to bedside and back

15:10–16:00 **Coffee break**

16:00–17:40 **Session 2** **Mechanisms relevant to retinal degeneration**

16:00–16:25 Solon Thanos, Münster
Retinal microglia and regeneration

16:25–16:50 Peter Vandenabeele, Gent
Molecular mechanisms of regulated necrosis, an attempt at an overview

16:50–17:15 Kari Alitalo, Helsinki
Vascular growth factors, angiogenesis and lymphangiogenesis

17:15–17:40 J. Emanuel de Carvalho, Amsterdam
The role of the proteasome in age-related macular degeneration

17:40 **Dinner**

19:00–20:00 **Session 3** **The PRO RETINA Professorship: A story of success**

19:00–19:20 Antje Grosche, Regensburg
Perspectives on targeting Müller glia cells as a treatment option for complex retinal diseases

19:20–19:40 Peter Charbel Issa, Bonn
ABCA4-related retinopathy – linking phenotype and therapeutic strategies

19:40–20:00 Thomas Langmann, Köln
How innate immunity and genetics meet in the retina

20:00–open **Swingin' poster session**

PROGRAMME

Saturday, March 28th 2015

09:00 – 10:40	**Session 4**	**Diagnostic advances**
	09:00 – 09:25	Wolf Harmening, Bonn *The potential of adaptive optics for retinal degenerations*
	09:25 – 09:50	Johannes de Boer, Amsterdam *Beyond structural OCT imaging in the human retina*
	09:50 – 10:15	Michael B. Hoffmann, Magdeburg *Assessment of visual function with multifocal electrophysiology*
	10:15 – 10:40	Hanno Bolz, Ingelheim *Opportunities and pitfalls of high throughput DNA testing in retinal dystrophies*
10:40 – 11:15	**Coffee break**	
11:15 – 12:55	**Session 5**	**Strong in translation**
	11:15 – 11:40	Monika Fleckenstein, Bonn *Therapeutic approaches for dry age-related macular degeneration – hopes and constraints*
	11:40 – 12:05	Astrid Limb, London *Potential of Müller glia for developing therapeutic approaches to treat retinal degenerative diseases*
	12:05 – 12:30	Yvan Arsenijevic, Lausanne *The potential of stem cells/iPSC in the therapy of retinal degenerations and AMD*
	12:30 – 12:55	Stefanie Hauck, München *Neurotrophic factors and related therapeutical approaches for retinal degenerations*
12:55 – 13:00	**Concluding remarks**	
13:00	**Lunch and end of meeting**	

SPEAKER ABSTRACTS

A decade of progress in retinal degenerations research: Cage-side to bedside and back

Steven J. Fliesler

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The past decade has witnessed significant progress toward a better understanding of the biological processes and mechanisms that underlie retinal degenerations, whether caused by genetic mutations, environmental conditions, aging, or trauma. Some of these discoveries have made us rethink certain aspects of "conventional wisdom" in the field regarding fundamental premises, *e.g.,* "retinal remodeling". The development of relevant animal models that mimic, with fidelity, the key phenotypic hallmarks of such diseases has contributed greatly to this progress. So to, advances in human genetics, diagnostic testing and high-throughput screening, bioinformatics, and imaging methods have led to better definition of disease phenotypes, which in turn has informed the refinement of previously developed animal models and the creation of new models used to study these diseases in a laboratory setting. In addition, animal models have proven invaluable in the pre-clinical design and testing of new therapeutic interventions aimed at preventing, slowing or halting the progression of retinal degenerations, as a prelude to human subjects clinical trials, including the "repurposing" of drugs not originally developed or intended with ophthalmic applications in mind. Multiple discoveries and innovations in the broader scientific community, particularly in the fields of gene therapy, gene editing (*e.g.,* CRISPR/Cas9 and Talen technologies), vascular biology, stem cell biology and regenerative medicine, have started to be applied to the field of retinal degenerations research. These new approaches hold great promise for significantly impacting the trajectory of further fundamental scientific as well as clinical advances in understanding and treating retinal degenerations.

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Molecular mechanisms of axonal regeneration within the injured optic nerve and the role of microglia cells

Solon Thanos, Münster, Germany

Purpose: Injuries of the optic nerve, caused mechanically or by diseases, are still not reparable and result in retrograde and anterograde atrophy One of the strategies to support regeneration may be the implantation of pluripotent neural progenitor cells which might produce growth promoting factors and pave the microenvironment, thus allowing for nerve regeneration. An alternative strategy would be the pharmacological alteration of microglial cells. The object of this project was to initiate regeneration of axons by using both strategies.

Methods: We aimed to examine whether the application of pluripotent embryonic cells NPCs at the site of injury or within the vitreous body, supports axonal growth. NPCs from the prospective retina and the rostral neural tube were cultivated. In the first experimental group the rat ON was completely cut intraorbitally and resutured to adapt the proximal and distal optic nerve stump. In the second group the ON was only crushed. In both groups the NPCs were injected intravitreally. Regeneration was proved with ERG and VEP. In a second series of experiments, microglia cells were inhibited with microglia inhibiting factor (MIF) to inactivate these cells and study RGC survival. One week to one month postsurgery anterograde and immunohistochemical staining of the ON was applied. To get information about the quantitative aspects of regeneration regenerating ganglion cells were retrogradely labelled with Fluorogold or DiI-ASP.

Results: Cultured or implanted NPCs produce neurotrophic and neuroregenerative substances. Such cells can be implanted into the cut optic nerve (ON) to produce *en loco* neuroregenerative factors (neurotrophins, crystallins, trophic factors, metalloproteases), which can be secreted into the site of injury. So far NPCs are used as living *"cell biofarms"* to stimulate neuroregeneration. Cut axons intermingle with NPCs and pass through the zone of injury to enter the distal optic nerve over long distances. Immunohistochemical data obtained from serial sections through the proximal, injured and distal optic nerve permitted to count the axons and examine the incidence of axonal regeneration. Inactivation of microglial cells prolonged RGC survival and enhanced the number of RGCs regenerating axons *in vivo.*

Conclusions: We conclude that paving of the distal optic nerve environment with neurotrophins and inactivation of microglia are complementary strategies to improve survival and regeneration of optic nerve axons.

Molecular mechanisms of regulated necrosis, an attempt at an overview

Peter Vandenabeele[1,2,3]

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The discovery of regulated cell death has created many possibilities for gaining control over the life and death decisions made by cells during many inflammatory, degenerative and infectious diseases. For many years, apoptosis has been the focus of drug discovery. However, cell death research recently identified regulatory mechanisms and signaling pathways of non-apoptotic forms of regulated cell death, called regulated necrosis including necroptosis, parthanatos, cyclophilin-D-mediated necrosis, ferroptosis or oxytosis, MPT-dependent necrosis, pyroptosis and pyronecrosis, and NETosis/ETosis. Regulated necrosis (RN) is now defined as a genetically controlled cell death process morphologically characterized by cytoplasmic granulation, cellular swelling ("oncosis"), eventually resulting in plasma membrane permeabilization. Several of these cell death modalities share morphological features of necrosis, in particular the cellular swelling ("oncosis") and the eventual plasma membrane permeabilization. We will briefly discuss how several of these cell death modalities have distinct signaling pathways but also share common regulatory mechanisms. Several small molecules have been identified that can induce and inhibit these non-apoptotic forms of cell death. The availability of these drugs will allow to develop strategies for translating the fundamental understanding of cell death pathways and their targeting into new therapeutic strategies for inflammatory, degenerative and infectious experimental disease contexts.

The role of the proteasome in age-related macular degeneration

J. Emanuel Ramos de Carvalho, Reinier Schlingemann

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The ubiquitin-proteasome pathway is a non-lysosomal protein-degradation nanomachinery present in all types of eukaryotic cells. Formation of drusen and choroidal neovascularization appears to be etiologically related to local inflammation, oxidative stress, complement overactivation, dysregulation of angiogenesis and accumulation of damaged or postsynthetically modified proteins. Removal of aberrant proteins by the ubiquitin-proteasome pathway is essential for normal cellular function. Our group has shown that complement overactivation and ageing mechanisms lead to impairment of the ubiquitin-proteasome pathway which in turn may contribute to the accumulation of abnormal proteins, cytotoxicity in the retina and ultimately lead to age-related macular degeneration. Moreover, decreased proteasomal activity has been linked to photoreceptor degeneration, abnormal inflammatory responses, dysregulation of signal transduction and choroidal neovascularization. In the future, modulation of the proteolytic capacity of cells by means of a new generation of pharmacotherapies may contribute to diminish the burden of age-related macular degeneration and other age-related diseases.

Perspectives on targeting Müller glia cells as a treatment option for complex retinal diseases

Thomas Pannicke[1], Alexandra Hauser[2], Stefanie Hauck[3], Antje Grosche[2]

[1]Paul Flechsig Institute of Brain Research, Leipzig
[2]Institute of Human Genetics, Regensburg
[3]Helmholtz-Zentrum München, Neuherberg

Purpose: Glia cells are still a neglected stepchild in research on retinal degeneration. Müller cells, the principal retinal macroglia, undergo major changes in gene expression associated with partial loss of their functions in diseased retinae. Little is known about the mechanistic relevance of this process. The goal of our research is to understand the relevance of this glial reaction and to improve the neuronal survival in retinal diseases by manipulating Müller cell gene expression with gene therapeutic tools.

Methods: We investigated the gliotic activation of Müller glia in a model of transient ischemia evoked by elevation of the intraocular pressure in mice deficient in the nucleotide receptor P2Y1 (P2Y1R-KO) or lacking the intermediate filaments, GFAP and vimentin (GFAP/vimentin-KO). Information about gliosis and neuronal survival was assessed by immunohistochemistry, morphometric analyses and patch-clamp recordings. Moreover, a method to enrich intact Müller glia via magnetic activated-cell sorting was established enabling cell specific expression analyses.

Results: Typically, Kir4.1, the major glial potassium channel, is down-regulated in gliotic Müller cells. We found a stable Kir4.1 expression in P2Y1R-KO mice in gliotic Müller glia and a concomitant protective effect especially on neurons of the inner retina, while the postischemic photoreceptor loss was more pronounced. In GFAP/vimentin-KO mice, Müller glia displayed a reduced potassium conductance. An enhanced neurodegeneration was detected in these mice. To identify additional key players (apart from Kir4.1) in Müller cell gliosis, we established a method for cell type-specific expression analyses. First data comparing gene expression from enriched Müller glia with that from Müller cell-depleted (neuronal) fractions demonstrate the usibility of this method to collect unbiased, comprehensive gene expression data on transcript and protein level.

Conclusion: We identified the Kir4.1 channel as a first candidate gene to be modulated in Müller glia for therapeutic use. Since the effect of an altered Müller gliosis strongly depended on the respective neuronal cell type, future studies to improve our understanding about mechanisms involved in reactive gliosis are needed, before heading at the development of respective gene therapeutic strategies. Cell type-specific expression analyses will help to identify such candidate genes with the prime goal of stabilizing the neuron-supportive functions of Müller glia in the diseased retina.

ABCA4-related retinopathy – linking phenotype and therapeutic strategies

Peter Charbel Issa

Biallelic mutations in the ATP-binding cassette (ABC) transporter *ABCA4* are amongst the most common causes for inherited retinal disease and thus loss of vision early in life. *ABCA4*-related retinopathy is phenotypically heterogeneous and includes Stargardt disease, cone-rod-dystrophy and Bull's eye maculopathy. Despite such phenotypic heterogeneity, a common theme across most patients is increased accumulation of lipofuscin/bisretinoids in the retinal pigment epithelium (RPE), which occurs relatively early in the disease pathogenesis. Similar observations have been reported in the *Abca4*$^{-/-}$ mouse, which is an animal model for *ABCA4*-related retinopathy.

Therapeutic strategies currently pursued either aim at correcting the disease on a genetic level or aim at reducing the abnormally accelerated lipofuscin accumulation. In both cases, the ultimate goal would be to avoid loss of visual function due to RPE- and photoreceptor death. Thus, disease progression ideally is monitored using a parameter that precedes outer retinal atrophy.

Quantitative autofluorescence is a technique that for the first time allows assessing and monitoring lipofuscin-related autofluorescence intensity in vivo. We use this technique to differentiate patients with *ABCA4*-related retinopathy from patients with similar clinical presentations but different genetic causes, and to investigate the rate of lipofuscin accumulation over time. This will allow us to assess the potential usefulness of this novel imaging modality for future clinical trials. Also, the technique enables us to investigate genotype-phenotype correlations and to solve the question whether monoallelic *ABCA4*-mutations are disease causing or not. Importantly, qAF can also be assessed in mice, thus allowing to assess potential therapies for *ABCA4*-related retinopathy in a relevant animal model using the same outcome-parameters that may also be used in subsequent human clinical trials.

Any future therapy for *ABCA4*-related retinopathy will need to be assessed in a clinical trial. Only the right choice of adequate outcome measures will enable us to decide whether a therapy meets expectations or not. qAF might be a suitable outcome measure to assess forthcoming therapies.

How innate immunity and genetics meet in the retina

Thomas Langmann

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Our group was born in summer 2006 at the Institute of Human Genetics in the beautiful medieval city of Regensburg. At this time, Bernhard Weber decided to hire a group leader with some knowledge in innate immunity and genetics. His long standing expertise in retinal genetics rapidly stimulated my interest in transcription factors. This lead to a truly collaborative work that brought up some key aspects of retina-specific gene expression including the first targetome of a retinal transcription factor (Cone rod homeobox)[1] and the concept of cis-regulatory mapping that helped to identify the FAM161A gene as a genetic cause of retinitis pigmentosa[2]. At this very same time, Bernhard Weber showed me some images of large phagocytes that he spotted in the retinoschisin-deficient retina. Back then, microglia were a known but somehow neglected phenomenon present in some models of retinal damage[3]. However, the sequential events of their activation, specific markers and their potential utilization as therapy targets were not far developed. Luckily, the ProRetina foundation wisely guided by Franz Badura supported our concept of neuroprotection by microglia modulation with stipends and established an endowed professorship in Regensburg in 2010. With the progress of our studies in several different retinal disease models, visionary clinical partners showed interest and Bernd Kirchhof, an expert in retinal surgery, inspired the Cologne Stock foundation to finance an endowed chair for "Experimental Immunology of the Eye". Since 2012, the laboratory is established in the beautiful medieval city of Cologne and the goal of our research is to find treatment options for rare hereditary retinal diseases as well as age-related macular degeneration, which are both good examples of retinal disorders that combine genetic changes with activation of innate immunity[4]. I appreciate the support of my mentors and sponsors and especially thank those members of my laboratory that moved from one medieval city to another (Marcus Karlstetter, Alexander Aslanidis, Eva Scheiffert).

References:

[1]Corbo JC et al. CRX ChIP-seq reveals the cis-regulatory architecture of mouse photoreceptors. Genome Res. 2010; 20:1512-25.

[2]Langmann T et al. Nonsense mutations in FAM161A cause RP28-associated recessive retinitis pigmentosa. Am J Hum Genet. 2010; 87:376-81.

[3]Langmann T. Microglia activation in retinal degeneration. J Leukoc Biol. 2007; 81:1345-51.

[4]Karlstetter M et al. Retinal microglia: Just bystander or target for therapy? Prog Retin Eye Res. 2015; 45C:30-57.

The potential of adaptive optics for retinal degenerations

Wolf Harmening

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The eye with its transparent optical media offers a unique view onto its neurosensory tissue, the retina. Microscopic inspection of the living retina, however, is primarily limited by the quality of the eye's internal optics, as they introduce significant wavefront aberrations that spread the light as it enters and exits the eye beyond optical control of an imaging system. Adaptive optics is a set of tools first introduced in astronomy to compensate for wavefront aberrations that occur in atmospheric turbulence, and is now increasingly used in ophthalmic applications. Today, adaptive optics driven ophthalmic instrumentation is capable of imaging microscopic structures down to a lateral extent of about 2 microns in the living retina. Because of their waveguiding properties, mostly rod and cone photoreceptor cells contribute to the light reflected out of the eye during retinal imaging, and their structural characteristics have thus been extensively studied. However, adaptive optics ophthalmoscopy has revealed other microscopic structures that were not accessible before; among those are the smallest perifoveal capillaries, retinal pigment epithelial cells, the lamina cribrosa and axons of retinal ganglion cells, for example.

En face optical resolution of single photoreceptor cells in the living eye offers another unique possibility. Specifically, the adaptive optics scanning laser ophthalmoscope (AOSLO) can be combined with real-time eye tracking and fast optical switching to allow direct experimental access to single sensory receptors in humans. This sets the stage to probe visual function on the elementary level of single cells. In the ophthalmological clinic, functional testing of damaged cones may prove useful, because structural and functional information is often entwined, but cannot be readily inferred from one another. Cone-targeted visual stimulation may be a tool for better characterization of disease progression and offer a way to evaluate the efficiency of novel treatments at the microscopic level in the living eye.

Assessment of visual function with multifocal electrophysiology

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Since its initial development in the 90-ies the multifocal electroretinogram (mfERG) has been firmly established as one of the routine tools of clinical electrophysiology in vision. [1]. It is of particular relevance for a spatially resolved assessment of retinal function based on the determination of the activity of the retinal bipolar cells. Further, the application of the multifocal technique to other electrophysiological measurements such as the visual evoked potentials (mfVEP) supports an objective visual field perimetry and the localisation of patho-mechanisms along the visual pathway [2]. Here an overview of the multifocal approaches and its application for the investigation of the interplay between retinal pathology and visual function impairment will be given [3].

References:

[1] Sutter E, Tran D (1992)
The field topography of ERG component in man – I. The photopic luminance response.
Vision Research 32:433-446

[2] Hoffmann MB (2008)
Investigating visual function with multifocal visual evoked potentials.
In: Essentials in Ophthalmology: Pediatric Ophthalmology, Neuro-Ophthalmology, Genetics 139–157

[3] Herbik A, Geringswald F, Thieme H, Pollmann S, Hoffmann MB (2014)
Prediction of higher visual function in macular degeneration with mfERG and mfVEP
Ophthalmic and Physiological Optics 34:540-551

Opportunities and pitfalls of high-throughput DNA testing in retinal dystrophies

Hanno J. Bolz[1,2]

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Purpose: To point out benefits, challenges and pitfalls of NGS-based testing in patients with retinal dystrophies (RD).

Methods: Samples from more than 200 patients with different forms of RD (mainly RP, CRD and LCA) were analyzed by high-throughput sequencing (next-generation sequencing, NGS). Bioinformatic processing included analysis for structural mutations (copy number variations, CNVs), and identified potentially pathogenic variants were verified by Sanger sequencing and (if applicable) segregation analysis. Besides diagnostic NGS, results from research will be shown.

Results: We were able to identify the causative mutations in the majority of patients with the above indications. "Hidden mutations" included CNVs and mutations in non-coding gene regions. Unexpected findings reversed the clinical diagnoses in some patients. In some cases, categorization of variants was challenging and only possible with in-depth analysis including animal studies.

Conclusion: The recent advent of NGS in routine diagnostics has transformed the field of medical genetics, making genetically heterogeneous entities like the RDs accessible to routine genetic testing. The diagnostic yield has increased tremendously, with mutations in the known RD genes explaining the vast majority of cases. Moreover, large NGS panels have the potential to detect coexistent mutations in related conditions. Despite the sometimes predictive nature of such results, this will be increasingly helpful in genetic counseling and patient management (shift of diagnoses, carrierships). Erroneous categorization of pathogenicity is probably an underestimated source of pitfalls, as is demonstrated by apparently unambiguous "mutations" of established disease genes that represent rare non-pathogenic variants. Therefore, even loss-of-function variants require careful evaluation.

Therapeutic approaches for dry age-related macular degeneration – hopes and constraints

Monika Fleckenstein, Frank G. Holz

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In contrast to wet AMD, where loss of vision is typically acute and anti-VEGF-treatment leads to a relatively rapid reduction in retinal fluid and subsequent improvements in visual acuity, disease progression and vision loss in geographic atrophy (GA) due to age-related macular degeneration (AMD) are gradual processes. Although GA can result in significant visual function deficits in reading, night vision, dark adaptation and produce dense, irreversible scotomas in the visual field, the initial decline in visual acuity (VA) may be relatively minor if the fovea is spared. Since best-corrected VA does not correlate well with GA lesions or progression, alternative clinical endpoints are being sought. These include reduction in drusen burden, slowing the enlargement rate of GA lesion area, and slowing or eliminating the progression of intermediate to advanced AMD. Among these considerations, slowing the expansion of GA lesion area appears to be a clinically suitable primary efficacy endpoint. Since GA lesion growth is characterized by loss of photoreceptors, it is considered a surrogate endpoint for vision loss. Detection of GA can be achieved with a number of different imaging techniques, including color fundus photography, fluorescein angiography, fundus autofluorescence (FAF), near infrared reflection, and spectral-domain optical coherence tomography. Previous studies have identified predictive characteristics for progression rates including abnormal patterns of FAF in the perilesional retina. Currently there is no approved or effective treatment to prevent either onset or progression of GA. However, in recent years, significant progress has been made in understanding the pathogenesis of GA, which has led to a number of new potential therapies currently undergoing clinical trial evaluation including complement-inhibitors (anti-factor D, anti-C5), neuroprotectants, visual cycle inhibitors, and anti-inflammatory agents.

Stem cell based therapeutic approaches to treat retinal degenerative disease

Prof. G. Astrid Limb

UCL Institute of Ophthalmology, London, UK

Müller glia are responsible for the regenerative ability observed in the zebrafish retina throughout life. Although Müller glia with stem cell characteristics are also present in the adult human retina, there is no evidence of spontaneous regeneration occurring in humans. However these cells can be isolated from normal donor eyes, become spontaneously immortalized, and can be differentiated into enriched populations of retinal ganglion cells (RGC) or photoreceptors (PR) *in vitro*. Our laboratory investigates the potential therapeutic use of these cells to treat retinal degenerative diseases, either by transplantation to replace damaged neurons and provide neurotrophic support, or by promotion of endogenous regeneration.

Intravitreal transplantation of Müller-derived RGC into a rat model of RGC depletion results in attachment of the grafted cells to the host's RGC layer with local extension of neural-like projections and partial restoration of the negative scotopic threshold response of the electroretinogram (ERG), an indicative measure of RGC function. Similarly, subretinal transplantation of Müller-derived photoreceptors into the P23-H rat eye (a model of photoreceptor degeneration) resulted in migration and integration of these cells into the outer nuclear layer, where they expressed rhodopsin, a marker of mature photoreceptors. This was also accompanied by a significant improvement in the A-wave amplitude of the electroretinogram, an indicative of photoreceptor function. These results suggest that human Müller glia has the potential for development of cell transplantation therapies to repair visual function. However, further work is needed to refine methods for cell delivery into the retina as well as to promote integration and long term survival of the grafted cells.

In examining mechanisms for potential induction of endogenous regeneration, we observed that Müller stem cells cannot be derived from the gliotic human retina, for which we have proposed that inflammatory factors released during gliosis may play a role in the inhibition of endogenous proliferation and differentiation of these cells *in vivo*. Our studies showed that the Wnt signalling pathway, which is indicative of neural stem cell progenicity, operates in human Müller stem cells and can be modified by TGF-β1, a cytokine that is highly upregulated during retinal gliosis. Furthermore, we showed that TGF-β1 inhibits photoreceptor differentiation of hMSC, an event which found to be mediated by activation of the canonical Wnt signalling pathway. Further investigations into the mechanisms of regulation of Wnt signalling in hMSC by TGF-β1 may help identifying factors that could be potentially targeted to promote endogenous regeneration of the adult neural retina.

The potential of stem cells/iPSC in the therapy of retinal degenerations and AMD

Yvan Arsenijevic

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Retinal dystrophies have different gene mutation origins leading to dysfunctions of the rod photoreceptors, cones or cells of the retinal pigment epithelium (RPE). So far, no cures exist to treat the great majority of these diseases. The understanding of the pathological mechanisms necessitates adequate animal models as well as cell culture systems to investigate some intrinsic mechanisms and develop innovative therapeutic approaches.

In that context, the use of stem cells is a promising tool to approach such developments. We previously investigated the possibility to derived retinal stem cells from the newborn mouse retina and the adult mouse and human ciliary margin zone. Despite these cells show several retinal progenitor characteristics, they never lead to the full differentiation of retinal cells although several specific markers of retinal neurons were observed. We thus investigated the potential of embryonic mouse stem cells (mES) to generate fully differentiated photoreceptors. Optimizing a protocol developed by the Sasai's team (Univ. of Kyoto) we generated a mES line expressing the GFP specifically in the photoreceptors by the activation of the Crx gene. Culture in 3D leads to the formation of a high rate of optic-cup forming retina-like tissues with multilayered photoreceptors rows presenting several specific markers of photoreceptors, but without outersegments. After cell sorting and transplantation into normal mice, the rods formed fully differentiated outersegments and synapses. A similar approach is now pursued to generate ES-cone lines.

Because RPE are degenerating in age-related macular degeneration, a protocol to rapidly generate RPE from human induced pluripotent cells (hiPS) was developed. After isolation of pigmented cells, the RPE form a cobble-stone like monolayer expressing many specific RPE genes.

These different cell sources of differentiated cells can now serve to investigate the biology of the cells, to develop in vitro disease models, to perform large scale drug screening and to study the potential of these cells to repair the retina.

Neurotrophic factors and related therapeutical approaches for retinal degenerations

Stefanie M. Hauck[1], The HOPE I / HOPE II consortium

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Purpose: In a large group of rare inherited retinal degenerations, protecting photoreceptors from degeneration can be regarded as the key objective for future therapeutic strategies. Despite the diversity of mutations causing neurodegenerations, the final, unifying event leading to blindness is apoptosis of retinal neurons. Even though restoration of specific gene function tailored to the respective causative mutation in each patient seems the ideal treatment, the genetic heterogeneity of Retinitis Pigmentosa (RP) and often late diagnosis is a downside for this particular approach. Thus, the first goal for any therapeutic intervention should be to prevent disease progression. Application of known neurotrophic factors initially characterized to protect brain neurons have been applied as single molecules to retinal neurons, demonstrating that protection of functionally impaired retinal neurons *in vitro* and *in vivo* works (LaVail, 2005). An important source of neurotrophic factors are retinal Müller glial cells (RMG) and an in depth understanding of their contributions to support retinal neurons in health and disease will provide a basis for future therapeutic applications.

Methods and Results: In order to systematically analyse RMG reactivity to retinal degeneration, we have developed a combined genomics and proteomics approach based on profiling primary RMGs from mouse and pig. This led to the identification of several proteins that are induced and secreted by RMG in response to GDNF such as osteopontin (delRio et al., 2011) and Cyr61 (Kucharska et al., 2014) which in turn support survival of photoreceptors. In addition RMG secretomes are neuroprotective for photorecpetors (Hauck et al., MCP, 2008) and differential SILAC labelling of RMG over time in culture resulted in the identification of differentially expressed and neurotrophic proteins, including osteopontin, transferrin and LIF as well as a yet undiscussed molecule, CXCL10, that exert neurotrophic effects on isolated photoreceptors and retinal explants of rd1 mice (von Toerne et al., 2014).

Conclusion: While understanding RMG in the context of retinal health and disease with respect to their ability to provide neuroprotection and the functional analyses of those RMG-derived neurotrophic factors will be key to develop rational therapies, a major hurdle to translate neuroprotection into clinical application remains. The therapeutic use of neurotrophic molecules does depend on its continuous supply, long lasting expression of such factors must be ascertained to generate a meaningful therapeutic benefit. In order to effectively, locally and continuously supply neurotrophic factors to the degenerating retina, implantation of encapsulated producer cells has been proposed and functionally evaluated. An important goal for future neuroprotective therapies is the long term survival of transplanted cells and sustained production of a cocktail of relevant and effective neurotrophic factors by the transplant.

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POSTER ABSTRACTS

Activated Microglia/Macrophage Whey Acidic Protein (AMWAP) inhibits NFκB signaling and induces a neuroprotective phenotype in microglia

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Background: Microglial reactivity is a hallmark of degenerative diseases of the brain and retina. We have previously identified Activated Microglia/Macrophage Whey Acidic Protein (AMWAP) as a marker of reactive microglia and counter-regulator of pro-inflammatory response. Here, we studied its mechanisms of action in microglia with a focus on nuclear factor κB (NFκB) signaling and tested its neuroprotective effects.

Methods: Recombinant AMWAP was produced in E. coli and HEK293 EBNA cells and purified by affinity chromatography. AMWAP uptake was monitored by fluorescent labeling and pro-inflammatory markers were measured by qRT-PCR. NFκB pathway proteins were assessed by immunocytochemistry, Western blot and immunoprecipitation. A 20S proteasome activity assay was used to investigate the antipeptidase action of AMWAP. Microglial neurotoxicity was estimated by nitrite measurement and quantification of caspase 3/7 levels in 661W photoreceptors cultured in the presence of microglia-conditioned medium. Microglial morphology was analyzed by Phalloidin-TRITC staining. Microglial proliferation was investigated using FACS and their phagocytic capacity was monitored by the uptake of fluorescently labeled apoptotic 661W photoreceptor debris.

Results: AMWAP was secreted from LPS-activated BV-2 microglia and recombinant AMWAP dose-dependently reduced the TLR4-mediated expression of IL6, iNOS, CCL2 and CASP11 in reactive microglia. Further, AMWAP reduced TLR2- and partially TLR9-mediated pro-inflammatory gene expression. Microglial uptake of exogenous AMWAP effectively inhibited TLR4-dependent NFκB activation by preventing IRAK-1 and IκBα proteolysis. No inhibition of IκBα phosphorylation or ubiquitination and no influence of AMWAP on overall 20S proteasome activity were observed. Functionally, both microglial NO secretion and 661W photoreceptor apoptosis were significantly reduced after AMWAP treatment. AMWAP promoted filopodia formation and ramification of microglia and increased their recognition and phagocytic uptake of apoptotic 661W photoreceptor cells.

Conclusions: AMWAP is secreted from reactive microglia and acts in a paracrine fashion to counter-balance their reactivity through NFκB inhibition. AMWAP also induces a neuroprotective microglial phenotype with increased phagocytosis and strongly reduces neurotoxicity. We therefore hypothesize that anti-inflammatory whey acidic proteins could have a therapeutic potential in neurodegenerative diseases of the brain and the retina.

Delivery of recombinant proteins to rod photoreceptors via lipid nanovesicles: Toward a protein therapy of retinal diseases

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Purpose: Liposomes are increasingly used to encapsulate and deliver a variety of therapeutic molecules. The anatomical and morphological features of the eye constitute an ideal benchmark for testing the potential of delivering well-defined and precisely structured proteins into the cell, in order to reach desired levels and functionally modulate the physiological response.

Methods: In this work, we evaluate the potential of liposomes to deliver functional proteins in retinal photoreceptors using two experimental approaches. First, we treated isolated mouse retinas with liposomes encapsulating either recoverin, an important endogenous protein operating in visual phototransduction, or antibodies against recoverin. The electrical response of single photoreceptors to specific light stimuli was measured by electrophysiological recordings using a perforated loose seal technique. We then intravitrally injected *in vivo* liposomes encapsulating either rhodamin B or recoverin and we investigated the distribution in retina sections by confocal microscopy.

Results: Electrophysiological properties of photoreceptors in retina slices showed that the two types of liposomes, namely those encapsulating either recoverin or its antibody release their content intracellularly and differentially modulate the phototransduction cascade. In line with the current model of phototransduction in mouse rod cells, sequestering of endogenous recoverin by antibodies resulted in lower photoresponse peak amplitudes to dim flashes and significantly reduced saturation times following saturating flashes. The opposite phenomena were observed when recoverin in excess was carried by the nanovesicles. Confocal microscopy suggested that the content of liposomes is released in higher amount in the photoreceptor layer compared to other regions of the retina

Conclusion: Our study sets the basis for quantitative investigations aimed at assessing the potential of intraocular protein delivery via biocompatible nanovesicles to restore impaired visual function in various retinal diseases affecting the photoreceptor layer.

PARP inhibitors partially rescue cell death in the retinal degeneration 1 (rd1) mouse model

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Purpose: Inhibition of the enzyme poly(ADP-ribose) polymerase (PARP) can partially rescue photoreceptor degeneration in rd1 mice, a mouse model for inherited retinal degeneration (RD). Recently, several PARP inhibitors have been successfully tested in late phase clinical trials for cancer therapy. Here, I investigated whether some of these clinically tested inhibitors are able to rescue photoreceptor cell death in *rd1* mice.

Methods: Retinas of *rd1* mice were explanted at P5 and cultured *in vitro* under serum-free, entirely controlled conditions until P11 (i.e. 6 days in vitro; DIV). From DIV2 on, they were treated with PARP inhibitor concentrations ranging from 10 µM to 50 µM or corresponding concentrations of DMSO as solvent control. On cryosections, cell death was assessed using the TUNEL assay, while PARP activity was determined by immunohistochemical DAB staining against PAR-residues.

Results: The outer nuclear layer (ONL) of treated retinal explant cultures displayed significantly reduced PAR positive and TUNEL positive cells. This effect was dependent on the inhibitor concentration used, with some inhibitors showing protective effects at submicromolar concentrations.

Conclusion: These results confirm the importance of PARP activity for *rd1* retinal degeneration and point to the ambiguity of PARP actions for cell death and survival in neurodegeneration and cancer. The efficacy of clinically tested PARP inhibitors highlights their potential for a rapid translation into a therapy for RD.

An augmented *ABCA4* screen targeting non-coding regions reveals a deep intronic founder causal variant in Belgian Stargardt patients

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Purpose: Autosomal recessive Stargardt disease (STGD1) is hallmarked by a large proportion of patients with a single heterozygous causative variant in the disease gene *ABCA4*. Braun *et al.* (2013) reported deep intronic variants of ABCA4, prompting us to perform an augmented screen in 131 Belgian STGD1 patients with one or no ABCA4 variant to uncover deep intronic causal ABCA4 variants.

Methods: All 131 prescreened patients underwent resequencing of four deep intronic *ABCA4* regions using next-generation sequencing (NGS) (Miseq, Illumina). Haplotype analysis was performed on patients with variant c.4539+2001G>A (known as V4) and family members. Analysis and comparison of clinical and molecular data allowed us to establish phenotype-genotype correlations of V4. The cohort without an identified second mutation currently undergoes targeted NGS with a customized Haloplex panel that includes the entire *ABCA4* gene and its regulatory domain, and the entire genomic regions of *BEST1*, *RDH12* and *PRPH2*.

Results: Our augmented screen revealed a second variant in 28.6 % of cases. Twenty-six percent of these carry the same causal variant V4. Haplotyping showed a common region of 63 kb, suggestive of a founder mutation. Genotype-phenotype correlations indicate a moderate-to-severe impact of V4 on the STGD1 phenotype. The remaining patients in whom no second *ABCA4* mutation was identified undergo NGS of the entire genomic regions of *ABCA4, BEST1, RDH12* and *PRPH2*. Preliminary sequence data of five patients show that the standard filtering yields approximately 130 variants per patient that require further investigation.

Conclusions: Causal variant V4 occurs in a high fraction of Belgian STGD1 patients and represents the first deep intronic founder mutation in *ABCA4*. This emphasizes the importance of augmented molecular genetic testing of *ABCA4* in Belgian STGD1 patients. Finally, more extensive resequencing in the remainder of the STGD1 patients without an identified second *ABCA4* mutation, will allow us to identify novel deep intronic and non-coding mutations.

Natural disease progression and symmetry in X-linked retinitis pigmentosa

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Background and Purpose: In the X-linked form of Retinitis Pigmentosa (XLRP), mutations in the Retinitis Pigmentosa GTPase Regulator (RPGR) and Retinitis Pigmentosa 2 (RP2) genes are causative for photoreceptor death and lead to blindness in affected patients. The purpose of this study is to analyse disease progression of XLRP, assess symmetry between eyes and determine which clinical endpoint has the lowest variability. This disease characterization lays the groundwork for future XLRP treatments by ascertaining the most effective time of intervention, the best form of treatment control and the outcome measure which best determines success of the treatment.

Materials and Methods: This retrospective, cross-sectional study comprises 51 male, genetically confirmed patients with mutations in the RPGR or the RP2 genes. Outcome measures included best corrected visual acuity (BCVA), refractive error, optical coherence tomography (OCT), perimetry, electroretinography (ERG), multifocal ERG (mfERG), tonometry and colour vision tests.

Results: We analysed 155 data points of BCVA (0.93 ± 0.12 logMAR; mean ± 95 % confidence interval), 101 of IOP (13.72 ± 0.52 mmHg), 109 of refraction (-3.05 ± 0.68 spherical equivalent) and 81 of foveal thickness (150 ± 12.26 µm). The degeneration measured with BCVA showed good symmetry between both eyes (R_2= 0.65), indicating that the non-treated eye can effectively be used as control in a gene therapy trial. BCVA (logMAR) and age of patient at time of examination was poorly correlated (best fit $R_{2\ cubic}$ = 0.17).

Conclusion: This study provides important evidence of the natural disease progression and symmetry between eyes of XLRP patients and thus enables effective planning of interventional trials.

Gene panel diagnosis for retinitis pigmentosa – Phenotypic characteristics of unresolved cases

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Purpose: Retinitis pigmentosa (RP) is a genetically and clinically heterogeneous disease. Numerous RP genes have been described, but in a considerable number of cases, no disease-causing mutations are detected in these genes. Potential reasons are deep intronic mutations, changes in regulatory regions, mutations in as yet unknown genes, or phenocopies of RP. To detect potential clinical differences between mutation-positive and -negative patients, we investigated the phenotype and associated diseases in a retrospective, observational clinical study.

Methods: To determine the underlying molecular changes in RP patients, targeted next-generation sequencing on an Illumina Hiseq1500 system was carried out for 60 arRP and 23 adRP genes after enrichment using NimbleGen sequence capture technology. Thirty six patients were analyzed in a tertiary referral center in Germany based on clinical and demographical aspects. All patients underwent standardized clinical examination and imaging, including spectral domain optical coherence tomography, wide field fundus autofluorescence imaging and fundus photography. To assess retinal function, best corrected visual acuity, electroretinography and visual field testing were performed.

Results: Pathogenic mutations where identified in 25 (69 %) out of 36 patients. Of the 11 patients without detected disease-causing mutation, only one had an affected family member and none had confirmed parental consanguinity, 6 showed no bone spicule pigmentation (RP sine pigmento) and 5 had an additional systemic autoimmune disease, such as hashimoto's thyroiditis or rheumatoide polyarthritis. None of these two clinical findings applied to any patient in the group defined by a specific genetic defect. Age of disease onset (first symptoms) was above 40 years in 7 out of 11 (64 %) patients without and 4 out of 25 (16 %) with a mutation in a known RP gene.

Conclusion: Phenotypic and demographic difference between RP patients might be used as a tool to stratify patients with and without detectable disease-causing mutations. The high rate of autoimmune diseases in patients without molecular genetic confirmation for a hereditary retinal disease points to a multifactorial disease process in a subgroup of RP patients.

Towards the functional characterization of the WD repeat domain protein WDR17 in the retina

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Purpose: Due to its abundant retinal expression and regulation by photoreceptor key transcription factor CRX, the gene encoding WD-repeat domain protein 17 (WDR17) is believed to play an important role in retinal physiology. In a first step towards the functional characterization of WDR17, we focused on its cellular localization and the elucidation of the β-propeller structure of its twelve WD repeat motifs.

Methods: Antibodies towards WDR17 peptides from the N-terminal part of mouse WDR17 were purified by affinity chromatography. Immunological methods were used to characterize the specificity of these antibodies. A series of different WDR17 deletion constructions were generated, overexpressed in mammalian cells and detected by immunocytochemistry.

Results: We showed that purified peptide antibodies specifically recognized WDR17 in cells overexpressing recombinant WDR17 and in Western blots of retinal extracts. The native protein in the retina, however was not labeled in immunohistochemical analyses. Immunocytochemical staining of cells overexpressing full-length WDR17 and WDR17aa1-762, containing all twelve WD repeat motifs as well as WDR17aa362-1322 with seven WD repeat motifs revealed a common punctual vesicle-like distribution in the cytoplasm. In contrast, heterologously expressed WDR17aa1-369 and WDR17aa1-393, both harboring the first five WD repeat motifs, and WDR17aa694-1322 with no WD repeat domains were found to be localized in the cytoplasm and nucleus in a net-like manner.

Conclusion: Our specific WDR17 N-terminal peptide antibodies represent a valuable tool for protein analysis by immunoblotting and immunocytochemistry, but are not suited for immunohistochemical studies. Amino acids 393-762, harboring seven of the twelve WD-repeat motifs in WDR17, are crucial for cytoplasmic localization of WDR17 and most likely fold into a classical β-propeller.

Conditional deletion of endothelial TGF-β signaling reduces severity of oxygen induced retinopathy

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Mice carrying an ocular deletion of TGF-β signaling develop a phenotype that essentially mimics the retinal changes seen in patients suffering from diabetic retinopathy (Braunger et al., American Journal of Pathology, in press). In the present study, we focused on the distinct role of endothelial TGF-β signaling in vascular repair following oxygen-induced retinopathy (OIR) as a model of retinopathy of prematurity.

To interrupt endothelial TGF-β signaling, we generated mice with a conditional deletion of the essential Tgfbr2 in endothelial cells and provoked neo-angiogenesis via oxygen-induced retinopathy (OIR). For this purpose, we crossed a Cdh5-CreERT mouse line carrying an endothelial specific Cre recombinase under a tamoxifen responsible promoter, with Tgfbr2fl/fl mice. The resulting Cdh5-CreERT Tgfbr2fl/fl mice (*Tgfbr2*$^{\Delta ec}$) were used as experimental mice and Tgfbr2fl/fl littermates served as controls. OIR was performed from postnatal day 7 to 12, followed by topical application of tamoxifen resulting in the activation of the cre recombinase. The successful deletion of Tgfbr2 in endothelial cells was confirmed by PCR and immunohistochemistry. FITC-Dextran perfused retinal whole mounts were used to visualize the retinal vasculature at P12, 15 and 17. Following OIR, the avascular zone was significantly smaller in *Tgfbr2*$^{\Delta ec}$ mice compared to littermate controls. Moreover, *Tgfbr2*$^{\Delta ec}$ mice showed a significant increase in the area of pre-retinal tufts and the deep vascular plexus compared to controls. In addition, we frequently observed cell-rich retinal microaneurysms in *Tgfbr2*$^{\Delta ec}$ mice.

Taken together, our results strengthen the hypothesis of a pro-proliferative, pro-migratory and pro-angiogenic phenotype following conditional deletion of Tgfbr2 in endothelial cells. Two distinct type I receptors can be activated in endothelial cells following binding of TGF-β to the TGF-β type II receptor (Tgfbr2): the EC-restricted type I receptor anaplastic lymphoma receptor tyrosine kinase (ALK1), which stimulates EC proliferation and migration, or the broadly expressed type I receptor ALK-5, which inhibits EC proliferation and migration. Therefore, our results might additionally indicate a Tgfbr2-ALK 5 dominated signal transduction of TGF-β signaling in retinal endothelial cells.

Evidence for *de novo* gene conversion underlying the occurrence of rare *OPN1LW/MW* exon 3 haplotypes associated with X-linked Cone Dysfunction Syndromes

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Purpose: Defects in the *OPN1LW/MW (LW/MW)* gene cluster can lead to Cone Dysfunction Syndromes such as Blue Cone Monochromacy (BCM). We investigated a new category of *LW/MW* mutations in subjects in which structural aberrations and known pathogenic point mutations were excluded. This third category is characterized by rare combinations of common SNPs in exon 3 of *LW/MW*. These rare haplotypes are thought to interfere with proper transcript processing and may originate from gene conversion between *LW* and *MW* genes.

Methods: We genotyped the LW/MW opsin gene cluster in patients from 19 independent families with a clinical diagnose of BCM or BCM-like cone dysfunction. Sequencing of *LW/MW* exon 3 revealed a variety of rare novel haplotypes. Minigene constructs carrying these haplotypes were generated and transiently expressed in HEK293 cells to evaluate transcript splicing. FAM-labeled RT-PCR products were separated by capillary electrophoresis for semi-quantitative analysis. Microsatellite markers were used to investigate integrity/recombination of the Xq28 region in the family in which gene conversion was assumed.

Results: A total of 13 different exon 3 haplotypes were found and their impact on splicing analyzed applying the minigene assay. The so-called 'LIAVA', 'MIAVA$^{c.465C}$', 'MIAVA$^{c.465G}$' and 'LVAVA' haplotypes (termed according to the composite amino acid variants), led to a high fraction of aberrantly spliced transcripts. Other haplotypes were less deleterious with intermediate or minor proportion of aberrantly spliced transcripts. We observed a family with distinct color vision phenotypes of the grandfather (deutan) and the grandson (BCM). We could establish that in this family the deleterious 'LIAVA' haplotype in the grandson's *LW* gene was introduced through a *de novo* gene conversion event in the grandfather's germline.

Conclusion: We established that a variety of rare *LW/MW* exon 3 haplotypes are associated with BCM or BCM-like cone dysfunction syndromes. These haplotypes induce partial or fully penetrant splicing defects. The eight nucleotide positions that constitute these haplotypes may interfere with several exonic splicing regulatory sequences of the opsin genes. Within a single pedigree, we demonstrated for the first time that a pathogenic exon 3 haplotype can occur in *LW* by gene conversion.

Effect of AMD patients sera on ARPE-19 cells in comparison to normal human serum

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Exposing RPE cells with normal human serum (NHS) leads to an increase in intracellular free calcium ($[Ca^{2+}]_i$), caused by active complement components. Altered systemic complement activity in sera of AMD patients has already been shown by different studies. Thus we investigated if exposing ARPE-19 cells with sera of AMD patients leads to a modified increase of $[Ca^{2+}]_i$ compared to NHS.

Using Ca^{2+} imaging, we investigated the effect of sera from 16 AMD-patients (age 72.1 ± 8.7 years; 12 female, 4 male) with known polymorphisms in the CFH and/or ARMS2 gene on human ARPE-19 cells. Differential gene expression was assessed by q-PCR.

Exposure to NHS and AMD patients sera led to a biphasic increase in $[Ca^{2+}]_i$, consisting of an initial peak and a subsequent phase of sustained Ca^{2+} increase. The response to sera of AMD patients showed a significantly reduced peak amplitude compared to NHS. However, the amplitude of the late phase and the ratio of late phase amplitude to peak amplitude revealed an accentuated late phase of $[Ca^{2+}]_i$ increase in AMD patients. Furthermore the late phase/peak ratio was significantly increased in smokers and increased with the number of risk alleles in the CFH and ARMS2 genes. Also the amplitude of the initial peak and the cumulative Ca^{2+} increase was significantly enhanced in patients with 3 or 4 risk alleles compared to those with 2 or less risk alleles. Stimulation with AMD patients sera for 24 h led to an upregulation of C5, C5a-receptor, CFH, CD55, CD59 and a significantly reduced C3-gene expression compared to stimulation with NHS.

For the first time we investigated the effect of AMD patients sera on ARPE-19 cells using Ca^{2+} imaging. We assume that the modified increase in $[Ca^{2+}]_i$ observed in ARPE-19 cells in response to AMD patients sera is caused by an altered complement profile in AMD patients, especially in smokers and those with risk alleles in the CFH and ARMS2 gene. Hence this modified increase in $[Ca^{2+}]_i$ could function as a biomarker for pathological complement profiles.

Activation of Erk1/2 signaling pathway by complement serum in UV-POS pretreated ARPE-19 cells

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Purpose: Retinal pigment epithelial (RPE) cells undergo functional changes upon complement stimulation, which play a role in the pathogenesis of age-related macular degeneration. These effects are in part enhanced by pre-treating ARPE-19 cells with UV-irradiated photoreceptor outer segments (UV-POS) *in vitro*. The aim of this study was to investigate the effects of human complement serum (HCS) treatment on p44/42 mitogen-activated protein kinase (ERK1/2) activation in ARPE-19 cells pretreated with UV-POS.

Methods: ARPE-19 cells were pretreated three times with 10 µg/ml UV-POS in 1 % FCS containing DMEM/F12 medium. Subsequently, cells were thoroughly washed and were stimulated with 5 % HCS or heat-inactivated HCS (HI-HCS) as a control in FCS-free medium for 24 hours. Total protein was analyzed for phosphorylated (activated) and non-phosphorylated ERK1/2 and for Bcl-2 protein expression by western blotting. Cell culture supernatants were analyzed for the concentration of IL-6, IL-8, MCP-1, and VEGF via ELISA. The content of reactive oxygen species (ROS) in the cell culture supernatants was determined photometrically by the dissolved end product of the reaction with nitroblue tetrazolium salt.

Results: The amount of phosphorylated Erk1/2 was increased in UV-POS pretreated ARPE-19 cells and especially after stimulation with HCS when compared to non-pretreated ARPE-19 cells incubated with HCS alone or HI-HCS. This observation was paralleled by the Bcl-2 expression. In contrast, the expression of non-phosphorylated Erk1/2 and the β-actin control did not differ between all treatment groups. Furthermore, a slight raise of ROS in cell culture supernatants of UV-POS pretreated ARPE-19 cells was detected. The ELISA data revealed the trend of an elevated production of IL-6, IL-8, MCP-1, and VEGF in response to HCS in both UV-POS pretreated and non-pretreated ARPE-19 cells.

Conclusions: Erk1/2 activation in ARPE-19 cells was induced by UV-POS pretreatment and further enhanced by combining UV-POS pretreatment with HCS stimulation. The effect of pronounced cytokine production could be attributed to HCS alone. Thus, we conclude that Erk1/2 does not represent the crucial signaling pathway mediating the functional changes of RPE cells in response to complement stimulation. Erk1/2 activation in ARPE-19 cells may represent a protective mechanism in response to cellular and oxidative stress inducing survival factors as Bcl-2.

Systemic influences on the integrity of the blood retina barrier: Diabetes and hypertension

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The retinal pigmented epithelium (RPE) plays an important role in maintaining the function of the photoreceptors and is the main component of the blood retina barrier (BRB). As the RPE is exposed to the choroid thus the blood stream, systemic influences might directly affect the RPE and therefore its function as part of the BRB. For a better understanding of the consequences of these influences in the RPE, we approached two scenarios: systemic hypertension and diabetes.

To investigate systemic hypertension we used a double transgenic rat (dTGR) model harboring both rat and human renin and angiotensinogen genes for diabetes we used a rat model with a tetracycline inducible knock-down of the insulin receptor (TetO). Age-matched Sprague-Dawley rats (SD) were used as controls. To assess RPE features, we used immunodetection (in eye whole-mounts and sections), scanning laser ophthalmoscopy (SLO) and quantitative PCR. Functional analysis was performed by GanzfeldERG

In the dTGR we observed a reduction in expression of tight junction protein Claudin-1 in the RPE whereas ZO-1, remained unchanged. In GanzfeldERG both the scotopic a-wave and the scotopic b-wave were significantly decreased in the dTGR animals, whereas the b/a-wave ratio remained unchanged. Thus the dTGR rats show a mild loss of photoreceptor function. The dTGR reveals very high local Ang2 levels, but we did not see any upregulation of Renin in the RPE.

In the TetO rat we observed abnormalities in cell size of the RPE and tight junction protein expression of ZO-1. SLO reveals focal atrophic RPE areas. GanzfeldERG showed a significant reduction of the a-wave and subsequently b-wave. b/a-wave ratio increased significantly, depicting a reduced photoreceptor function. Immunohistochemical analysis suggests an upregulation of renin in the RPE.

Systemic hypertension and diabetes affect the integrity of the RPE and thus the BRB. Our model for diabetes shows a more pronounced photoreceptor function reduction while in the hypertensive model, changes remain more subtle.

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Loss of acid sphingomyelinase activity causes changes in retinal microglial morphology and function in mice

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Background: Niemann Pick disease type A and B are lysosomal storage disorders which are caused by loss of function mutations in the acid sphingomyelinase (aSMase) encoding gene causing symptoms like hepatosplenomegaly and rapid neurodegeneration. Intracellular loss of aSMase activity in aSMase- knockout mice leads to accumulation of sphingolipids in lysosomes, especially in macrophages. Because microglial cells are critically involved in retinal health we aimed to determine the consequence of aSMase deficiency in these immune cells.

Methods: Spectral-domain optical coherence tomography (SD-OCT) and infrared reflectance (IR) were used to characterize the integrity of the aSMase-deficient retina. Furthermore, retinal whole-mount and cross- section analysis of aSMase-KO mice were performed to determine the localization and immune status of retinal microglial cells by ionized calcium-binding adapter molecule 1 (Iba1) and translocator protein (18kDa) (TSPO) immunostaining. Intracellular accumulation of lipids was determined by Nile red staining. Quantitative real-time PCR was used to measure inflammatory gene expression.

Results: Although SD-OCT showed no changes in architecture of retinal layers, IR fundoscopy revealed an increased number of hyperreflective spots in the ganglion cell layer of aSMase-deficient mice. Histological examination of Iba1-stained whole-mounts and retinal cross-sections showed an even distribution of microglia throughout the retina. However, we detected an increased microglial cell number with significantly enlarged cell bodies in the aSMase-deficient retina. Specifically, aSMase- decifient microglial cells showed enhanced proliferation and elevated expression of TSPO which points towards increased microglial reactivity. Additional qRT-PCR analysis revealed increased expression of the microglial reactivity markers CD68 and AMWAP in the aSMase-deficent retina. Microglial cells also displayed strong accumulation of lysosomal lipids as detected by Nile red.

Conclusion: These results suggest that aSMase deficiency and hence disturbed lipid metabolism affect the morphology and function of microglia in the retina. Elevated TSPO protein levels and increased expression of CD68 and AMWAP suggest inflammation in aSMase-defiecient retinae.

Use of laser capture microdissection coupled mass spectrometry to analyze the proteome of photoreceptor outer segments

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Purpose: The combination of laser capture microdissection (LCM) and liquid chromatography-mass spectrometry (LC-MS/MS) provides a means to study proteins that are specific for cell subpopulations or anatomic regions. This proof-of-concept study examined whether this approach can achieve separation of photoreceptor outer segments from the surrounding tissue and detect proteins characteristic to these specialized structures.

Methods: Eyes of wild-type mice were removed and fixed with cross-linking (10% buffered formalin) or denaturing (methanol or acetone) fixatives. We examined the ability of these commonly used fixative regimens to preserve both cell morphology and protein quality. Laser capture microdissection was used to isolate minute amounts of outer segments (~200,000 μm^3) from cresyl violet stained sections for subsequent proteomics analysis. Catapulted tissue patches were directly collected in CapSure® caps and either processed directly or subjected to alternating cycles of ambient to high pressure (up to 35,000 psi), in order to facilitate enzymatic digestions of samples. Mass spectrometry analysis was conducted after trypsinization of the samples.

Results: Methanol was the best fixative for tissue preservation, while acetone fixation increased the number of identified proteins substantially. Nevertheless, tissue morphology was inadequate for proper isolation of outer segments in this latter case. Formalin fixation gave satisfactory results in preserving tissue morphology and also in protein identification. Furthermore, this procedure also preserves tissue lipids for an optional parallel analysis. Using formalin fixation, more than 300 proteins of the outer segments were identified without barocycling. Importantly, using high pressure assisted sample preparation not only abridged processing, but vastly improved protein identification (>700).

Conclusions: LCM and molecular analysis from tissue specimens can be complex and challenging due to numerous issues related with the tissue processing and its impact on the integrity of biomolecules in the specimen. The results presented here suggest that the combined use of LCM and LC-MS/MS technologies may be effective in detecting protein profiles within the eye specific to a

Spatial characteristics of an AOSLO-based micro display for in vivo retinal function testing on single photoreceptor level

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Purpose: Adaptive optics scanning laser ophthalmoscopy (AOSLO) can resolve single photoreceptors in vivo. Combined with real-time eye tracking and correction for chromatic aberrations the AOSLO can be used as a microscopy platform to make single cells optically accessible for functional testing. Due to its scanning nature, visual stimuli can be encoded into the system's beam with high-speed acousto-optic modulation (AOM), thereby creating an acute visual display directly on a subject's retina. We here characterize the limits and possibilities of such a micro display for the studies of visual function on the level of individual receptor cells in the living eye.

Methods: Measurements were performed with a multi-wavelength AOSLO, with 840 nm for imaging and 543 nm light for stimulation. Since space and time are interlinked in a scanning system, spatial characteristics can be inferred by recording temporal beam intensity modulation. Modulation accuracy for benchmark visual stimuli (gratings, lines, dots, Gabor patches, complex gray scale images) was measured using a high speed Si analogue photo detector sampled at 1.25 – 5 Giga samples per second with a digital oscilloscope. A simple light capture model was used to calculate nominal light delivery under experimental conditions. Stimulus fidelity and visible contrast was validated psychophysically under foveal inspection when higher order aberrations were compensated for.

Results: The smallest full contrast stimuli presentable were on the order of 3 pixels across in raster scanning coordinates (≈ 150 ns). This corresponds to about 2 µm on the retina for the typical raster scanning excursion of 1.2 deg of visual angle. Optical modelling confirms that this size would be small enough to drive single cells in isolation. Maximum light intensity contrast for extended stimuli achieved in our setup was ~0.99 (Michelson, or about 150:1), a level that is limited by the extinction ratio of the acousto-optic device used for switching. Residual light leak through these switches produces background illumination levels (~4.3 cd/m^2 at 543 nm, around 4100 isomerizations per second) that are too bright to pick up rod photoreceptor contribution, thus AOSLO-based visual psychophysics is currently limited to cone photoreceptor responses.

Conclusion: AOSLO-based micro-stimulation is accurate enough to drive individual cone photoreceptors in the living eye, ultimately enabling a direct relationship between retinal structure and visual function on single cell level. This technique promises to be useful for a host of fundamental and clinical vision research applications.

Quantitative fundus autofluorescence in age-related macular degeneration and other retinal diseases associated with drusen

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Purpose: Lipofuscin accumulation in the retinal pigment epithelium (RPE) has been suggested to play a pathophysiological role in various monogenetic and complex retinal diseases including age-related macular degeneration (AMD) with direct evidence for this assumption in Stargardt disease. The aim of this study was to quantitatively measure lipofuscin-related fundus autofluorescence (AF) in early or intermediate AMD and other retinal diseases associated with drusen.

Methods: Fundus AF images were acquired with a modified scanning laser ophthalmoscope equipped with an internal fluorescent reference (modified Spectralis HRA-OCT, Heidelberg Engineering, Heidelberg, Germany). All patients were younger than 65 years of age. The disease spectrum included early or intermediate AMD, reticular pseudodrusen with or without geographic atrophy (GA), basal laminar drusen (BLD), colloidal drusen and membranoproliferative glomerulonephritis (MPGN) type 2. For every subject, the mean gray value of a defined circular region at an eccentricity of approximately 7° to 9° centered on the fovea was determined. The quantitative AF (qAF) value was calculated after adjustment to the reference, the optical magnification, the density of the ocular media, the laser offset and a device-specific correction factor. Data was compared to normative qAF values derived from 90 healthy subjects.

Results: Thirty-nine patients were investigated. Mean±SD age was 52±6 years (range: 35-61 years). All patients with early and intermediate AMD (n=7) revealed qAF levels within the 95 % confidence interval (CI) of age-matched controls. In patients with BLD (n=11), colloidal drusen (n=3) and RPD without GA (n=12), qAF levels were within the 95 % CI in 91 %, 67 % and 75 %, respectively. The remaining patients of these groups as well as all patients with GA (n=4) and the patient with MPGN type 2 (n=1) had qAF values below the age-matched 95 % CI. No patient showed a qAF value above the age-matched 95 % CI.

Conclusions: QAF measurements showed no increased lipofuscin-related fundus-AF in early or intermediate AMD or other diseases associated with drusen which may serve as model diseases for AMD. Lower qAF-levels in certain subgroups may point to subnormal lipofuscin concentration in the RPE or disease-specific limitations to detect true RPE lipofuscin content.

Derivation of retinal neurons and (retinal) pigmented epithelial cells from JNCL patients' iPSCs

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Introduction: Neuronal Ceroid Lipofuscinosis (NCL) is a group of progressive neurodegenerative lysosomal storage disorders. The most common form is the juvenile (JNCL or Batten disease), caused by a mutated version of *CLN3* gene. JNCL leads to retina degeneration, followed by CNS neurodegeneration and premature death. Interestingly, whereas in JNCL patients retina is affected first, retinal defects are not severe and appear only later in JNCL mouse models. Therefore, human cell based-models might enable new insight into pathomechanisms and therapy development.

Methods: JNCL hiPSC have been generated from fibroblasts of two JNCL patients and one healthy donor carrier for the common mutation. Taking advantage of protocols established in the last years, we investigated JNCL hiPSCs potential to differentiate into neuronal lineage, retina and (retinal) pigmented epithelium.

Result: Here we show that, following dissociation to single cells, JNCL hiPSCs efficiently reaggregated when plated onto low adhesion 96-wells plates. During the following days aggregates became more compact, acquired patterning (translucent/brighter vs darker areas) and formed potential optic vesicle(OV)-like epithelial evaginations. OV-like structures excised and cultured in retina maturation conditions continued to grow, developed pigmentation and expressed Rax eye-field transcription factor. After one month in culture we observed formation of neural retina (including ganglion cells [Brn3+] and photoreceptor precursor [Crx+]) and pigmented epithelial structures (potentially RPE or ciliary epithelium).

Conclusion: We conclude that JNCL hiPSCs can be used to obtain JNCL patients'-specific neural retina and pigmented epithelium samples. It has been already shown that JNCL iPSCs-derived neurons display progressive storage material accumulation and organelle abnormalities typical of JNCL disease. Because of its early retinal disease onset, progressive neurodegeneration and potential involvement of different retinal cell types, we hypothesize that iPSCs-derived retinal cells will be a great tool to model JNCL disease, study retinal cell specific pathomechanisms and possibly discover/validate therapies.

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A candidate gene association study identifies *DAPL1* as a female-specific susceptibility locus for age-related macular degeneration (AMD)

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Age-related macular degeneration (AMD) is the leading cause of blindness among white caucasians over the age of 50 years with a prevalence rate expected to increase markedly with an anticipated increase in the life span of the world population. To further expand our knowledge of the genetic architecture of the disease, we pursued a candidate gene approach assessing 25 genes and a total of 109 variants. Of these, synonymous single nucleotide polymorphism (SNP) rs17810398 located in *DAPL1* (death associated protein-like 1) was found to be associated with AMD in a joint analysis of 3,229 cases and 2,835 controls from five studies (combined $P = 1.15\times10^{-6}$, OR=1.332 [1.187-1.496]). This association was characterised by a highly significant sex difference ($P_{diff} = 0.0032$) in that it was clearly confined to females with genome wide significance ($P_{ADJ} = 2.62\times10\text{-}8$, OR = 1.541 [1.324-1.796]; males: $P_{ADJ} = 0.382$, OR = 1.084 [0.905-1.298]). By targeted resequencing of risk and non-risk associated haplotypes in the DAPL1 locus, we identified additional potentially functional risk variants, namely a common 897bp deletion and a SNP predicted to affect a putative binding site of an exonic splicing enhancer. We show that the risk haplotype correlates with a reduced retinal transcript level of two, less frequent, non-canoncical *DAPL1* isoforms. *DAPL1* plays a role in epithelial differentiation and may be involved in apoptotic processes thereby suggesting a possible novel pathway in AMD pathogenesis.

ARL3 regulates transport of prenylated and acylated proteins to photoreceptor outer segment in mouse retina

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Purpose: Based on in-vitro experiments, ARL3 (Arf-like protein 3) functions as a GDF (GDI displacement factor) for lipidated proteins. We generated photoreceptor- and retina-specific *Arl3* deletions to investigate the function of ARL3 with focus on trafficking of lipidated proteins.

Methods: We generated $Arl3^{+/GT}$ mice containing a EUCOMM gene trap (GT) in intron 1 of the mouse *Arl3* gene. Rod- and retina-specific *Arl3* conditional knockout mice were obtained by crossing with Flp-mice followed by iCre75 and Six3Cre transgenics. Photoreceptor function was assessed by ERG and optomotry, and progress of retinal degeneration among littermate mice was ascertained by optical coherence tomography, confocal immunohistochemistry and histology.

Results: ERGs and retina histology of PN15 rod-specific knockout mice were indistinguishable from those of the WT littermates, suggesting normal photoreceptor development. OCT revealed rapid photoreceptor loss in retinas of $Arl3^{flox/flox}$;iCre75+ mice after PN15. At PN20, scotopic ERG a-wave amplitudes were reduced 70–80 % with the photopic ERG unaffected. In retinas of one month-old knockout mice, scotopic ERGs were extinguished and cone ERGs were attenuated; histology showed 4-5 rows of nuclei in the ONL. The two month-old $Arl3^{flox/flox}$;iCre75+ retina was ~100 µm thinner and only one ONL row, presumably of cone nuclei, was present. Six3Cre-mediated knockout of *Arl3*, deleting ARL3 during embryonic retina development, revealed enhanced photoreceptor degeneration: PN15 scotopic and photopic ERGs are moderately attenuated and OCT at PN15 confirmed faster retina degeneration with much thinner retina of Six3Cre-mediated knockout than with iCre75. PN20 scotopic (80–90 %) and photopic (70-80 %) ERGs were reduced significantly; 4-5 rows of nuclei in the ONL are left. Immunohistochemistry performed using retina sections of PN15 and one month-old rod- and retina-specific knockout mice revealed that rhodopsin, GC1 and CNGA1/3 trafficking appear normal. In contrast, prenylated PDE6 and GRK1, and acylated transducin-α mislocalize in the IS and ONL.

Conclusion: Rod- and retina-specific knockout of *Arl3* revealed a rapidly-progressing rod degeneration followed by cone defects, resembling RP. Absence of ARL3 affected trafficking of peripheral membrane proteins, but not transmembrane proteins consistent with its function as a GDF.

Personalized medicine: PARP inhibition preserves photoreceptors in compound heterozygous *Pde6a* mutant retina genotype matched to human Retinitis Pigmentosa

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Purpose: Retinitis Pigmentosa (RP) relates to a heterogeneous group of blinding diseases, which collectively affect about 1 in 4000 individuals worldwide. The enzyme poly(ADP-ribose) polymerase (PARP) has recently been shown to be involved in the degeneration of photoreceptors in a variety of animal models for RP.

Methods: To test whether PARP was also responsible for photoreceptor loss caused by mutations in the *PDE6A* gene, we utilized two mouse models homozygous for the *V685M* and *R562W* mutation in *Pde6a*. Since homozygosity for mutant alleles is extremely rare in human RD patients, we also used compound heterozygous *Pde6a*^{V685M*R562W} animals, which were genotype matched to human RP.

Results: In all three *Pde6a* mutant situations, we found an excessive activation of PARP which correlated in time with the progression of photoreceptor degeneration. We then used organotypic retinal explant cultures treated with the PARP specific inhibitor PJ34 to confirm the causal involvement of PARP activity in the neurodegenerative process. The neuroprotective effect of PARP inhibition was evaluated for the different genetic insults, at different treatment time-points and durations, to establish an optimal treatment regime for later in vivo application.

Conclusion: Our results highlight PARP as target for neuroprotective interventions in RP caused by Pde6a mutations and are a first attempt towards personalized, genotype matched therapy development for RP.

Keywords: Retinitis Pigmentosa; *PDE6a* mutants; TUNEL; DAPI; PARP activity; Neuroprotection

Gene trapping of the Fam161a locus in mice leads to a unique ciliopathy phenotype

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Purpose: Mutations in the FAM161A gene were previously identified as the genetic cause for autosomal-recessive retinitis pigmentosa 28 and *in vitro* characterization analyses suggested a role of FAM161A at the photoreceptor connecting cilium. To study the functional role of Fam161a *in vivo,* we gene-trapped Fam161a GT/GT mice by disruption of the highly conserved UPF0564 domain.

Methods: Retinal Fam161a expression of Fam161 GT/GT and wild type mice was analyzed by Western blotting and RT-PCR. Retinal morphology and integrity was studied by light microscopy of semithin retinal sections and retinal function was assessed by ERG measurements. Microglial activation was determined by immunohistochemical analysis of retinal flatmounts. Morphology, function and ultrastructure of photoreceptors were studied by co-immunolabeling of ciliary proteins and electron microscopy.

Results: We confirmed the absence of full-length Fam161a protein in the retina of Fam161a GT/GT mice and showed weak immunoreactivity of a truncated Fam161a protein. Histological characterization demonstrated disorganized photoreceptor outer segments in young Fam161a GT/GT mice with the outer retina being completely degenerated at 6 months of age. Retinal degeneration was associated with profound microgliosis and electroretinography revealed an early loss of photoreceptor function in 4-months-old Fam161a GT/GT mice. Ultrastructural analysis of Fam161a GT/GT photoreceptor cells showed a remarkable phenotype with a significantly shortened connecting cilium, spread ciliary microtubules and amorphous disk organization. Co-immunolabeling experiments demonstrated reduced expression of centrin3 and disturbed localization of the Fam161a interactor proteins lebercilin and Cep290. Furthermore, Fam161a-gene trapping caused misrouting of the outer segment cargo proteins opsin and rds/peripherin 2 to the outer nuclear layer and photoreceptor synapses.

Conclusion: Our results suggest a critical role of Fam161a for the functional integrity of the photoreceptor connecting cilium. Fam161a is required for the molecular delivery of cargo to the outer segment cilium, a function which is essential for outer segment formation and ultimately visual function. We conclude that the Fam161a GT/GT mouse model may serve as valuable tool to develop genetic and immune-modulatory therapies for Fam161a-associated ciliopathy.

Anoctamin 4 is a bonafide Ca^{2+}-dependent non-selective cation channel

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Anoctamins (TMEM16) are a family of transmembrane proteins which are known to function as Ca^{2+}-dependent ion channels and scramblases. Anoctamin 1 and 2 (ANO1 and ANO2) have been identified as molecular correlate of Ca^{2+}-dependent Cl-channels. Anoctamin 6 has been shown to be involved in phospholipid scrambling. However the ion channel function of other anoctamins remains less well understood. Aim of the study is to investigate the channel function of anoctamin 4 (ANO4) in a heterologous expression system and the endogenous expression in the retinal pigment epithelium (RPE).

Using the whole-cell patch-clamp technique, currents of heterologously expressed ANO4 in HEK293 were recorded. ANO4 sequence was modified by site-directed mutagenesis. Ano4 expression in ARPE-19 cells and murine RPE was determined by immunohistochemistry. Scramblase activity was assessed by FACS analysis. ANO4 expression was knocked down in ARPE-19 by siRNA.

ANO4 expression in HEK cells resulted in Ca^{2+}-dependent non-selective cation currents that could be blocked by niflumic acid. The currents showed a weak Eisenmann I-VI selectivity for monovalent cations. ANO4 expression did not induce scramblase activity in HEK293 cells, neither under resting Ca^{2+} conditions nor after application of ionomycin. Using sequence homology analysis, we identified in the assumed pore region between transmembrane domain 6 and 7 of anoctamins a negatively charged amino acid in ANO4. In the same region ANO1 and 2 contain positively charged amino acids. To demonstrate that the membrane currents in transfected cells result from ion channel activity of ANO4, we induced two mutations in ANO4: exchanging the negative amino acid at position 775 into a neutral one (E-775-G) and a positively charged one (E-775-K). The E-775-G mutation did not show Ca^{2+}-dependent increase in conductance, whereas the E-ANO4-K variant led to Ca^{2+}-dependent Cl- channel currents. This indicates that the negatively charged amino acid, which we identified, is a major determinant for ion selectivity. We detected endogenously expressed ANO4 in the RPE of adult C57/Bl6 mice as well as in the human RPE cell line ARPE-19. SiRNA mediated knockdown of ANO4 led to a reduction of the endogenous expression of ANO4 about 50 % and of the cation conductance of ARPE-19 cells.

We identified ANO4 as a bonafide Ca^{2+}-dependent non-selective cation channel by using heterologous expression and site-directed mutagenesis. ANO4 is responsible for Ca^{2+}-dependent cation conductance in the RPE.

Depletion of microglia prevents vasoregression in a rat model of retinal neurodegeneration

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The transgenic polycystic-kidney-disease rat (PKD) develops neurodegeneration and subsequent vasoregression morphological similar to diabetic retinopathy. Prior to the vasoregression an activation of the innate immunity system is observed involving CD74-positive microglia. This project addressed the question whether microglial activation is a bystander effect or causally involved in vasoregression.
PKD-rats received an intravitreal injection of clodronate-coated liposomes (5mg/mL) at either 4 or 8 weeks of age to deplete retinal microglia. After 7 days animals were sacrificed and the retinas isolated. Untreated and sham-treated PKD-rats served as controls. For the quantification of microglia we performed immunofluorescence stainings for CD11b (total microglia), CD74 (activation marker) and Lectin in retinal whole-mount preparations. Acellular capillaries were quantified with retinal digest preparations. Gene expressions of cytokines and retinal activation markers were determined with Taqman gene expression analysis of whole retinal lysates.
At an age of 4 weeks clodronate injection reduced the total amount of microglia in the superficial capillary layer by 17 % ($p<0.05$). The amount of CD74 positive cells showed an 8.8 fold increase in the superficial layer ($p<0.01$) and a 50 % decrease in the deep layer ($p<0.05$). Clodronate injection reduced the number of acellular capillaries in the deep layer by 31 % at the age of 4 weeks ($p<0.01$) and by 28 % at the age of 8 weeks ($p<0.05$) without a significant affection of the superficial layer. Gene expression analysis showed an increase of CD11b by 15 % after clodronate injection at the age of 4 weeks ($p<0.05$). The proinflammatory cytokine IL-6 was decreased by 35 % ($p<0.05$) but the complement components complement factor 3 and complement factor B were significantly increased by 110 % and 72 % respectively ($p<0.05$).
We demonstrate that depletion of CD74-expressing microglia protects capillaries from degeneration, indicating an involvement of microglia in vasoregression. By which mechanisms microglia contribute to vasoregression remains unclear due to contrary results in gene expression changes. There is a downregulation of IL-6 but on the other side there is an upregulation of complement components which might be explained by the structure of the liposomes but needs to be further addressed.

Pre-treatment characteristics of patients with *PDE6A*-mediated retinitis pigmentosa

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Purpose: Mutations in the *PDE6A* gene-encoding the alpha-subunit of the rod cGMP-phosphodiesterase-account for 1–4 % of autosomal recessive retinitis pigmentosa(arRP) by impairing the regulation of cGMP levels in the rod outer segment. This study aims for a detailed clinical characterization of patients with *PDE6A* mutations in preparation of a clinical gene replacement study (phase I/II safety trial).

Methods: In this prospective observational study, we collected data from 24 eyes of 12 patients aged 22-79 (9f, 3m) with genetically confirmed homozygous or compound-heterozygous *PDE6A* mutations. Besides psychophysical tests (visual acuity, visual field, color vision, dark adaptation) a detailed electrophysiological examination was performed including Ganzfeld and multifocal ERG(mfERG). Furthermore, fundus photography, autofluorescence imaging and spectral domain OCT imaging was carried out for an in-depth morphological characterization.

Results: All patients with *PDE6A* mutations presented with typical features of arRP, however, depending on the genotype, severity of disease varied considerably. BCVA was 0.38 ± 0.15 logMAR (mean ± 95 % confidence interval; median 0.3) without significant refractive error (0 ± 0.43 spherical equivalent). The majority of patients (62 %) had severe visual field constrictions (≤ 10°) but significant heterogeneity was observed in the rate of progression over age, in some cases with a remarkably slow time course. A high degree of left to right eye symmetry was found with a higher correlation efficient for foveal retinal thickness (r = 0.87) than for visual acuity (r = 0.71). Although Ganzfeld ERGs were mostly extinguished, mfERGs could detect residual responses in the milder cases.

Conclusion: Mutations in the *PDE6A* gene cause arRP, but with highly heterogenous clinical disease courses depending on the genotype. These findings will be useful for the identification of patients concerning future therapeutic trials. Furthermore, the observed good intra-individual symmetry is highly relevant for any interventional trial as the second eye will be able to serve as an internal control.

Effects of cGMP accumulation on calcium dynamics of cone photoreceptor terminals

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Purpose: Cone photoreceptors are the main source of human sight, enabling color and high-resolution daylight vision. In inherited retinal diseases, loss of cones may occur primarily or secondarily, depending on whether the initial genetic defect affects cone or rod photoreceptors. It has been speculated that cone degeneration may be caused by a cytotoxic overload of Ca^{2+}. Here, we set out to study cone Ca^{2+} dynamics in degenerating cones using 2-photon imaging and transgenic mice with cone-specific expression of the HR2:1-TN-XL Ca^{2+} biosensor *(Wei et al.,* J Neurosci. 32:6981–94; 2012).

Methods: Ca^{2+} imaging was performed on wild-type/TN-XL mice, as well as on *cpfl1/TN-XL* and *rd1/TN-XL* crossbred animals. Different staining techniques (TUNEL assay, cGMP immunofluorescence, calpain activity assay) were used to further characterize cone degeneration.

Results: Abnormal cGMP increases in cones, a presumed telltale for Ca^{2+} accumulation, occurred only during primary, but not during secondary cone degeneration. At post-natal day 30, genetically intact cones in *rd1/*TN-XL retina showed no Ca^{2+} response when stimulated by light, while a substantial number of mutant *cpfl1/*TN-XL cones still displayed light responses. Accordingly, significant difference in relative Ca^{2+} levels distribution was detected between *cpfl1/*TN-XL and *rd1/*TN-XL cones. Currently we perform calibrated Ca^{2+} measurements to evaluate absolute cone Ca^{2+} levels in the three mouse lines.

Conclusions: The HR2.1: TN-XL Ca^{2+} biosensor mouse line allows studying cone Ca^{2+} regulation, not only with pharmacological manipulations but also in genetic mutants. The observed light-evoked responses in *cpfl1/*TN-XL suggest residual cone PDE6 activity. Our preliminary data suggest dysfunctional Ca^{2+} regulation during primary (*cpfl1*) cone degeneration. Therefore, modulation of Ca^{2+} signaling may be a suitable target for neuroprotection therapy in primary cone degeneration.

The inhibition of soluble epoxide hydrolase protects retinal vasoregression via Notch signaling pathway

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Background: Retinal degeneration is accompanied by glia activation. Glial cells play an important role in the cross-talk between neurons and vascular cells. The impaired neurovascular coupling results in retinal neurodegeneration and vasoregression. Müller glia undergo reactive gliosis following acute injury or chronic neuronal stress [1]. Inflammatory responses, in particular microglia, are involved in retinal degeneration [2–3]. Recruitment and activation of microglia are regulated by Notch signaling and lipid metabolic products such as docosahexaenoic acid (DHA) [4-5]. Additionally, retinal vessel maturation and quiescence as well as mural cell recruitment are linked to Notch signaling [6]. Recently, a critical role of sEH plays a critical role in regulation of vascular formation and inflammatory biology was described. Moreover, ω-3 polyunsaturated fatty acids (PFA) such as docosahexenoic acid (DHA) can attenuate early retinal degeneration [7]. However, the role of sEH in retinal vasoregression is still unclear.

Aim: We want to know if sEH inhibition can prevent retinal vasoregression in rats.

Methods: The transgenic polycystic kidney disease (PKD) rat is a model of ciliopathy, which expresses a truncated human polycystin-2 gene. Male homozygote PKD rats were daily treated with intraperitoneal injection of a sEH specific inhibitor (30mg/kg) for four weeks. Retinal digestion preparation was used for quantitative morphometry. sEH expression and microglial activation were quantified upon using cryosection and whole mount immunofluorecent staining, respectively. sEH activity was measured using a mass spectrometer. Inflammatory and immune-related cytokines were detected using real time PCR. Activation of the Notch signaling pathway was analyzed using western blotting.

Results: sEH was upregulated in Müller cells of PKD rats. Retinal vasoregression was prevented by sEH inhibition. sEH inhibition was able to effectively reduce retinal pericyte loss and to repress pericyte migration. sEH inhibition decreased the activation of microglia and altered microglial distribution in PKD rats. The inflammatory cytokines were down-regulated. Moreover, Notch signaling pathway was involved in regulation of microglial recruitment.

References:

1. *Vogler, S., et al., Muller cell reactivity in response to photoreceptor degeneration in rats with defective polycystin-2. PLoS One, 2013. 8(6): p. e61631.*
2. *Mirza, M., et al., Progressive retinal degeneration and glial activation in the CLN6 (nclf) mouse model of neuronal ceroid lipofuscinosis: a beneficial effect of DHA and curcumin supplementation. PLoS One, 2013. 8(10): p. e75963.*
3. *Zeng, H.Y., W.R. Green, and M.O. Tso, Microglial activation in human diabetic retinopathy. Arch Ophthalmol, 2008. 126(2): p. 227-32.*
4. *Outtz, H.H., et al., Notch1 controls macrophage recruitment and Notch signaling is activated at sites of endothelial cell anastomosis during retinal angiogenesis in mice. Blood, 2011. 118(12): p. 3436-9.*
5. *Lu, D.Y., et al., Docosahexaenoic acid suppresses neuroinflammatory responses and induces heme oxygenase-1 expression in BV-2 microglia: implications of antidepressant effects for omega-3 fatty acids. Neuropsychopharmacology, 2010. 35(11): p. 2238-48.*
6. *Ehling, M., et al., Notch controls retinal blood vessel maturation and quiescence. Development, 2013. 140(14): p. 3051-61.*
7. *Ebert, S., et al., Docosahexaenoic acid attenuates microglial activation and delays early retinal degeneration. J Neurochem, 2009. 110(6): p. 1863-75.*

Age-related maculopathy susceptibility protein 2 (ARMS2) is expressed in human monocytes and microglia cells

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Background: The formation of drusen at the macula and the degeneration of RPE cells is characteristic for AMD and represents the most common cause of blindness in developed countries. The *ARMS2* variant at 10q26 (A69S, rs10490924) has been repeatedly significantly associated with AMD. This polymorphism is linked to a mutation in the 3' untranslated region in the *ARMS2* gene, which introduces an instability motif into the transcribed mRNA and subsequent protein deficiency.

Methodes: 56 patients with neovascular AMD were sequenced and the polymorphisms rs2736911, rs10490924 or del443ins54 in *ARMS2* were evaluated. Monocytes isolated from whole blood of patients with defined genotypes, were stained with ARMS2 antiserum and ARMS2 expression was followed by laser scanning microscopy. Furthermore ARMS2 protein expression and location was evaluated in human retinal sections from *ARMS2* genotyped patients.

Results: We identified *ARMS2* expression in human blood derived monocytes by gene expression and laser scanning microscopy using ARMS2 specific antiserum. Expression of ARMS2 in monocytes, as well as microglia cells was confirmed by co-staining of retinal sections with ARMS2 antiserum and CD68, a specific marker for monocytic cells. Interestingly ARMS2 was absent in monocytes derived from AMD patients homozygous for the ARMS2 risk variant (A69S, rs10490924). Also retinal sections from individuals homozygous of the *ARMS2* risk variant lacked ARMS2 reactivity. Thus, the risk variant of *ARMS2* leads to ARMS2 protein deficiency in monocytes and microglia cells.

Kinetics of visual acuity loss in patients with foveal sparing geographic atrophy due to AMD

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Purpose: To investigate visual acuity loss in patients with a "foveal-sparing"-shape geographic atrophy (GA) secondary to Age Related Macular Degeneration

Methods: Patients from the prospective natural history "Fundus-Autofluorescence imaging in Age-related Macular Degeneration" study (FAM, NCT00393692) were examined longitudinally with fundus autofluorescence (FAF), and near-infrared reflectance (IR) imaging (Spectralis HRA+OCT or HRA2, Heidelberg Engineering). Eyes with a contiguous well-demarcated area of GA either in a complete ring around the spared fovea or in a horseshoe pattern >270° encompassing the fovea were included in the analysis. Areas of foveal sparing and GA were measured using a semi-automated software tool allowing for combined IR- and FAF-image grading (RegionFinder™, Heidelberg Engineering). Best corrected visual acuity (BCVA) was acquired using ETDRS-charts. Changes in foveal sparing area and BCVA were analyzed using a linear mixed-model approach. To investigate minimal areas of spared fovea that were required to hold certain visual acuity maximum likehood estimations (MLE) were performed for undercutting defined BCVA levels.

Results: A total of 42 eyes (35 patients, 29 female, mean age at baseline: 72.79±7.89 y) were examined longitudinally over a period of 25.8±18.7 months. During the review the size of the intact foveal island decreased from 1.54±0.86 mm^2 at baseline to 1.01±0.69 mm^2, and BCVA decreased from 0.36 ±0.29 logMAR to 0.50±0.31 logMAR units. In nine eyes, BCVA remained stable over the entire observational period. Modelling changes in BCVA in all patients with a linear mixed-effects model using time and sparing size as fixed effects revealed a decrease of BCVA by 0.08 logMAR per year and 0.05 logMAR per mm^2 loss of spared fovea. Highest values for MLE were obtained for undercutting a BCVA of 0.2 logMAR occurring when sparing size falls below 1.2 mm^2. Odds-ratio to decrease below a BCVA of 0.2 was 3.3 for a sparing sizes of <1.2 mm^2.

Conclusions: The results demonstrate a dependency of BCVA on the size of the residual fovea island. A foveal sparing area of 1.2 mm^2 appears to be necessary to maintain good visual acuities of <0.2 logMAR. The results indicate that there is no clear critical size for the maintenance of lower BCVA. These natural history data may be helpful in the design of future interventional clinical trials in affected individuals with GA.

IFNß-treatment as a therapy targeting microglia in a murine model of retinal degeneration

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Purpose: Age-related macular degeneration (AMD) is a leading cause of vision loss in the elderly. Typical hallmarks of AMD are chronic activation of the innate immune system and reactive microglial cells in the retina. Here, we analyzed the role of interferon beta (IFNß) signaling and the effect of IFNß therapy on microglial activation and choroidal neovascularization in a murine model of AMD-like retinal damage.

Methods: Laser-rupture of Bruch's membrane was used as a murine model for AMD. Retinal inflammation and choroidal neovascularization (CNV) were analyzed in IFN-alpha/beta receptor knockout ($IFNAR^{-/-}$) mice, IFNß-treated C57BL6/J mice and C57BL6/J wild type controls using fundus fluorescein angiography (FFA), lectin staining and optical coherence tomography (OCT). Microglial morphology in laser-induced lesions was analyzed by Iba1 and Tspo staining of flat-mounted retinas and retinal pigment epithelia (RPE).

Results: Laser-induced lesions in $IFNAR^{-/-}$ animals showed increased vessel leakage as well as CNV compared to control animals, indicating that $IFNAR^{-/-}$ deficiency enhanced inflammation. In contrast, IFNß-treated animals showed reduced vessel leakage and CNV compared to untreated controls. OCT-analysis of IFNß-treated and untreated wild type mice 7 and 14 days after induction of the retinal damage revealed diminished edema formation in IFNß-treated animals. Immunohistological analysis of flat-mounted laser-damaged retinas displayed both, a higher number and a longer presence of activated microglial cells at the sites of damage in $IFNAR^{-/-}$ mice compared to controls. The amount of activated microglia cells in IFNß-treated animals was lower than in respective control groups. Iba1 staining of flatmounted RPE revealed microglial cells in the subretinal space of laser-treated $IFNAR^{-/-}$ and C57BL6/J wild type animals but not in laser- and additionally IFNß-treated mice.

Conclusion: Knockout of IFNAR leads to enhanced retinal inflammation and microglial reactivity. In contrast, IFNß therapy significantly prevented vessel leakage, CNV and microglial activation. We conclude that IFNß signaling dampens microglial reactivity and is a protective mechanism in retinal degeneration.

Biophysical and biochemical characterization of two novel GCAP1 mutants associated with cone-rod dystrophy

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Purpose: Guanylate Cyclase Activating Protein 1 (GCAP1) is a Ca^{2+}-sensor protein involved in the regulation of Guanylate Cyclase (GC) during the phototransduction cascade, which initiates the visual process. An increasing number of GCAP1 mutants has been found to be associated with degenerative retinal diseases such as cone dystrophy (COD) and cone-rod dystrophy (CORD). This study is focused on the structural and functional characterization of two novel CORD-associated GCAP1 mutants, namely I107T and L84F.

Methods: Circular dichroism spectroscopy was employed to investigate changes in protein secondary and tertiary structure and in thermal stability both in the absence and in the presence of physiological 1 mM Magnesium (Mg^{2+}) and saturating Ca^{2+} concentration. Variations in hydrodynamic radius of the mutants in the aforementioned conditions was monitored by dynamic light scattering. Ca^{2+}-binding constants were estimated by a chromophoric chelator assay. The conformational transition range upon Mg^{2+} or Ca^{2+} binding was investigated by monitoring the tryptophan fluorescence in titration experiments.

Results: I107T-GCAP1 exhibited similarities with the wild type in terms of conformational and hydrodynamic radius changes upon Mg^{2+} or Ca^{2+} binding, while its Ca^{2+} affinity was severely impaired and its stability was increased independently on the presence of Ca^{2+}. Ca^{2+} fluorescence titrations showed a biphasic pattern similar to the COD-associated G159V mutant. L84F-GCAP1 showed structural features significantly different from the wild type, with small differences in secondary structure but major differences in tertiary structure upon Ca^{2+} binding. Moreover this mutant showed higher thermal stability than the wild type particularly in the presence of Ca^{2+} and appeared to be oligomeric both in the presence and in the absence of Ca^{2+} or Mg^{2+}.

Conclusion: Our results suggest that these two novel CORD-associated GCAP1 mutants could affect GC regulation via different processes. Indeed I107T-GCAP1 might alter the Ca^{2+} regulation of GC by its impaired Ca^{2+}-sensitivity, while L84F-GCAP1 may lead to different supramolecular assemblies as a consequence of its oligomeric state.

Retinal in vivo imaging of anti-VEGF antibodies in an animal model

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Purpose: To investigate fluorescent molecular probes linked to antibodies directed against VEGF for retinal in vivo imaging of VEGF.

Methods: Bevacizumab (monoclonal antibody against $VEGF_{165}$), B20-4.1.1 (polyclonal antibody against $VEGF_{165}$ and $VEGF_{164}$) and AF564 (polyclonal antibody against $VEGF_{164}$) were conjugated with a novel indocyanine green label (6S-ICG maleimide). Binding properties were assessed by BIAcore and proliferation assays. Molecular in vivo imaging was performed in rats that had undergone argon laser photocoagulation to induce choroidal neovascularization. Retinal uptake and fluorescence were recorded following injection of the dye conjugates. Distribution and accumulation of the probes were determined by immunohistochemistry and flow cytometry analysis.

Results: Comparable affinities of $VEGF_{165}$ and $VEGF_{164}$ to labeled bevacizumab were measured by BIAcore. Antibody B20-4.1.1 was able to bind VEGF from both species. Antibody labeling with 6S-ICG maleimide showed no influence on binding properties. In vivo imaging showed a strong fluorescence immediately following injection. After 24 hours, a general observation after probe application was both the occurrence of fluorescence within the laser lesions (choroid) and multiple hyperfluorescent spots (retina).Over time, a continuous decrease of fluorescence intensity was observed for all probes. Bevacizumab-6S-ICG showed longest accumulation within the laser lesions and highest number as well as longest duration of detectable fluorescent spots. Lowest values were recorded for AF564-6S-ICG. Immunohistochemistry showed double-staining of fluorescent spots with macrophages and/or microglia cells. Analysis of cell sorting by flow cytometry suggested double staining of bevacizumab-6S-ICG with retinal microglia cells.

Conclusions: Ex vivo analysis demonstrated successful binding of antibody conjugates with ICG while affinity to VEGF isoforms and cell viability were both preserved. Pharmacokinetics of fluorescent-labeled anti-VEGF probes can be investigated in vivo following intravenous and intravitreal injection. Strong accumulations within the laser lesions were observed for all antibody-conjugates. Also fluorescent spots were visible that might represent macrophages and/or microglia cells. This novel molecular imaging approach of VEGF may be applicable in patients for earlier diagnosis and more refined individualized anti-VEGF therapies with the aim of optimizing functional outcomes.

Bestrophin 1 is indispensable for volume regulation in human retinal pigment epithelium cells

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Background: BEST1 encodes an integral membrane protein, specifically expressed in the RPE. Mutations in the gene have been associated with several maculopathies including Best vitelliforme macular dystrophy (BVMD) which is characterized by striking lipofuscin accumulation in the central retina and abnormalities in the electro-oculogram. To clarify the functional role of BEST1, we have focused on RPE cell culture models established via human induced pluripotent stem cell (hiPSC) technology.

Methods: hiPSC cells from normal probands but also from patients with pathologic mutations on BEST1 were generated and differentiation to RPE (hiRPE) by standard methods. RNA and protein expression was analyzed by immunohistochemistry. Whole-cell patch-clamp recordings in hiRPE cells were done upon hypotonic cell swelling.

Results: Immunostaining of control hiRPE cells confirmed correct BEST1 localization to the basolateral plasma membrane, whereas mutant protein appeared reduced (BEST1-Q238R) and/or mislocalized (BEST1-A243V). BEST1 showed highest RNA expression in hiRPE cells relative to other known calcium- or volume-activated chloride channels. Upon cell swelling, whole-cell patch-clamp recordings in hiRPE cells exhibit an outwardly rectifying chloride current with characteristic functional properties of a volume-regulated anion channels (VRACs). This current is severely reduced in patient-derived hiRPE cells carrying mutant BEST1.

Conclusion: The identification of BEST1 as an essential component of VRAC in human RPE has led us to propose a scenario where VRAC is not a ubiquitous channel (e.g. composed solely of LRRC8 isoforms), but instead is a cell type- or tissue-specific complex likely composed of subunit others than LRRC8.

Monoallelic *ABCA4*-mutations are not sufficient to cause retinopathy

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Background: Bi-allelic mutations in the *ABCA4* gene cause Stargardt disease and cone-rod dystrophy. A very early finding in *ABCA4*-related retinopathy is a diffuse increase of lipofuscin in the retinal pigment epithelium (RPE), which can now be assessed using quantitative fundus autofluorescence (qAF) imaging. We used this sensitive technique to investigate if mono-allelic *ABCA4* mutations would result in a retinal phenotype.

Methods: Individuals with mono-allelic *ABCA4* mutations were selected by investigating genotyped parents (n = 23) of patients with retinal disease due to bi-allelic *ABCA4* mutations. Fundus AF images were acquired with a scanning laser ophthalmoscope (Spectralis HRA-OCT, Heidelberg Engineering, Heidelberg, Germany) equipped with an internal fluorescent reference. For every subject, the mean gray value of a circular region at an eccentricity of approximately 7° to 9° centered on the fovea was determined. The qAF value was calculated after adjustment to the reference, the optical magnification, the density of the ocular media, the laser offset, and a device-specific correction factor. Data were compared to normative qAF-values derived from 90 healthy subjects.

Results: In each parent, segregation analysis revealed one of the two pathogenic mutations identified in the index patient. The parents' mean ± SD age was 52 ± 8.3 years. The spectrum of mutations included frequent missense mutations (p.Gly1961Glu, p.Gly863Ala), and 7 out of the 23 parents carried a null mutation. All index patients with bi-allelic mutations revealed qAF intensity measures above the age-related 95 % confidence interval (CI) of the control group. All parents with mono-allelic *ABCA4* mutations showed normal findings on ophthalmoscopy, OCT- and conventional fundus AF-imaging. Lipofuscin-related qAF levels were not significantly different between carriers of mono-allelic *ABCA4* mutations and controls, independent from the type of mutation. QAF levels were within the age-adjusted 95 % CI of normal controls in 19 subjects, and were slightly above or below this range in 1 subject each.

Conclusions: Absence of a phenotype based on qAF in parents of patients with bi-allelic *ABCA4* mutations makes it unlikely that mono-allelic *ABCA4* mutations are disease causing. The finding that one normal *ABCA4* allele is sufficient to prevent retinal disease may be important for the required transduction efficiency in gene therapy for *ABCA4*-related retinopathy.

A European young investigators network for Usher syndrome

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Purpose: The Usher syndrome (USH) is the most common form of inherited deaf-blindness. It is a complex disorder divided into three clinical types, which are genetically heterogeneous, making diagnosis and treatment challenging. Molecular analyses revealed that all USH1 and 2 proteins are organized in protein networks in the eye and inner ear; however the exact pathomechanisms in the eye remain unclear. EUR-USH is a collaboration of scientists with backgrounds in medicine, genetics, cellular and molecular biology aiming to combine their expertise to provide new insights towards the understanding of USH.

Methods: We devolved an USH patient database for data comprising state-of-the-art clinical examination and molecular analyses (e.g. next generation sequencing), analysed the disease mechanisms and evaluated gene-based therapy options for the retinal degeneration, namely exon-skipping and read-though of nonsense mutations.

Results: In component A, we have compiled a multinational clinical protocol for a prospective observational USH cohort study. The data of the clinical examinations and molecular analyses are uploaded in the EUR-USH database. We analysed 41 DNA samples (24 families, 29 USH patients). We identified the two causative USH mutations in 55 % of patients (62.5 % of families). In component B, we validated USH protein expression and interaction of USH proteins with different imaging methods in human donor and murine retinas. Within component C, we generated an *ush2a* mutant zebrafish for the development of exon-skipping therapy for two frequently found mutations in *USH2A* patients and tested antisense oligonucleotides for redirecting splicing of *USH2A* pseudoexon40. Furthermore, we detected read-though of nonsense mutations in *USH1C*, *USH1G* and *USH2A*. Comparing the biocompatibility of the designer aminoglycosides NB84, NB124 and Ataluren in murine organotypic retina cultures revealed good biocompatibility for all compounds tested.

Conclusion: The current EUR-USH consortium has established an interdisciplinary collaborative network. We have shown significant potential towards improving early diagnosis, unravelling pathogenesis and potentially delivering novel therapies for USH patients.

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Activated human microglia increase pro-inflammatory mediators and affect cytoskeleton organisation in ARPE-19 cells

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Background: Inflammatory processes play a crucial role in the development of age related macular degeneration (AMD), which is a main cause for legal blindness of the elderly in industrialized nations. Here, we studied the role of reactive human microglia on retinal pigment epithelial (RPE) cell physiology.

Methods: Human induced pluripotent stem cell derived microglia (iPSdM) were stimulated with Lipopolysaccharide (LPS) to induce pro-inflammatory activation. The conditioned supernatant was collected and added to human ARPE-19 cells for 24 h. Thereafter, expression levels of several pro-inflammatory mediators were analyzed by qRT-PCR. Moreover, the morphology and cytoskeleton organisation of ARPE-19 cells were analyzed by phalloidin-TRITC staining and fluorescence microscopy.

Results: Transcript levels of the pro-inflammatory markers IL-6, IL-8, IL-1β, IL-18, CCL-2, GM-CSF, IFN-γ, Cybb and TSPO increased in ARPE-19 cells after treatment with conditioned medium from LPS activated iPSdM cells.
ARPE-19 cells, that were stimulated with LPS activated iPSdM conditioned medium, showed bloated intracellular structure and a dysmorphic cytoskeletal network.

Conclusions: ARPE-19 cells treated with conditioned medium from activated microglia showed an increased pro-inflammatory response and exhibited a characteristic appearance of stress response.
We conclude that microglial cells may significantly impair RPE function and structure and perpetuate the inflammatory response of the RPE.

A novel *ex vivo* assay to evaluate the role of Müller cells in retinal drug delivery

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Purpose: We aim to develop a bovine *ex vivo* retinal model that closely mimicks the human ocular physiology by maintaining the vitreoretinal barrier intact. This would allow us to assess if Müller cells represent a gateway for intravitreally injected nanomaterials into the retina. At the same time, it could be applied as a model to evaluate the influence of particle physicochemistry on their ability to cross the vitreoretinal interface.

Methods: ***Model set-up:*** fresh bovine eyes are obtained from the local slaughterhouse and kept ice cold during transport. After removing extraocular tissue and the anterior part of the eye, the retina – with vitreous attached – is detached from the retinal pigment epithelium and placed on a Transwell® filter with the vitreous side facing up. Neurobasal medium supplemented with 10 % FBS is added below the filter after which the explant is incubated at 37 °C and 5 % CO_2. ***Staining:*** After explant isolation, the explant medium is supplemented with 5 µM Mitotracker Deep Red to selectively stain Müller cells and 20 µg/ml of FM 1-43 lipid dye. In addition, MitoTracker Deep Red (20 µM), FM 1-43 (20 µg/ml) and carboxylated polystyrene particles are intravitreally injected and incubated overnight to allow diffusion from above. ***Imaging:*** A z-stack of confocal images (Nikon C1si confocal microscope) of the retina is recorded using a Nikon 60x NIR Apo water dipping objective that is brought into contact with the vitreous layer on top of the retina.

Results: The Mitotracker staining of the retinal explant confirms that the ganglion cell layer is virtually completely occupied by Müller cell endfeet. The lipid dye FM 1-43 stains the cell membranes and thus outlines the Müller cells in this layer. In addition, it clearly distinguishes veins running through the retina. We further confirm that, in contrast to rodent species, the vitreoretinal interface of bovine eyes greatly resembles the human physiology. Following intravitreal injection of polystyrene particles we found that some colocalize with Müller cells, suggesting that these cells could form a transport route across the retina. However, the distance of injection from the retina and nanoparticle concentration should be optimized.

Conclusion: A novel bovine *ex vivo* retinal model was developed that, in contrast to existing models, keeps the vitreoretinal barrier intact, which is highly relevant for drug delivery via intravitreal administration. We suggest that this model can serve as a set-up to aid in the evaluation and design of ocular nanomedicines with the retina as their target.

Retinoschisin and its influence on the ion pump function of the Na/K-ATPase

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Background: X-linked juvenile retinoschisis (XLRS), a macular dystrophy predominantely affecting males, is caused by mutations in the *RS1* gene. Expression of the *RS1* gene is limited to the retina, where its protein product retinoschisin is secreted from and binding to photoreceptor cells. The role of retinoschisin in the molecular pathology of XLRS is yet to be elucidated although it was shown that the protein is crucial for maintaining the structural integrity of the retina (Weber et al., 2002). In previous work, the retinal Na/K-ATPase consisting of subunits ATP1A3 and ATP1B2 has been identified as a direct interaction partner of retinoschisin, facilitating its anchorage to the plasma membrane (Molday et al., 2007, Friedrich et al., 2011). The aim of this work is to investigate whether retinoschisin binding influences the functionality of the Na/K-ATPase. Specifically, here we focus on the ATP-dependent ion transport activity of the Na/K-ATPase.

Methods: Plasma membranes isolated from wildtype and *Rs1h*$^{-/Y}$ mice at postnatal day 8 as well as membranes from a human retinoblastoma cell line (Weri-Rb) were subjected to an enzymatic assay to assess the effect of retinoschisin on the Na/K-ATPase catalyzed ATP hydrolysis. To examine whether retinoschisin influences the active transport of Na^+ and K^+ ions by the Na/-ATPase *Xenopus leavis* oocytes are established as a cellular model system. Oocytes are injected with cRNA encoding ATP1A3, ATP1B2, and retinoschisin. Two Electrode Voltage Clamp is used to record stationary K^+-dependent currents as a measure for ATPase ion pump function.

Results: The presence of retinoschisin did not affect the Na/K-ATPase mediated ATP hydrolysis in membranes from murine retinae or Weri-Rb cells. It also had no effect on the Ouabain mediated inhibition of the Na/K-ATPase catalyzed release of inorganic phosphate. *Xenopus leavis* oocytes expressing ATP1A3 and ATP1B2 after cRNA injections were measured for stationary K^+ currents. Co-expression of retinoschisin and its influence on K^+-dependent currents is ongoing.

Conclusion: The current results do not suggest an influence of retinoschisin on the ATP cleavage by the Na/K-ATPase. Data about its impact on the ion transport activity of the Na/K-ATPase will be available. Further studies, addressing other known functions of the Na/K-ATPase including steroid hormone mediated signal transduction and intercellular adhesion, as well as the effect of retinoschisin on Na/K-ATPase localization and turnover will also be performed. These attempts shall elucidate the effect of retinoschisin (deficiency) on the functionality of the retinal Na/K-ATPase and the influence of this interaction on retinal integrity.

Alteration of serum lipids in late age-related macular degeneration

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Purpose: Age-related macular degeneration (AMD) has a multifactorial etiology that includes genetic and environmental factors. Its early form is characterized by accumulation of extracellular deposits (drusen) under the retinal pigment epithelium (RPE). These drusen contain lipid particles with a particular composition of phospholipids, cholesteryl esters, and apolipoproteins. Several lipid-related genes have been associated with AMD risk, such as apolipoprotein E and hepatic lipase LIPC, and high dietary intake and plasma concentration of n-3 polyunsaturated fatty acids have been associated with a reduced risk for AMD. Analysis of cholesterol (total, HDL and LDL fractions), triglycerides, and total phospholipid contents in serum samples showed contradictory results in several studies. None of the earlier studies included an analysis of sphingolipids, glycerophospholipids and cholesteryl esters (CE). Here, we carried out a comparison of serum lipid classes and species between late AMD stages and healthy controls. Our aim was to find specific alterations in circulatory lipids that may be associated with the pathology of the disease.

Methods: Serum samples from controls (n=96) and AMD patients with choroidal neovascularization (CNV, n=96) and geographic atrophy (GA, n=39) were used. Lipids were extracted according to the Bligh and Dyer method, and lipid species were quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS). Statistical significance was determined by the Kruskal-Wallis test.

Results: Analysis of lipid classes showed a significantly higher sphingomyelin (SM) concentration in GA compared with controls (p=0.03), and higher hexosylceramide (HexCer) levels in CNV compared with controls (p=0.02). All other total lipid classes showed no statistical differences. Regarding molecular species, GA presented higher levels of SM species (SM 34:1, SM 34:2, SM 42:2, SM 42:3, SM 42:4), and ceramide Cer d18:1/16:0, and lower levels of phosphatidylcholine species (PC 36:0, PC 38:2) than controls. For most of these species CNV patients had intermediate concentrations. We also observed for CNV higher levels of SM 42:0, SM 34:1, HexCer d18:1/16:0, and CE 18:0 compared with controls.

Conclusion: Serum lipidomic profiles showed alterations in specific lipid species between AMD patients and controls, with differential changes for GA and CNV. Additional studies are needed to evaluate the implication of these changes, and possible associations with genetic polymorphisms.

Interaction of $Ca_V1.3$ and disease associated, mutant bestrophin-1

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Mutations of BEST-1, the gene encoding for bestrophin-1, are associated with an inherited autosomal dominant form of macular degeneration, Morbus Best. The pathophysiology of Morbus Best is not fully understood. Bestrophin-1, which is expressed in the basolateral membrane of the retinal pigment epithelium (RPE), is known to function both as a Cl-channel and a modulator of $Ca_V1.3$ L-type channels.

By means of the whole cell configuration of the patch-clamp technique, we analyzed electrophysiological properties of Ba^{2+} currents in CHO cells co-expressing $Ca_V1.3$ and different mutant bestrophin-1 (T6P, F305S, R218C, F80L kindly provided by Prof. Dr. Bernhard Weber). Surface expression of $Ca_V1.3$ and bestrophin-1 was assessed by means of immunohistochemistry and confocal imaging. Pearson's correlation coefficient (PCC) was calculated using α5β1-integrin as a surface marker in CHO. Physical interaction between $Ca_V1.3$ and bestrophin-1 was examined by immunoprecipitation and Western Blot.

Co-expression of T6P, F305S and F80L constructs with $Ca_V1.3$ led to a reduced surface expression of the $Ca_V1.3$ channel subunits $Ca_V1.3$ and β4, compared to wildtype bestrophin-1. F80L and F305S significantly decreased the current density and shifted the voltage dependence of $Ca_V1.3$ currents towards more positive values. Although the co-immunoprecipitation efficacy of $Ca_V1.3$ and bestrophin-1 was weaker with all the mutant bestrophin-1 proteins than compared with wild type bestrophin-1, there was a co-immunoprecipitation and thus interaction between $Ca_V1.3$ and all the mutant bestrophin-1 proteins.

As the mutations used in our study are identical to mutations found in Best patients, our results open new ways of understanding the pathophysiology of Morbus Best.

Physiology of explanted human retina: A comparative study

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Purpose: Little is known about information processing in human retina and its similarities or differences to retinal function in other species. To study human retinal function, we obtained both ex-vivo and post-mortem human donor tissue. We recorded ganglion cell activity in explanted ex-vivo human retina in response to various light stimuli, and compared response properties to pig and mouse ganglion cells. Due to variable ischemic conditions during the donation process (clamping of optic nerve in ex-vivo donations; time from death to retina extraction in post-mortem cases), the quality of explanted human retinas might be unpredictable. We thus analyzed the influence of various durations of ischemia on the quality and survival of pig and human retina.

Methods: We analyzed light responses of individual ganglion cells (human post-mortem donors: n = 920, domestic pig: n = 355, wild-type mouse: n = 77) with 60-electrode multi-electrode-array recordings. Ischemia was simulated in mini-pig eyes by storing them without oxygen and nutrient supply for different times at 37, 21 or 4 °C. Survival of ganglion cells in ischemia experiments was assessed by the number of electrodes showing spiking activity.

Results: Functional response properties (such as temporal frequency and speed tuning) differed between human, pig, and mouse ganglion cells. After controlled ischemic conditions at 37 °C, ganglion cell activity in mini-pig retina was measurable for up to 4h of ischemia. Lowering the temperature prolonged survival to 14h (21 °C) and >30h (4 °C). Light responses were measurable for up to 5h of ischemia at 4 °C. These results are consistent with the properties of human post-mortem retinas obtained 25–27h after death, which still showed ganglion cell activity.

Conclusion: Differing light response properties in fresh human, pig, and mouse retina hint at species-specific circuit properties. The long survival of the retina after more than 30h of ischemia makes post-mortem human donor retina a suitable experimental tissue. Light responses after ischemia indicate that the full synaptic pathway from photoreceptors to ganglion cells remains functional for at least 5h. This shows that post-mortem human retina is a promising tool for in-vitro optogenetic tests.

Acknowledgements: We thank Prof. Bartz-Schmidt (Eye-Hospital Tübingen) for the collaboration during the donation process, the Institute for Experimental Surgery Tübingen for domestic pig eyes, and the Department for Urology Tübingen for mini-pig eyes.

Conceptional considerations to investigate the pathomechanism of age-related macular degeneration via iPSC-based technology

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Purpose: To establish an *in vitro* disease model for age-related macular degeneration (AMD). Specifically, we want to gain insight into processes of complement dysregulation on a genetic background of high- and low-risk AMD predisposition.

Methods: Risk alleles at genetic loci CFH (rs1061170, rs800292, rs6677604), CFB (rs4151667, rs438999) and C3 (rs2230199) were determined in blood lymphocytes from eight AMD patients and disease-free controls, respectively. Probands were recruited at the University Eye Hospital Regensburg. A genetic risk score (GRS) was calculated according to Grassmann et al. 2012. From an independent blood sample of each proband, peripheral blood mononuclear cells (PBMCs) were isolated followed by activation of T-lymphocytes. Reprogramming of these cells into induced pluripotent stem cells (hiPSCs) will be done by lentiviral transduction with factors Oct3/4, Sox2, Klf4 and c-Myc (Takahashi et al. 2007). Subsequent differentiation into cells of the retinal pigment epithelium (hiRPE), the cell which is likely the source of primary disease pathology in AMD is well established as described earlier by Brandl et al. 2014.
Subsequent testing for functional consequences due to various genetic backgrounds will follow a scheme based on unchallenged and challenged hiRPE cells. Challenges will include physiological stimuli like feeding of photoreceptor outer segments (POS) and carboxyethylpyrrole (CEP) adducts while stimulation of components of the complement system will be achieved by applying zymosan, lipopolysaccharide (LPS) or H_2O_2 in the presence of human serum (NHS). To monitor changes in complement activity a hemolysis assay, which assesses the lytic potential of the induced complement components is performed and activation products iC3b and C3a as well as the terminal complement complex (TCC) are measured by an enzyme-linked immunosorbent assay (ELISA).

Results: In total, five AMD patients with highly increased GRS intervals (>3.44) as well as six AMD controls with low GRS (≤-1.79) have been recruited. Somatic reprogramming of stimulated PBMCs into hiPSCs followed by differentiation into hiRPE cells is under way. Concepts and experimental setups to monitor changes in complement activity with and without stimulation will be presented.

Conclusion: A major difficulty with investigating the pathomechanisms of a complex disease such as AMD includes the definition of suitable read-out assays. There is the issue of sensitivity and reproducibility. Here we propose a coherent concept to assess functional consequences of a complex combination of risk alleles in an adequate cellular system closely reflecting the idea of "a patient in a dish".

References: Brandl et al. (2014). Neuromolecular medicine, 16, 551–564. Grassmann et al. (2012).PLoS One, 7, e37979. Takahashi et al. (2007). Cell, 131, 861–872.

PARG: A therapeutic target for hereditary retinal degeneration?

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Purpose: Poly(ADP-ribosyl)ation turnover is involved in many cellular processes such as DNA repair, transcription, cell differentiation and cell death and is regulated by the synthesizing enzyme poly(ADP-ribose) polymerase (PARP) and the degrading enzyme poly(ADP-ribose) glycohydrolase (PARG). Previously, excessive PARP activation was found to be involved in photoreceptor degeneration in the *rd1* mouse for hereditary retinal degeneration (RD). However, there is no data about the relevance of PARG in RD. To address this, we studied retinal morphology and response to pharmacologically induced retinal degeneration in PARG knockout (KO) mice.

Methods: Mutant mice lacking the 110 kDa isoform of PARG (PARG 110 KO) and corresponding wild-type *(wt)* mouse retinae at post-natal day (P) 11 and P30 were analyzed using TUNEL staining for the detection of cell death and PAR immunohistochemistry. PARG antibody, which detects PARG 110 and PARG 56 kDa proteins, was used to detect expression in *wt* and PARG 110 KO retinae. Characterization of PARG expressing cells was performed by co-staining with antibodies for calbindin and PKCα. To emulate *in vitro* a situation comparable to the *rd1* mouse model, PARG 110 KO and wt mouse retinal explants were cultured with/without zaprinast, a selective inhibitor of cyclic GMP-specific PDE5/6.

Results: TUNEL assay and PAR immunohistochemistry showed very few positive cells in PARG 110 KO and *wt* retinae. Immunofluorescence for PARG showed positive staining in the outer plexiform layer (OPL), in the inner nuclear layer (INL), ganglion cell layer (GCL) and optical fibers. Immunofluorescence analysis showed that zaprinast treatment on PARG 110 KO and wt retinal cultures induced an accumulation of cGMP in photoreceptors. The TUNEL assay showed a zaprinast induced increase in the number of dying photoreceptors of both PARG 110 KO and wt retinae. However, the number of dying cells in PARG 110 KO was smaller than in corresponding wt.

Conclusions: Similar to PARP, PARG may have important functions in retinal photoreceptor cell death. PARG 110 KO retinae appeared morphologically normal. Additionally, in PARG 110 KO retina, there is no sign of any enzymatic activities associated with RD. PARG 110 and PARG 56 are prominently expressed in bipolar cells, amacrine cells, ganglion cells and optical fibers. These results suggest that neuroprotective strategies aimed at inhibiting PARG 110 would not negatively affect photoreceptor retinal cell survival and could hence be a novel target for drug developments.

Read-through strategies for therapeutic suppression of nonsense mutations in Usher syndrome

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Purpose: The Usher syndrome (USH) is the most frequent cause of inherited combined deaf-blindness, distinguished into three clinical and at least 10 genetic subtypes. So far no effective treatment for the ophthalmologic component of USH exists. Database search revealed that ~20 % of all USH cases are caused by nonsense mutations leading to the expression of truncated non-functional protein. Translational read-through mediates over-read of nonsense mutations and thereby induces the expression of full-length proteins. The read-through of nonsense mutations by translational read-through inducing drugs (TRIDs) has become a promising pharmacogenetic strategy for degenerative diseases. Here we aim to analyse the read-through efficacy of TRIDS on mutations from different USH causing nonsense mutations.

Methods: We generated a bidirectional reporter construct (PBI-CMV-HA) coding for dsRed as transfection reporter and the expression of the HA-tagged gene of interest. We transfected HEK293T cells with cDNA of USH genes with and without selected nonsense mutations and compared the read-through efficiency of different TRIDs, namely PTC124 and the designer aminoglycosides NB84 and NB124 via quantitative immunofluorescence and Western blotting for all nonsense mutations and tested functionality of recovered proteins e.g. by GST-pull downs. Retinal toxicity of TRIDs was assessed in mouse retinal explants by TUNEL-assays.

Results: Our studies revealed the recovery of protein expression after TRIDs applications for all analyzed nonsense mutations, namely SANS/USH1G (S243X), USH3A (p.Y63X; p.Y176X), and the USH1C isoforms harmonin a1 (p.R155X) and harmonin b3 (p.R155X). We observed various read-through efficacies in HEK293T cells transfected with nonsense mutations in the analyzed genes after application of different TRIDs. Application of TRIDs restored scaffold protein function of harmonin a1 , harmonin b3 and SANS. Furthermore, we observed a good retinal biocompatibility of PTC124 and NB84.

Conclusion: The high retinal compatibility of PTC124 and NB84 combined with their transcriptional read-through efficacy, provide a promising therapy strategy for USH patients suffering from debilitating nonsense mutation-mediated retinal degeneration. Moreover translational read-through could serve as a treatment option for retinal disorders caused by in-frame nonsense mutation in general.

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Newly generated antibodies indicate CFHR3 function in the human complement system

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Purpose: The CFH-related protein family (CFHR) comprises a group of five plasma proteins structurally and functionally related to the main negative complement regulator, factor H (CFH). Mutations within the *CFH/CFHR* gene cluster are associated with various human diseases such as age-related macular degeneration (AMD), the leading cause of irreversible vision loss in the elderly. Deletion of the *CFHR3/1* genes goes along with a lower risk for AMD although the precise functions of the CFHR proteins still remain unclear. Based on our previous work, which resulted in CFHR3 monoclonal antibodies (mAbs), we aim to elucidate the role of CFHR3 in complement regulation in degenerative diseases.

Methods: Generated mAbs were tested for specificity and avidity against human CFHR3 in indirect enzyme-linked immunosorbent assay (ELISA) and Western blot. MAbs were used for immunoprecipitation from human serum and following mass spectrometry analysis. Complement modulation was analyzed in functional *in vitro* hemolysis assays and complement ELISA. Sandwich ELISA for detection of CFHR3 from human serum was established. Further, the interaction of CFH and CFHR3 with the oxidative stress marker CEP (ω-(2-carboxyethyl)pyrrole) and the complement component 3b (C3b) was tested.

Results: Newly generated mAbs were highly specific for human CFHR3. MAb 269-5 showed the highest avidity in indirect ELISA and detected CFHR3 in human serum. Furthermore, mAb 269-5 immunoprecipitated CFHR3 in complex with alternative and terminal complement components from human serum. Preliminary *in vitro* analyses showed an inhibitory effect of high anti-CFHR3 mAb concentrations on the alternative pathway. Promising data exposed a binding of CFH as well as CFHR3 to an oxidative stress epitope (CEP) and to C3b. Both proteins competed for binding to these molecules, and mAb 269-5 interfered with this competition by inhibiting CFHR3 interaction. MAbs 269-5 and 552-3 detected specifically CFHR3 from human serum in an optimized sandwich ELISA.

Conclusions: With this work we generated and characterized highly specific monoclonal antibodies against CFHR3. First results for a mAb-based *in vitro* modulation of the complement system encourage further investigation into the functional role of CFHR3 in complement and AMD.

Light damage induced changes in mouse complement expression

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Purpose: Millions of individuals older than 50-years suffer from retinal degeneration. Recent studies suggest an important role of the complement system in these disease progressions. However, the underlying mechanisms by which complement proteins contribute to degeneration remain unclear. There is uncertainty about whether the complement system is a local or systemic player involved in the pathogenesis. The goal of this study was a systematic overview to estimate the role of complement involvement in light damage induced retinal degeneration in a mouse model.

Methods: We used two different mouse models for the analysis of complement involvement in retinal degeneration: acute and chronic light-induced retinal damage. Complement activation was analyzed either in the retina or in the complex of retinal pigmented epithelium (RPE) and choroid on transcriptional (qPCR) and translational level (Western Blot). We compared tissue samples of treated and control mice.

Results: Complement proteins were detected both in the retina and RPE-chorid complex independent from analyzed mouse model. The complex data still set needs to be interpreted, but as a first examples we can specify the following data: Activated MASP1 was elevated in acute light damage in both tissues in western blots. Whereas significant protein detection of complement factors B was only possible in the RPE-choroid complex but not in the retina of mice with no difference between treated and untreated mice. In contrast, mRNA for all tested complement factors was mostly detectable in both tissues. A local detection of the protein in the retina involves a local complement activation and a detection in the RPE/choroid could suggest a role of the systemic complement.

Conclusions: This comprehensive study reveals detailed information for a list of complement proteins in the healthy and damaged mouse eye. We intend to provide essential preliminary data for mouse model interpretation as well as for the testing interventional, perspective strategies for degenerative retinal diseases.

Deletion of ocular TGF-β signaling mimics essential characteristics of diabetic retinopathy

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The vascular phenotypes of diabetic retinopathy are important causative or contributing factors of vision loss and blindness. However, its molecular causes are not sufficiently clear. To identify the role of TGF-β signaling for maintenance and proliferation of retinal vessels, we generated mice with a conditional deletion of the TGF-β type II (TβRII) receptor which is essential for TGF-β signaling.

Floxed Tgfbr2 mice were crossed with CAG-Cre mice with the coding sequences of Cre recombinase under control of a tamoxifen-responsive chicken actin promoter. The successful deletion of TβRII was confirmed by real time RT-PCR, Western blotting and immunohistochemistry. Retinal structure and function were studied by light and electron microscopy, immunohistochemistry, fluorescence angiography, real time RT-PCR, and electroretinography.

Treatment of newborn *Tgfbr2*$^{-/-}$;CAG-Cre mice with tamoxifen resulted in a substantial and significant deletion of TβRII throughout the entire retina. Lack of TGF-β signaling led to the formation of abundant microaneurysms, leaky capillaries, and retinal hemorrhages. Retinal capillaries were not covered by differentiated pericytes, but by a coat of vascular smooth muscle-like cells and a thickened basal lamina. Reactive microglia was found in close association with retinal capillaries. In older animals, loss of endothelial cells and the formation of ghost vessels were observed, findings that correlated with the induction of angiogenic molecules such as VEGF-A, FGF-2, ANGPT2 and IGF and the accumulation of retinal HIF-1α indicating hypoxia. Consequently, retinal and vitreal neovascularization occurred, a scenario that led to retinal detachment, vitreal hemorrhages, neuronal apoptosis and impairment of sensory function.

TGF-β signaling is critically required for the differentiation of retinal pericytes during vascular development of the retina. Lack of differentiated pericytes initiates a scenario of structural and functional changes in the retina that mimics those of diabetic retinopathy and strongly indicates a common mechanism. We conclude that *Tgfbr2*$^{-/-}$;CAG-Cre mice constitute an animal model to study the molecular pathogenesis of retinal diseases associated with neo-angiogenesis such as diabetic retinopathy.

Characterization of the cellular and humoral immune response in the eye following light induced photoreceptor degeneration in mice

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Study objectives: The cellular immune system of the eye comprises microglial and macroglial cells e.g. Müller cells. The complement system is a part of the humoral immune system. Both immune responses play a crucial role in the pathogenesis of retinal neurodegenerations. A common endpoint of retinal neurodegenerative disorders, including age related maculopathy and inherited photoreceptor degenerations, is the apoptosis of photoreceptor cells. In the current study, we used the light damage model to induce photoreceptor apoptosis and characterized the immune response of the cellular and humoral immune system in the eye.

Methods: We exposed albino Balb-c mice to a light intensity of 5000 lux for 30 minutes. TUNEL labelling was used to verify the induction of photoreceptor apoptosis. To characterize the cellular immune response, we performed immunohistochemical staining using antibodies against glial fibrillary acidic protein (GFAP), which labels reactive Müller cells, ionized calcium-binding adapter molecule 1 (IBA-1), which labels microglia cells and F4/80, a marker for macrophages and reactive microglia cells. To determine a potential activation of the humoral immune response following light exposure, we investigated the transcriptional expression levels of different components of the complement systems by realtime RT-PCR in samples of the neuroretina and a tissue complex containing the retinal pigment epithelium (RPE) and the choroid.

Results: Light exposure resulted in numerous apoptotic cells in the outer nuclear layer (ONL). Immunohistochemical staining for GFAP showed a characteristic stripe-shaped reaction throughout the retina indicating a reactivation of Müller cells. Staining for IBA-1 confirmed the reactivation of microglia cells and F4/80 staining further confirmed the reactivation of microglia cells and additionally showed numerous macrophages in the choroid. Furthermore, following light exposure, we frequently observed a translocation of IBA-1 or F4/80 positive cells into the ONL and into the inner/outer segments of the photoreceptors. Realtime RT-PCR data confirmed an increased expression of factors of the complement system following light exposure. Here, we observed a robustly elevated expression of the factors C1qa, C1s, C4, CFH, CFB and C3 in the retina and the choroid/RPE.

Conclusions: Both, cellular and as well as humoral components of the ocular immune system are activated following light induced photoreceptor degeneration.

Targeting the translocator protein (18kDa) (TSPO) prevents microglia reactivity and protects from light induced retinal degeneration

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Purpose: Microglia activation is a common hallmark of several retinal degenerative diseases. Our previous work showed that TSPO is as marker for reactive retinal microglia and that the selective TSPO ligand XBD173 exerts strong anti-inflammatory effects on microglia in vitro. In the present study, we investigated whether XBD173 has the capacity to modulate retinal microglia in vivo and thereby protects from light induced retinal degeneration.

Methods: BALB/C mice were treated with vehicle or 10 mg/kg XBD173 by intraperitoneal injection prior to exposure to 15.000 lux white light for one hour. Daily XBD173 treatment was continued for four consecutive days. After this time period, retinal flat- mounts and sections were prepared to analyze microglia morphology, localization and reactivity using Iba1 and TSPO protein expression. Optical coherence tomography, morphometric measurements of retinal thickness and TUNEL stainings were used to to determine the extent of retinal degeneration and photoreceptor apoptosis.

Results: In control eyes that were not exposed to light, Iba1 staining revealed that microglia were located in the inner and outer plexiform layers and showed a ramified morphology. Light exposed animals that were sham treated displayed a severe thinning of the photoreceptor layer and prominent photoreceptor apoptosis which was accompanied by the migration of amoeboid microglial cells into the outer nuclear layer and the subretinal space. TSPO staining revealed a strong TSPO expression in these microglia, indicating a highly reactive status. In contrast, light exposed mice that received XBD173 injections showed a well preserved photoreceptor layer and strongly reduced apoptosis. Significantly fewer numbers of amoeboid microglial cells were present in the ONL and subretinal space and nearly all of them displayed a ramified cell shape. Furthermore, these microglia showed much less staining for the activation marker TSPO.

Conclusion: TSPO-specific XBD173 treatment of mice challenged with intense withe light reduced the number of reactive microglia and protected retinal photoreceptors from light induced apoptosis. We conclude that TSPO and its ligands represent promising targets for neuroprotective and anti-inflammatory therapy of retinal degenerative diseases.

Molecular interaction between Bestrophin-1 and Rab27a

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Mutations in BEST1 result in several different retinal degeneration diseases, among them Best macular dystrophy. The gene product of BEST-1, bestrophin-1, can be detected at the basolateral membrane of the retinal pigment epithelium (RPE). There is evidence for bestrophin-1 as a calcium dependent chloride channel, as well as a modulator of L-type calcium channels. Since the physiological function of bestrophin-1 is not yet fully described, patho-mechanisms of Best disease are still largely unknown. Unpublished data from own preliminary work point towards a possible interaction with the small GTPase Rab27a.

This study aims to investigate whether there is a direct interaction between Rab27a and bestrophin-1, what is the underlying molecular basis of this interaction and whether mutant bestrophin-1 show reduced interaction.

Therefore, co-immunoprecipitation and subsequent Western Blot analysis of Rab27a and wild-type bestrophin-1 as well as several mutant forms was performed. The plasmids with mutant bestrophin-1 were kindly provided by the group of Prof. Bernhard Weber, Regensburg.

Some of the mutated bestrophin-1 are associated with Best's disease (amino acid exchanges: T6P, F80L, R218C, F305S) in human subjects, whereas others were cloned to identify a possible binding domain, strategically leaving out parts of the amino acid sequence of bestrophin-1 (PxxP motifs and parts of the C-terminus respectively).

Co-immunoprecipitation could be detected between Rab27a and all bestrophin-1 constructs. However, the efficacy determined by densitometric analysis of the Western Blots was diminished in T6P, F305S, as well as in the bestrophin-1 constructs lacking the PxxP motifs or parts of the C-terminus.

These results allow the conclusion that there is clearly an interaction between Rab27a and bestrophin-1. The possible role of the PxxP motifs for Rab27a binding imply that it may be mediated by another interacting molecule.

Multimodal assessment of choroideremia patients defines pre-treatment characteristics

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Purpose: Choroideremia (CHM) is a X-chromosomal disorder leading to blindness by progressive degeneration of choroid, retinal pigment epithelium (RPE), and retinal neurons. A current clinical gene therapy trial (NCT01461213) showed promising safety and efficacy data in a carefully selected patient population. The present study was performed to shed light on pre-treatment characteristics of a larger cohort of CHM patients using a high resolution multi-modal approach.

Methods: In a retrospective cross-sectional study, data from 58 eyes of 29 patients with clinically confirmed CHM were analysed including best-corrected visual acuity (BCVA), refractive error, spectral-domain optical coherence tomography (SD-OCT), fundus autofluorescence (FAF), perimetry, and tonometry. Residual retinal volume, area of residual RPE, and foveal thickness were quantified to further define natural disease progression and assess symmetry.

Results: We evaluated 98 data points of BCVA [0.34 ± 0.06 (logMAR); mean ± 95 % confidence interval], 80 of IOP (14.6 ± 0.6 mmHg), and 98 of refraction (-2.16 ± 1.08 spherical equivalent). Visual fields (n = 76) demonstrated variable degrees of concentric constriction (54 % <10°, 25 % 10–30°, 21 % >30°). Mean residual RPE area on FAF (n = 64) measured 8.47 ± 1.91 mm^2 (range 0.30–38.5 mm^2), while mean neuroretinal volume (n = 42) was found to be 1.76 ± 0.12 mm^3. Age at examination was exponentially associated with BCVA, while logarithmic functions best described progressive loss of retinal area and volume. A high degree of left to right symmetry was found in all modalities with structural markers showing the best correlation ($r^2_{area} = 0.83$; $r^2_{volume} = 0.75$).

Conclusion: Analysis of these widely available clinical data defines the natural disease characteristics of a relevant patient population eligible for gene therapeutic intervention. In the wake of preliminary reports on safety and efficacy of CHM gene therapy (NCT01461213), this multi-modal assessment of a cohort of CHM patients provides important evidence of the natural rate of disease progression and degree of symmetry between eyes.

Minocycline protects from light-induced-retinal-degeneration

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Background: Minocycline is a semi-synthetic tetracycline derived antibiotic that is commonly used in the therapy of acne vulgaris and several human infections. It was shown, that minocycline exerts neuroprotective effects by dampening neuroinflammation through the inhibition of microglia. The aim of this study is to evaluate the impact of minocycline on BV-2 microglia cells and on retinal degeneration in a mouse model of light induced retinal degeneration.

Methods: Gene expression of microglial pro-inflammatory markers (IL-6, CCL2, iNOS, AMWAP and TNF-α) was determined at different time points after minocycline (50µg/ml), LPS (50ng/ml) and minocycline and LPS co-treatment using qRT-PCR. 2 month old Balbc mice were exposed to 15.000 lux bright white light for one hour to induce light damage. The animals were treated daily intraperitoneal with minocycline (45µg/kg body weight) starting one day before induction of light damage for five consecutive days. Mice were injected twice at day one and once at the following days. At day five we performed OCT (optical coherence tomography) analysis of all eyes. Then eyes were removed and retinas were prepared to measured gene expression or to analyse retinal damage by immunohistochemistry.

Results: Minocycline significantly reduced gene expression of pro-inflammatory markers (IL-6, CCL2, iNOS) in LPS-activated BV-2 microglia cells. *In vivo* the expression of AMWAP, CD68, Caspase8 and TSPO were decreased by minocycline treatment. The OCT scans revealed a thicker retina in minocycline treated light damage mice compared to control animals with light damage. Especially the ONL (outer nuclear layer), which is thinned by cell death, was preserved. Immunohistochemistry showed that the retinal structure was preserved and microglial activation and migration were decreased by minocycline treatment.

Conclusion: We conclude that minocycline could provide a new possibility to preserve retinal structure during damage via inhibition of microglial activity.

The SANS-USH2a-whirlin ternary complex of Usher syndrome proteins is disrupted by pathogenic mutations

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Purpose: Usher syndrome (USH) is the most common form of combined deaf-blindness. Among three clinical types (USH1-3), USH1 is the most severe and USH2 the most common type. Previous *in vitro* studies have indicated that USH genes encode for proteins of diverse families which interact within an interactome. Furthermore, we have described a USH1-USH2 protein network in the periciliary compartment of rodent photoreceptor cells. Here we aimed to further characterize USH1-USH2 protein networks in photoreceptor cells and to get insights into the molecular pathomechanisms underlying USH by testing the affects of diverse USH causing mutation on these networks.

Methods: We analysed mutual direct binding *in vitro* (GST-pull downs), and in cell culture (membrane targeting assays). Ternary complexes assembly was accessed by co-precipitation experiments. USH mutations were introduced by site-specific mutagenesis. PTC124 and gentamicine were applied as translational read-through drugs (TRIDs) targeting nonsense mutations. The subcellular localization of complex partners was analyzed by immunofluorescence and electron microscopy in mouse and human retinas.

Results: We demonstrate for the first time the mutual direct interaction between the scaffold protein SANS (USH1G) and the transmembrane adhesion protein USH2A. Furthermore, we identified a ternary USH1-USH2 protein complex of SANS, USH2A and the PDZ-domain protein whirlin (USH2C) mediated by direct interactions and dimerization. Co-localization pattern emphasize that the SANS-USH2A-whirlin complex assembles in the periciliary region of human and rodent photoreceptor cells and in calyceal processes of human photoreceptor cells. USH causing mutations in *USH1G* severely affected complex formation. TRIDs treatment targeting the $USH1G_{C728A}$ nonsense mutation restored SANS scaffold and complex function.

Conclusions: Present data revealed that the USH1 and USH2 proteins are interwoven in protein complexes and indicate that pathogenic mutations in complex partners disrupt these complexes which can be targeted by gene-based treatments. Our results further suggest that the ternary SANS-USH2A-whirlin protein complex participate in functional modules of photoreceptor cilia and support the structure of calyceal processes in human photoreceptor cells.

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Fingerprint profile of cone dystrophy related GCAP1 mutants

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Purpose: Photoreceptor cells efficiently respond to changing light conditions on a millisecond timescale by a well-balanced interplay of two second messengers, cGMP and calcium. Calcium sensor proteins like the guanylate cyclase-activating proteins (e.g. GCAP1 and GCAP2 in mammalians) control the synthesis of cGMP in a calcium-dependent manner and in a step- by-step calcium relay mode of action. Mutations in the gene GUCA1A encoding GCAP1 correlate with human cone dystrophies and are known to cause an imbalance of the calcium and cGMP homeostasis. Here we investigate the biophysical and biochemical properties of the GCAP1 mutants E89K, D100E, L151F and G159V, which are constitutive activators of photoreceptor guanylate cyclase (GC)[1].

Methods: GCAP1 wildtype (WT) and mutants were heterologously expressed and purified. Hydrodynamic properties and calcium-binding parameters of GCAP1 variants were investigated by dynamic light scattering, isothermal titration calorimetry and size exclusion chromatography. Calcium-induced conformational changes were monitored by surface plasmon resonance. Catalytic parameters were determined by enzymatic assays using the target guanylate cyclase.

Results: Calcium-binding studies revealed three functional EF-hand calcium-binding sites in all mutants, but two EF-hands showed a several-fold lower affinity in the mutants than in WT GCAP1. Interestingly, the EF-hand with the highest affinity remained nearly unchanged. Changes in protein conformation correlated with data from dynamic light scattering and size exclusion chromatography showing a rearrangement of the protein hydration shell and a change of the dielectric constant of the protein-water interface. All mutations decrease the catalytic efficiency in regulating the target GC[2,3].

Conclusion: Point mutations of the calcium sensor GCAP1 have strong, but differential impacts on the biophysical and biochemical properties enabling the formulation of a fingerprint profile of each mutant. Thus, we further tested the consequences of dystrophy-related mutations in a kinetic model of phototransduction, in which we can assess the cGMP synthesis rate resulting from either GCAP1 or GCAP2 during a photoresponse. The computational analysis revealed that the synthesis rate controlled by GCAP1 remains at a constant level, but it would not at all contribute to the shaping of the photoresponse. The latter would prominently be regulated by GCAP2[3].

References:
[1] Behnen et al., Involvement of the calcium sensor GCAP1 in hereditary cone dystrophies, Biol Chem, 2010, 391(6):631-7.
[2] Sulmann et al., Conformational changes in calcium-sensor proteins under molecular crowding conditions, Chemistry, 2014, 20(22):6756-62.
[3] Dell´Orco et al., Impact of cone dystrophy-related mutations in GCAP1 on a kinetic model of phototransduction, Cell Mol Life Sci., 2014, 71(19):3829-40.

The retinal output changes qualitatively with every change in ambient illuminance

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Purpose: Healing blindness has always been a dream of human kind. In the last decade, we have come very close to making this dream come true. Several teams world-wide have developed electrical retinal implants for patients suffering from retinal degeneration, with two products (Second-Sight, Retina Implant) being approved for patient treatment. Similarly, optogenetic approaches hold promise to restore light sensitivity to blind retinal tissue. Building on such success stories, the goal of vision-restoring technologies should now be to not only restore light sensitivity to the diseased retina, but to restore – as much as possible – the "natural" activity pattern of the retina. For example, one aim has always been that the response polarity of retinal cells should be preserved: when artificially stimulated, ON cells and OFF cells should be stimulated differentially. This is a prerequisite for their responses to faithfully represent light increments and light decrements, respectively.
Here, we characterized the "natural" response pattern of retinal ganglion cells under different environmental conditions. We asked if the responses of a given cell would be stable when probed at different ambient luminance (scotopic, mesopic, photopic), or if the response pattern would be different.

Methods: We recorded explanted mouse and pig retina with 60-electrode multi-electrode-arrays. Visual stimuli were focused onto the photoreceptors. Neutral density filters inserted into the stimulation light path set the overall luminance level, spanning 5 log units of intensity across scotopic, mesopic and photopic regimes.

Results: Responses of retinal ganglion cells in mouse and pig retina depended on ambient luminance. With each change of brightness, responses changed qualitatively. For example, ON cells gained (or lost) OFF responses, and OFF cells gained (or lost) ON response. Such changes were also observed in-vivo in the dLGN of anaesthetized mice. Response changes did not require rod-cone interactions, nor center-surround processing. They were also observed in the responses to natural movies.

Conclusion: The natural response pattern of the retina changes qualitatively with each change of ambient luminance. In particular, the response polarity of ganglion cells is not fixed. This may be advantages for prosthetic devices that have poor cellular specificity, such as electrical retinal implants. The implications of these findings for retinal prosthesis require further investigation of how higher visual brain centers deal with the changing retinal code.

In vivo protection of degenerating cones in the cpfl1 mouse by HDAC inhibition

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Purpose: Contrary to the general view that photoreceptor cell death in inherited retinal degeneration is governed by "classical" apoptosis, a new line of evidence suggests the execution of non-apoptotic mechanisms (Arango-Gonzalez *et al.,* Plos One., 9(11):e112142, 2014). Non-apoptotic cell death is also the driving force behind primary cone death in *cpfl1* mice, which, also show impaired cone migration during retinal development. *Cpfl1* cones suffer from a mutation in *Pde6c*, which leads to cGMP accumulation as well as increased activity of histone deacetylase (HDAC) (Trifunović *et al.,* J Comp Neurol., 518(17):3604-17, 2010). Previously, we found that HDAC inhibition attenuated *cpfl1*-cone death in retinal explant cultures, *in vitro* (Trifunović *et al.* ARVO Abstracts 55:4035, 2014). In the present study, we investigated whether HDAC inhibition could prevent *cpfl1* cone loss also *in vivo*.

Methods: We tested the *in vivo* neuroprotective properties of Trichostatin A (TSA), injected intravitreally at the onset of cone degeneration in *cpfl1* animals and assessed the effects at the peak of degeneration, quantifying cone photoreceptor survival vs. degeneration, as well as cone migration.

Results: *In vivo* treatment with a single injection of two different TSA concentrations resulted in a significant improvement of cone survival. Percentage of cones compared to the wt retinas in non-treated animals was ~80%, while in TSA treated the number of cones reached 96% of wt cones number. At the same time, HDAC inhibition also significantly improved developmental cone migration, from the outer plexiform border to the outer parts of the outer nuclear layer from ~71% of ONL tickness for non-treated to ~77% after the treatme compared to to ~86% in wt retinas.

Conclusions: Together with earlier results (Sancho-Pelluz *et al.,* Cell Death Dis., 1:e24, 2010), our findings suggest that in inherited diseases of the retina, pharmacological inhibition of HDAC can prevent both rod and cone photoreceptor degeneration. This highlights the feasibility of targeted neuroprotection *in vivo,* in an animal model for primary cone degeneration, and in turn creates hope to maintain vision in patients suffering from both, cone as well as rod-cone dystrophies.

Simulating diabetic retinopathy in vitro

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Purpose: Diabetic retinopathy (DR) is one of the most common complications of diabetes and is considered to be a leading cause of blindness in working age patients. The disease is characterized by an initial degeneration of both retinal neurons and endothelial cells followed in extreme cases by an abrupt neovascularization, macular edema and severe visual dysfunctions. The underlying mechanisms through which neurodegeneration occur are not completely understood. We propose the use of organotypic retinal explant cultures derived from healthy wild-type mice to reproduce DR in vitro (e.g. low insulin and/or hyperglycemia) to study how neurodegeneration occurs and to screen for DR drugs.

Methods: Retinal explants from P5 wild-type mice were cultured for 6 days (till P11) in R16 medium with supplements at 37 °C. Treatments consisted of adding glucose, removing insulin from the culture medium or applying the glycolysis inhibitor 2-deoxy-glucose (2-DG) to alter glucose metabolism. To quantify the percentage of dying cells in the retina we used the TUNEL assay. Immunohistochemistry was used to identify changes in the expression of glucose transporter 1 (Glut-1). GFAP (Müller cells and astrocytes) and Glycogen phosphorilase (cones) were used to co-label specific cell types with TUNEL to identify if cell death was cell-specific.

Results: We found increased cell death in the outer nuclear layer (ONL) under high glucose conditions and glycolysis inhibition but not in the conditions without insulin and/or high glucose. In the inner nuclear layer (INL) cell death was increased under high glucose and high glucose minus insulin. Removing insulin from the medium alone did not produce an increase in cell death. Immunolabeling for Glut-1 revealed an increased expression in the ONL under high glucose and glycolysis inhibition. When co-immunolabeling with TUNEL and GFAP or Glyphos, we found that cell death was present in a broad range of different cell types.

Conclusion: These preliminary results suggest that high glucose by itself is sufficient to produce increased cell death in a non-cell-type specific manner in both the INL and ONL. The upregulation observed in the expression of glucose transporter 1 requires further study using more precise protein quantification techniques.

SF3B2, a novel candidate gene for autosomal dominant retinitis pigmentosa, encodes a component of the U2 small nuclear ribonucleoprotein.

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Five affected and five unaffected individuals of a Belgian adRP family in which known adRP loci were excluded, were enrolled. They underwent genome-wide (GW) linkage analysis (BeadChip, Illumina). Whole exome sequencing was carried out in two affected individuals (HiSeq, Illumina; CLC bio). Segregation analysis of variants was done using Sanger sequencing and testing of 300 controls by HRM (LightScanner). Targeted resequencing of *SF3B2* was performed (Miseq, Illumina) in 472 unrelated adRP patients. *SF3B2* expression was tested using a commercial cDNA panel. Localization studies were carried out in 661W mouse cells using commercial anti-SF3B2 antibodies. *Sf3b2* knockdown in *Xenopus* was done using targeted injection of a splicing blocking morpholino (MO) (GeneTools).

GW linkage analysis revealed two novel candidate loci with a maximum LOD score of 1.7. In the 11q13 region, a missense variant c.2417A>G p.(Tyr806Cys) was found in *SF3B2* encoding the splicing factor 3b, subunit 2. The Tyr residue is highly conserved, and predictions suggest an effect on protein function. The change is predicted to disrupt a phosphorylation site. The variant co-segregates with adRP and is absent in 300 controls. No additional mutations were found in a large adRP cohort. Ubiquitous expression of *SF3B2* was demonstrated in human tissues, including retina and RPE, and localization in perinuclear and nuclear areas was shown in 661W mouse cells. Targeted MO knockdown in *Xenopus* showed gross developmental anomalies affecting the retina. Rescue experiments are ongoing.

SF3B2 is required for binding of the U2 small nuclear ribonucleoprotein (snRNP) to the branchpoint and is involved in early spliceosome assembly. Interestingly, protein-protein interactions have been identified between SF3B2, SNRNP200 and PRPF8, the latter being two proteins implicated in adRP. So far, of the seven known adRP genes involved in splicing, six encode components of the U4/U6-U5 triple snRNP (tri-snRNP) complex. Our study potentially involves other components of the spliceosome apart from the tri-snRNP complex in adRP.

Homozygous deletion of glutamate receptor gene *GRID2* causes new hotfoot mutant phenotype, characterized by early-onset cerebellar ataxia and retinal dystrophy

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Purpose: To identify the genetic cause of early-onset autosomal recessive cerebellar ataxia (ARCA) associated with retinal dystrophy in a consanguineous family.

Methods: An affected six-months old child underwent neurological and ophthalmological examinations. Genetic analyses included homozygosity mapping, copy number analysis, conventional PCR, Sanger sequencing, qPCR, whole exome sequencing (WES). Expression analysis of GRID2 was performed by qPCR and immunohistochemistry.

Results: The child had truncal and appendicular ataxia, tonic upgaze and nystagmus. Magnetic resonance imaging showed cerebellar atrophy, particularly of the vermis region. Fundoscopy revealed no pigmentary abnormalities and electroretinography demonstrated reduced scotopic and photopic amplitudes, pointing to early-onset retinal dystrophy.

A homozygous deletion of exon 2 of GRID2 (p.Gly30_Glu81del) was found in the proband, compatible with mouse hotfoot mutant *ho15J. GRID2* encodes an ionotropic glutamate receptor known to be selectively expressed in cerebellar Purkinje cells. Here, we demonstrated *GRID2* mRNA expression in human adult retina and retinal pigment epithelium. In addition, *Grid2* expression was demonstrated in different stages of murine retinal development. GRID2 protein expression was observed in both murine and human retina. More specifically, GRID2 localized to photoreceptor inner segments, the outer plexiform layer and ganglion cell layer in human and mouse. Faint Grid2 immunoreactivity was also observed at the inner nuclear layer/inner plexiform layer margin in the mouse retina. WES in the proband did not provide arguments for other disease-causing mutations, supporting the idea that the phenotype observed represents a single clinical entity.

Conclusions: We identified *GRID2* as underlying disease gene of early-onset ARCA with retinal dystrophy, expanding the clinical spectrum of *GRID2* hotfoot deletion mutants in humans. We demonstrated, for the first time, GRID2 mRNA and protein expression in human and murine retina, providing evidence for a novel functional role of GRID2 in the retina. Finally, to the best of our knowledge *GRID2* is the second glutamate receptor gene, apart from *GRM6,* leading to retinal disease when mutated.

Dysfunction of cGMP signaling in photoreceptors by a Macular Dystrophy related mutation in the calcium sensor GCAP1

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Purpose: Point mutations in the cGMP signaling unit in photoreceptor cells consisting of guanylate cyclases and guanylate cyclase-activating proteins (GCAPs) are known to cause hereditary retinal diseases like Cone-Dystrophies (CD), Leber's Congenital Amaurosis (LCA) and Retinitis Pigmentosa (RP).(1,2,3) A recent mutational screening of patients diagnosed with Macular Dystrophy unveiled a so far unknown point mutation in the GUCA1A gene causing a L176F amino acid exchange in the encoded protein GCAP1. It was the aim of the present work to investigate the cellular and biochemical consequences of the L176F mutation in GCAP1.

Methods: Site-directed mutagenesis was performed to create the L176F GCAP1 mutant using standard cloning techniques. For functional studies the protein was expressed in *E.coli*, purified and its operational performance as a calcium-sensitive regulator was compared with wildtype GCAP1.

Results: Members of three different families in which the mutation was found showed paracentral and central soctomas in the 30° visual field and family history indicated an autosomal dominant mode of inheritance. Functional analysis of the protein revealed no severe disturbance in calcium sensing properties or calcium-induced conformational changes as detected by gel-shift analysis and tryptophan fluorescence spectroscopy. However, a moderate, but significant disturbance was observed in the calcium-dependent regulation of target guanylate cyclase. The mutation caused a lower Ca^{2+}-affinity leading to a later inhibition of the guanylate cyclase and therefore to a delayed restoration of the photoreceptor dark state.

Conclusion: The biochemical profile of the GCAP1 L176F mutant indicates a moderate disturbance of cGMP signaling and calcium homeostasis in rod and cone cells. These findings are different from previous results obtained with GCAP1 or guanylate cyclase mutations that correlate with CD, LCS and RP.(1,2,3) Therefore, the less severe dysfunction in GCAP1 operation would be consistent with a delayed onset of macular dystrophy as observed in the clinical studies.

References:
(1) Behnen et al. (2010) Biol Chem 391, 631-637
(2) Kitiratschky et al. (2009) Hum Mutant 30; E782-96
(3) Zägel and Koch (2014) Front Mol Neurosci 7; A4

Müller cell reactivity in response to photoreceptor degeneration in rats with defective polycystin-2

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Purpose: Retinal degeneration in transgenic rats that express a mutant cilia gene polycystin-2 (CMV-PKD2$_{(1/703)}$HA) is characterized by initial photoreceptor degeneration and glial activation, followed by vasoregression and neuronal degeneration (Feng et al., 2009, PLoS One 4: e7328). It is unknown whether glial activation contributes to neurovascular degeneration after photoreceptor degeneration. We characterized the reactivity of Müller glial cells in retinas of rats that express defective polycystin-2.

Methods: Age-matched Sprague-Dawley rats served as control. Retinal slices were immunostained for intermediate filaments, the potassium channel Kir4.1, and aquaporins 1 and 4. The potassium conductance of isolated Müller cells was recorded by whole-cell patch clamping. The osmotic swelling characteristics of Müller cells were determined by superfusion of retinal slices with a hypoosmotic solution.

Results: Müller cells in retinas of transgenic rats displayed upregulation of GFAP and nestin which was not observed in control cells. Whereas aquaporin-1 labeling of photoreceptor cells disappeared along with the degeneration of the cells, aquaporin-1 emerged in glial cells in the inner retina of transgenic rats. Aquaporin-4 was upregulated around degenerating photoreceptor cells. There was an age-dependent redistribution of Kir4.1 in retinas of transgenic rats, with a more even distribution along glial membranes and a downregulation of perivascular Kir4.1. Müller cells of transgenic rats displayed a slight decrease in their Kir conductance as compared to control. Müller cells in retinal tissues from transgenic rats swelled immediately under hypoosmotic stress; this was not observed in control cells. Osmotic swelling was induced by oxidative-nitrosative stress, mitochondrial dysfunction, and inflammatory lipid mediators.

Conclusion: Cellular swelling suggests that the rapid water transport through Müller cells in response to osmotic stress is altered as compared to control. The dislocation of Kir4.1 will disturb the retinal potassium and water homeostasis, and osmotic generation of free radicals and inflammatory lipids may contribute to neurovascular injury.

Inhibition of Notch signaling increases photoreceptor genesis in mESC-derived retina organoids

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The differentiation of pluripotent stem cells into retinal cell types opens up new possibilities for studies on retinogenesis, retinal degeneration and regeneration. Here, we modified recently developed mouse embryonic stem cell (mESC) three-dimensional differentiation approaches, to make the protocol independent of a transgenic eyefield reporter, and investigated the efficiency of mESC derived retinogenesis at all steps of the protocol. Further, we investigated the effect of Notch signaling inhibition on retina organoid-genesis.

We achieved efficient eyefield induction (82 ± 12 SD% of aggregates, N = 7), as well as patterning into RPE and neural retina domains. Further, upon trisecting aggregates into three evenly sized pieces, the majority (87 ±3 SD% of aggregates, N = 4) generated big, stratified retinal tissue, reminiscent of early postnatal retina in vivo. Each aggregate retina had between 0.41 to 1.9 mm (1.4 mm ± 0.4 SD) photoreceptor layer circumferential length. Inhibition of Notch signaling by DAPT treatment prevented the differentiation of retinal bipolar cells and Müller glia, reduced progenitors and organoid culture time-dependently increased Crx+ photoreceptors.

Our results suggest that our novel approach makes the generation of mESC derived, stratified retina organoids simpler and independent of an eyefield transgenic fluorescent reporter. Further, differential responses of retina organoids to Notch inhibition at different culture times suggests that progenitors in retinal organoids pass through different intrinsically regulated competence states.

Expression and function of Myocyte enhancer factor 2c (Mef2c) in the retina

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Background: Photoreceptor-specific genes are regulated by a hierarchical network of transcription factors, including the master regulators Cone rod homebox (Crx) and Neural retina leucine zipper (Nrl). Recent findings indicate that Myocyte enhancer factor 2c (Mef2c) is expressed in the retina and its transcription levels are downregulated in several mouse models of retinal degeneration. Mef2c was originally described as a muscle-enriched transcription factor but only little is known about its putative function in the retina. Therefore, we studied the expression and function of Mef2c in the retina.

Methods: To characterize the different Mef2c mRNA isoforms in the retina, murine Mef2c was PCR-amplified from cDNA from 2 month old C57BL/6 mice, cloned and sequenced. The retina-specific expression of Mef2 family members and the temporal expression of Mef2c in the developing retina was assessed by qRT-PCR. Mef2c protein levels and localization in the retina were determined by Western blot and immunohistochemistry. Mef2c Chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-seq) was used to identify genomic targets of Mef2c. Sono-seq was carried out to map locations of high chromatin accessibility in the retina.

Results: The Mef2 family members Mef2c and Mef2d were abundantly expressed in the mouse retina. Mef2c expression steadily increases during postnatal development with highest expression in adult animals. Both Mef2c isoforms present in the retina contain the mutually exclusive exon 7α1 as well the β domain and differ only in the presence of the inhibitory γ domain. Immunohistochemistry showed consistent Mef2c expression in the outer nuclear layer. Sono-seq revealed 13802 open chromatin regions in the mature retina and Mef2c ChIP-seq identified 3630 target regions.

Conclusions: Here we showed that Mef2c is strongly expressed in the adult retina, which indicates a potential role in photoreceptor homeostasis. The ChIP-seq results together with further experiments such as Mef2c knockdown could help to elucidate the function of Mef2c in the adult retina.

Correlation between foveal involvement of degeneration and fixation stability in patients with choroideremia

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Purpose: Choroideremia (CHM) is a X-chromosomal disorder leading to blindness by progressive degeneration of choroid, retinal pigment epithelium (RPE) and retinal neurons. At the same time it leads to a progressive reduction of visual field and retinal sensitivity. With foveal involvement, some patients loose fixation stability, which might reduce reliability of psychophysical outcome measures. This study was performed to characterise structural retinal biomarkers correlating with fixation stability in microperimetry.

Methods: Data from 25 eyes of 13 patients (age 21-58y) with clinically confirmed CHM were analysed including spectral domain optical coherence tomography (HRA+OCT, Heidelberg Engineering), fundus autofluorescence (FAF) and microperimetry. Choroideal and retinal thickness was quantified in the fovea. Locations of fovea, and residual RPE islands were set in reference to fixation stability (P1/P2) measured during microperimetry examination (MAIA, Centervue)

Results: Central retinal and choroideal thickness showed no significant correlation with fixation stability P1 [$R^2_{retina} = 0.08$; $R^2_{choroid} = 0.14$] or P2 [$R^2_{retina} = 0.04$; $R^2_{choroid} = 0.13$]. In a semiquantitative analysis of foveal location in relation to residual RPE area on FAF (n = 25) all nine eyes with island border running through the fovea ('split fovea') showed a fixation stability of < 95 %, whereas the other 16 eyes with foveal sparing (n = 14) or complete foveal atrophy (n = 2) always reached fixation stability of ≥ 95 % in P2.

Conclusion: Multimodal analysis of foveal involvement by FAF and SD-OCT is superior to predict fixation stability in CHM patients compared to central retinal or choroidal thickness measures alone. Acute involvement of the fovea ('split fovea') leads to poor fixation stability and is predicted to cause variability in other psychophysical outcome measures. This has important implications e. g. for patient selection in interventional trials where psychophysical outcome measures contribute to safety and efficacy analysis.

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