PRO RETINA FOUNDATION

15th PRO RETINA
RESEARCH-COLLOQUIUM POTSDAM

CONFERENCE REPORT

Retinal Degeneration

Update on Innovative Approaches

An Interdisciplinary Dialogue

April 09, 2021

THIS MEETING IS SUPPORTED BY

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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 (2021), PRO RETINA Deutschland e. V. counts more than 6,400 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, April 09, 2021

14:00 – 14:05 **Welcome remarks**
Franz Badura (Amberg, Germany)

14:05-14:45	**Session 1**	**Selected Poster Presentations**
		Eight abstracts to be selected
	14:45-15:15	**Keynote Lecture** Systems pharmacology for acquired and inherited retinal diseases *Krzysztof Palczewski (University of California, Irvine, USA)*

15:15-16:00 **Coffee break – Poster Session I**

16:00-16:45	**Session 2**	**Precision medicine – innovative approaches**
	16:00-16:15	Splicing modulation therapy for inherited retinal disease using antisense oligonucleotides *Rob W. J. Collin (Radboud UMCNijmegen, The Netherlands)*
	16:15-16:30	Gene therapy for RPGR-related RP– strategy and first results froma clinical trial *Yasmina Cehajic-Kapetanovic(University of Oxford, UK)*
	16:30-16:45	Pharmacotherapy for Stargardtdisease *Hendrik Scholl (Institute of Molecular and Clinical Ophthalmology Basel – IOB, Switzerland)*

16:45-17:30 **Coffee break – Poster Session II**

PROGRAMME

17:30-18:30	**Session 3**	**Innovative model systems and technologies**
	17:30-17:45	An introduction to retinal organoids *Patricia Berber (University of Regensburg, Germany)*
	17:45- 18:00	Human retina organoid model for macular degeneration *Mike Karl (DZNE, Dresden, Germany)*
	18:00-18:15	Using single cell RNA-seq to investigate retinal injury and disease *Joshua R. Sanes (Harvard University, USA)*
	18:15-18:30	Transcriptional engineering, epigenome editing and epigenetic screens *Stefan Stricker (Helmholtz Zentrum München, Germany)*

18:30-18:45 Concluding remarks

Announcement of the poster prize winners

POSTER ABSTRACTS

Regulation of neuroprotective factors in muller glia using artificial transcription factors

Cavit Ağca[1,2,], Mehri Ahmadian[1,2], Iskalen Cansu Okan[1,2], Yeşim Tütüncü[1,2], Halit Yusuf Altay[1,2]*

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**Corresponding Author*

Purpose: Visual impairment and blindness has a huge impact on a persons' quality of life. Irrespective of the genetic cause, photoreceptor and ganglion cell loss is the hallmark for majority of the blinding diseases in ophthalmology. The biggest challenge for any blinding disease is the availability of the treatment options. Therefore, the ongoing neuronal loss cannot be stopped and complete loss of vision is inevitable. In order to have a treatment, novel neuroprotective approaches that will slow down or halt neuronal cell loss and at the same time maintain the function of retinal neurons are required. The main aim of this project is to develop and validate multiplex gene regulation-based therapies for neuroprotection. This unique approach will exploit gene transactivators (ATAs) that aims for overexpression of neuroprotective genes in Muller glia at different doses in retina.

Methods: To generate repertoire of ATAs that overexpress *Fgf2, Lif* and other neuroprotective factors, we designed a gRNA screen that depends on expression of dCas9-VP64 in a stable Muller glia line and the corresponding vector that expresses gRNA and an eGFP reporter. This have advantages like reducing the size of the vector by several kbs and increasing the transfection efficiency of the vector that expresses gRNA. Transfected dCas9-VP64 Muller glia were FACS-sorted and overexpression levels of each gene was assessed using ddPCR method.

Results: We cloned gRNAs that were designed against *Fgf2* and *Lif* promoter regions. We further tested the two component system using *Fgf2* gRNAs and the Muller glia cell line that has 7 copies of dCas9-VP64. We identified several gRNAs that upregulated *Fgf2* at different doses. Similarly, we were able to find a gRNA that also overexpresses *Lif*. These results showed that it is feasible to control overexpression of neuroprotective factors using ATAs.

Conclusion: We previously anticipated to have different fold levels of upregulation for different gRNAs which was important to establish dosages for neuroprotective factors. Our findings for *Fgf2* was promising and suggested that the anticipated dosage adjustment is feasible at least for *Fgf2* overexpression. We are now cloning the corresponding *Fgf2* and *Lif* gRNAs into AAV vectors and plan to test in *vivo* efficiency of ATAs in retina.

Self-assembling lipid nanoparticles enhance compound delivery and increase neuroprotective efficacy of VCP inhibitors in Rho^{P23H} retinal explants

Blanca Arango-Gonzalez [1,], Merve Sen [1,2,*], Stefano Salmaso [3], Paolo Caliceti [3], MD Al Amin [3], Eva Kickova [3], Sylvia Bolz [1], Marius Ueffing [1]*

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** Both authors share equal authorship*

Purpose: The dominant P23H mutation in rhodopsin (Rho^{P23H}) causes misfolding, endoplasmic reticulum (ER) stress, and unfolded protein response (UPR), leading to rod photoreceptor degeneration and autosomal dominant retinitis pigmentosa (ADRP). We have recently demonstrated that pharmacological inhibition of the ATP-driven chaperone valosin-containing protein (VCP), a molecular checkpoint for protein quality control at the ER, can halt retinal degeneration in Rho^{P23H} transgenic rat retinal explants. To optimize the delivery of VCP inhibitors to the retina, we designed a drug delivery system that provides suitable drug availability and a sustained release.

Methods: We tested two different formulations to encapsulate the VCP inhibitor, ML240, namely micellar carrier obtained by self-assembling of methoxy-poly(ethylene glycol)$_{5kDa}$–Cholane (mPEG-CA) or methoxy-poly(ethylene glycol)$_{5kDa}$–Cholesterol (mPEG-CO) polymers. ML240 micellar release was evaluated by dialysis and analyzed by reverse-phase-HPLC. The neuroprotective effect of the released drug was evaluated using retinal organotypic cultures from Rho^{P23H} rats treated with both micelles and compared to the corresponding controls. Photoreceptor cell survival and functional and structural integrity were evaluated by cell row quantification and TUNEL assay. Rho distribution and microglia activation were assessed using specific antibodies.

Results: Both drug-loaded nanosystems provide for prolongued released of ML240. Application of ML240 using either of the two delivery systems in retinal organotypic cultures resulted in a similar increase of cell viability compared to VCP inhibitor treatment alone, however, at a lower dose. The percentage of TUNEL(+) cells in the outer nuclear layer (ONL) indicates that both VCP inhibitor-loaded micellar formulations significantly reduced the number of dying cells. More importantly, the ONL of treated RhoP23H retinas contained more cell rows than the controls. The treatment with either of the two drug delivery systems also corrected the aberrant distribution of Rho to the outer segment. mPEG-CA/ML240, however, resulted in significantly reduced activation and lower translocation of microglia to the outer neuroretina compared to free or mPEG-CO encapsulated ML240.

Conclusions: In summary, mPEG5kDa-Cholane encapsulation of ML240 proved superior over free ML240, or mPEG5kDa-Cholesterol mediated delivery, allowing a slower and sustained release of the drug at reduced dose and toxicity. Moreover, mPEG5kDa-Cholane micelles-mediated drug delivery reduced microglial activation in the explanted neuroretina.

Complement Factor H (CFH) dysregulation in Retinal Pigment Epithelium (RPE) cells leads to retinal degeneration in a novel human-porcine co-culture system

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[2] Translational Research Institute, University of Queensland, Australia

Purpose: Age related macular degeneration (AMD) is a progressive and degenerative disease of the macula, leading cause of blindness in the elderly population. AMD is a very complex disease, caused by an interplay of diverse risk factors (genetic predisposition, age and lifestyle factors). One of the main genetic risks corresponds to the Y402H polymorphism in the Complement Factor H gene (*CFH*/FH), an inhibitor of complement system activation. In our previous work, we show that FH holds additional functions and FH loss alters RPE cell homeostasis. In this study we investigated the impact of RPE cells damaged by FH loss on the retina in a novel co-culture system.

Methods: We established a co-culture model comprised of hTERT-RPE1 cells and porcine retinal explants, obtained from the visual streak of the porcine retina, rich in cone photoreceptors (PR). We used shRNA to silence *CFH* in hTERT-RPE1 cells (si*CFH*) prior to co-culture initiation (n=6). Cultures were maintained for 3 days, then fixed and sectioned for imaging or lysed for RNA extraction and qPCR analyses (fold change).

Results: Retinas cultured with si*CFH* RPE cells showed signs of retinal degeneration compared to retinas cultured with RPE controls (SEM and p-values are shown). In detail, we observed a reduction in retinal thickness (180±11 vs 140±9 µm, $p<0.05$), outer nuclear layer (ONL) thickness (50±5 vs 30±4 µm, $p<0.05$), number of PR cells in the ONL (11±3 vs 6±2 µm, $p<0.01$) and percentage of displaced cones (12 vs 20%). Retinas cultured with *CFH*-silenced RPE cells showed increased expression of the inflammatory cytokine Intercellular Adhesion Molecule 1 (ICAM1, 2.5±0.5, $p<0.05$) and increased expression of glucose (GLUT1, 1.6± 0.3, $p<0.05$) and lactate (MCT4, 2.3±0.4, $p<0.05$) transporters.

Conclusions: Our data support the hypothesis that RPE-derived FH plays a wider role in retinal homeostasis out with its known complement-regulatory function. As a result of *CFH* silencing, RPE cells are unable to properly support the neuroretina, causing changes in the inflammatory and metabolic profile of the retina, and ultimately leading to photoreceptor loss. These findings may help elucidate the function of FH in the retina and our co-culture system may provide a suitable model to test medical interventions.

Molecular properties of human GCAP2 and its retinal dystrophy-associated variant G157R

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Purpose: Phototransduction is a cascade of biochemical events that allow the conversion of a light stimulus into an electrical signal. The guanylate cyclase-activating proteins (GCAPs) are neuronal Ca^{2+} sensors which detect the light-induced decrease of intracellular Ca^{2+} and regulate Guanylate Cyclases (GCs) in a Ca^{2+}-dependent manner, thus restoring the dark-adapted state of photoreceptors. GCAP1 and GCAP2 are the most prominent GCAP isoforms in human photoreceptors and respond sequentially to variations in Ca^{2+} concentration according to a Ca^{2+}-relay model. While GCAP1 was extensively characterized in the last decades, no comprehensive biochemical investigation of the molecular proprieties of human GCAP2 was available. Here, we present a thorough characterization of human GCAP2 and of the effects of its inherited-retinal dystrophy (IRD)-associated G157R variant.

Methods: The conformational changes of human GCAP2 and G157R variant in the presence of Mg^{2+} or Ca^{2+}, corresponding to the GC-activating/inhibiting states respectively, were evaluated by Circular dichroism spectroscopy. Analytical Size Exclusion Chromatography was employed to evaluate the quaternary structure and the dimerization process of the variants, while enzymatic assays allowed to assess the regulation of GC by GCAPs.

Result: GCAP2 can bind both Mg^{2+} and Ca^{2+} ions, although each ion exerts specific effects. Indeed, Mg^{2+} is required for GCAP2 correct folding and formation of a compact dimer in solution, whereas Ca^{2+}-binding results in a conformational change and a shift in dimerization equilibrium towards the dynamic switch between monomers and dimers. The G157R variant, however, displayed secondary but not tertiary structure as in a molten globule state, with reduced cation affinity and remarkable propensity to form aggregates. Finally, we observed that GC1 activation by GCAP2 was significantly lower than that of GCAP1 and substantially Ca^{2+}-independent, while that of the G157R variant was even lower than wild-type GCAP2.

Conclusion: Our findings suggest that in human photoreceptors GCAP2 might not be directly involved in the phototransduction cascade, but rather in processes not occurring in the outer segment. However, such processes would be altered by the G157R mutation, causing misfolding, aggregation and possibly severe cell toxicity due to massive retention in the inner segment.

Role of complement receptors and microglia in age-related macular degeneration

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Purpose: Age-related macular degeneration (AMD) is a leading cause of blindness among the elderly within developed countries. Genetic variants in the complement system and overly activation of microglia are associated with retinal degeneration, which makes them a therapeutic target for AMD. Here we hypothesize, that microglia interact via receptors with the overactive complement system to sustain a chronic inflammatory state in AMD.

Methods: Full body *Knockout* of anaphylatoxin receptors *(C3aR/C5aR)* were exposed to 15,000 lux white light for 1 h. The effect of receptor knockout on microglia reactivity was analyzed by immunohistochemically staining of retinal sections or flat mounts and gene expression analysis. Spectral domain optical coherence tomography (SD-OCT) was performed to quantify retinal thickness and thereby retinal degeneration.

Results: Iba-1 staining of retinal flat mounts and sections showed migration of amoeboid-shaped microglia in in the subretinal space four days post light damage without differences in experimental groups. mRNA expression of complement factors *C3, C1qa* and *CFH* as well as pro-inflammatory markers *iNOS* and *TSPO* were elevated after light exposure. In *Knockout* animals elevated inflammation without light exposure was indicated. SD-OCT analysis showed no rescue of retinal degeneration by anaphylatoxin receptor depletion. Importantly, $C5aR^{-/-}$ mice showed a very high susceptibility to light damage with a significant thinner retina than wildtype animals following light exposure.

Conclusion: Partial unexpectedly, anaphylatoxin receptor deficient animals displayed no changes in microglia activity or retinal thickness after light damage. Elevated mRNA expression levels of complement components suggest a compensatory mechanism in complement pathways in these animals.

Towards the development of new biomarkers for retinal neurodegeneration

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Background: Inherited retinal degeneration (IRD), such as retinitis pigmentosa or Leber's congenital amaurosis, is a group of diseases characterized by progressive photoreceptor death, leading to blindness. These diseases are still untreatable. A key problem for therapy development is the lack of clinical biomarkers that would enable a rapid assessment of treatment efficacy. Techniques currently used in the clinic for diagnosis, monitoring of disease progression and detection of patient response to treatment are essentially quantifying a loss of signal over a high background, providing a poor signal-to-noise ratio. The aim of the present study is to promote the development of novel calpain-activity based biomarkers that could be used in combination with adaptive optics scanning laser ophthalmoscopy (AO-SLO), to indicate photoreceptor cell death in the living retina with single-cell resolution.

Methods: An amino-chloromethylcoumarin (CMAC)-based probe was used for calpain activity detection on live organotypic retinal explants from two retinal degeneration mouse models *(rd1 and Rho$^{P23H/+}$)*. CMAC was dissolved in the culture medium and incubated for different amounts of time (1h, 3h, 6h, and 24h). Calpain-activity-dependent fluorescence was visualized after tissue fixation and cryosectioning. Potential toxicity of the probe on live tissue was investigated using the TUNEL assay.

Results: In live organotypic retinal explants derived from *rd1* animals, an increasing number of calpain activity positive cells in the photoreceptor layer was detected when CMAC was incubated from 1h to 6h. Between 6h and 24h, a plateau seemed to be reached. In wild-type explants the number of calpain positive cells was relatively low. Some calpain active cells were also detected in the photoreceptor layer of explants derived from *Rho$^{P23H/+}$* animals. The TUNEL assay did not reveal an increase in dying cells after treatment with CMAC.

Conclusion and Outlook: CMAC may be used to assess calpain activity on live organotypic retinal explants and, when combined with appropriate imaging, may be promising for *in vivo* photoreceptor cell death detection. The numbers of TUNEL positive cells after treatment with CMAC suggests that it is not toxic for the retina. A novel CMAC-based biomarker could potentially detect a signal increase over a negative background and afford higher sensitivity both for early disease diagnosis and the rapid assessment of the treatment efficacy.

The role of *VTN* genetic polymorphism rs704 on vitronectin function and AMD-related processes

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Purpose: Vitronectin (VTN) is a secreted protein abundant in blood and in the extracellular matrix (ECM), where it participates in the regulation of cell adhesion and migration, innate immunity, ECM remodeling and angiogenesis. Fritsche et al. 2016 (doi: 10.1038/ng.3448) reported an association of non-synonymous variant rs704C>T in the *VTN* gene with age-related macular degeneration (AMD), a complex retinal degenerative disorder with abnormal extracellular sub-RPE and subretinal deposits (so-called drusen and subretinal drusenoid deposits, respectively) as a key feature. Of these characteristic deposits, VTN represents a major constituent. This study aims to clarify the role of VTN in choroidal neovascularization, a critical feature of late-stage AMD.

Methods: The effect of heterologously expressed *VTN* variants VTN_rs704:C (non-AMD-associated) and VTN_rs704:T (AMD-risk associated) on ECM deposition by ARPE-19 cells was studied *via* immunostaining of ECM proteins followed by confocal microscopy imaging. Besides ECM scaffolding components (laminin, fibronectin, elastin and collagen VI), the angiogenesis regulators VEGF and PAI-1 were also included in this analysis. In addition, the effect of the two recombinant VTN isoforms was investigated in cell adhesion, migration and formation of capillary-like tube structures in the endothelial cell line HUVEC.

Results: The ECM of ARPE-19 cells heterologously expressing VTN_rs704:T revealed increased VTN expression compared to ECM produced by VTN_rs704:C expressing cells. Furthermore, VTN expression increased the deposition of the ECM scaffolding proteins fibronectin, collagen VI and elastin, as well as of VEGF and PAI-1, with VTN_rs704:T showing a stronger effect than VTN_rs704:C. The presence of VTN in ECMs (produced by ARPE-19) promoted HUVECs adhesion to those matrixes. Both VTN isoforms decreased the ability of HUVECs to form capillary-like structures, while VTN_rs704:C but not VTN_rs704:T slightly reduced HUVECs migration.

Conclusions: Taken together, our experiments reveal an involvement of VTN in cellular processes linked to the pathology of choroidal neovascularization, with functional differences between the non-AMD and AMD-risk associated VTN isoforms. Further studies to elucidate the role of VTN and rs704 in the pathogenesis of AMD are ongoing.

Characterization of a novel GCAP1 variant found in an Italian family affected by Cone Dystrophy

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Purpose: The Guanylate Cyclase Activating protein 1 (GCAP1) is a neuronal Ca^{2+}-sensor protein which regulates the Guanylate Cyclase (GC) in a Ca^{2+}-dependent manner, thus triggering the shutoff of the phototransduction process. In recent years over 20 missense mutations have been related with autosomal dominant degenerative retinal diseases such as cone dystrophy (COD) and cone-rod dystrophy (CORD). The present study is focused on the structural and functional characterizationof the novel COD-associated GCAP1 variant N104H.

Methods: The Ca^{2+}-sensitivity of the mutant was evaluated by monitoring the electrophoretic mobility on SDS-PAGE under different cation-loading conditions and by a titration assay in the presence of a chromophoric chelator. Circular dichroism spectroscopy was employed to investigate changes in protein secondary and tertiary structure upon Mg^{2+} and Ca^{2+}-binding, while Dynamic light scattering was performed to monitor the variations in hydrodynamic radius. GC activity was assessed under activating and inhibiting Ca^{2+} concentrations.

Results: GCAP1 variant N104H did not show any hint of unfolding, as the near UV spectrum displayed small but appreciable differences in tertiary structure compared to the wild type. Dynamic light scattering measurements showed no signs of aggregation and highlighted a significantly higher hydrodynamic radius in the presence of Ca^{2+} with respect to the wild-type. The decrease in electrophoretic mobility exhibited by the variant suggested a reduction in Ca^{2+}-sensitivity which was confirmed by the Ca^{2+} titrations in competition with the chromophoric chelator 5-5'Br_2 BAPTA, showing a decreased affinity for Ca^{2+} in each binding site. Finally, GC activation by N104H variant was 1.5-fold higher than the wild-type at both activating and inhibiting Ca^{2+} concentrations, suggesting a constitutive cGMP synthesis by the enzyme.

Conclusion: Our results suggest that the novel COD-associated GCAP1 variant N104H is not prone to aggregation or unfolding, but it exhibits reduced Ca^{2+} sensitivity and a different conformation upon Ca^{2+}- binding. Nevertheless, such subtle differences are sufficient to render the Guanylate Cyclase constitutively active, thus altering Ca^{2+} and cGMP homeostasis and ultimately resulting in photoreceptors progressive degeneration.

Comparison of complement in different retinal degeneration models

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Purpose: Dysregulated retinal inflammation is a common feature of many blinding eye diseases. As part of the innate immunity, the complement system can be an expediting and aggregating factor that may promote retinal degeneration. Indeed, Stargardt disease type 1 (STGD1) is associated with the deposition of bisretinoid in retinal pigment epithelium (RPE) cells that causes a complement response leading to para-inflammation and to a slow degeneration of the RPE and, subsequently, of photoreceptors. Bisretinoid accumulation in STGD1 occurs because of a mutation in the ATP binding cassette subfamily A member 4 (ABCA4). In an ABCA4$^{-/-}$ mouse model, we assessed retinal complement accumulation and compared it to findings from the acute retinal degeneration model induced by sodium iodate ($NaIO_3$) administration. Preparing the translation of findings about retinal complement from mouse studies to human, we evaluated the complement deposition in human retinal explants treated with serum containing auto-antibodies known to elicit complement activation.

Methods: Immunofluorescence staining was employed to examine complement deposition and morphological changes in ABCA4$^{-/-}$, $NaIO_3$ treated mice and human retinal explants. Complement expression was assessed by quantitative real-time PCR (qPCR).

Results: Concluding from cell counts, neurodegeration was most pronounced in the $NaIO_3$ model, followed by that in human retinal explants and it was barely detectable in ABCA4$^{-/-}$ at 24 weeks of age. C3, a key player of the complement cascade irrespective of its mode of activation, was mainly located in the ganglion cell layer in ABCA4$^{-/-}$ mice, while it accumulated at photoreceptor outer segments and RPE in the $NaIO_3$ model. We found a prominent C1S staining of the outer nuclear layer three days after disease induction the $NaIO_3$ model only. qPCR data confirmed C3 upregulation in both mouse models, while surprisingly no changes were detected for C1s and *Cfh*. Interestingly, in all our models including the human retinal explants, microglia accumulated complement regulatory factor H (CFH) as determined by immunostaining.

Conclusion: While we observed a clear complement targeting to photoreceptors in the $NaIO_3$ model, similar findings were not observed in ABCA4$^{-/-}$ mice or human explants. The very slow progression of retinal degeneration in the ABCA4$^{-/-}$ model seems to be associated with mild changes that could only be measured via sensitive qPCR. In sum, we gained an extended view on the complement reaction in slow and acute retinal degeneration useful for future studies of complement modulating therapies.

Investigation into the neuroprotective effect of TSPO in Müller glia in a murine model of transient ischemia

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Purpose: The translocator protein (TSPO) is an integral membrane protein located in the outer mitochondrial membrane and is in the retina predominantly expressed by Müller glia, microglia, blood vessels and pigment epithelial cells. Treatment of damaged retinae with agonistic TSPO-ligands indicate a promising therapeutic potential. Described effects include dampened microglial reactivity and an improved preservation of retinal neurons. However, the mechanism by which TSPO-agonists mediate the neuroprotective effect remains largely unknown. Since TSPO appears to be involved in the import of cholesterol to the inner mitochondrial membrane - the initial place of steroidogenesis - we have the hypothesis that the beneficial actions of TSPO-agonists work via an increased production of neurosteroids in the damaged or diseased retina.

Methods: We are aiming to uncover the neuroprotective activity of TSPO by Müller glia specific conditional TSPO-knockout using Glast-Cre^{ERT2} in comparison with gain of function studies using TSPO agonists in a murine model of transient ischemia/reperfusion. To this end, measurement of the neurosteroid levels after lesion are performed to uncover the involvement of TSPO in steroidogenesis (collaboration with Michael Schumacher, Paris). Morphometric analysis and immunohistochemistry are providing insights into the TSPO-mediated preservation of retinal neurons. Moreover, alterations of the mitochondrial metabolism in TSPO-knockouts are assessed by measurements of the membrane potential (JC-1), fluorescence lifetime imaging (FLIM) of NADH and the respiratory chain via seahorse analysis (collaboration with Christian Wetzel, Regensburg). Possible changes in TSPO localization are examined by super-resolution microscopy (STED). Finally, analysis of intracellular calcium levels and the capacity to counteract hypoosmotic stress will be uncover of the consequences of TSPO-loss for Müller glia metabolism in the context of ischemia.

Results: TSPO appears to transport cholesterol to the inner mitochondria membrane; thereafter a metabolic cascade could process it to allopregnanolone – a potential neuroprotective steroid hormone – in Müller cells. Initial steroid hormone measurements show that major metabolites of this synthesis pathway are detected in native retinal tissues supporting this theory. In addition, TSPO agonists are shown to compensate barium induced hypoosmolar swelling of native Müller cells and support the integrity of the mitochondrial membrane potential under hypoosmolar and glutamine induced stress.

Conclusion: With this project, we will shed light on the endogenous function of Müller glia TSPO in the ischemic retina. To this end, we will use our Müller glia specific knockout model and the TSPO-ligands as a combinatory approach to uncover the involvement of TSPO in response to retinal damage in more detail. Finally, this study will allow a further evaluation of the therapeutic potential of TSPO ligands as a treatment for retinal diseases.

Targeted cyclosporin A nanoparticles enable cell-specific anti-VEGF therapy

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Purpose: The neovascular ocular diseases retinopathy of prematurity, proliferative diabetic retinopathy and age-related macular degeneration are all leading causes of blindness. Although the origin of neovascularization differs, they share the same pathomechanism with uncontrolled growth and delocalization of blood vessels. Mechanistically, the vascular endothelial growth factor (VEGF) was identified as a key factor for pathological angiogenesis. However, since VEGF is also an essential survival factor for various other cells in the retina, physiological VEGF levels are crucial for overall ocular homeostasis and integrity. Therefore, we reasoned that a cell-specific, VEGF modulating nanotherapy could be a major accomplishment in the treatment of neovascular ocular diseases.

Methods: For cell-specific drug deliver after i. v. injection, targeted nanoparticles that accumulate in endothelial and retinal pigment epithelial (RPE) cells were used and, for the intracellular anti-VEGF therapy, loaded with Cyclosporin A (CsA). CsA interferes with the VEGF signaling pathway in endothelial cells at different intracellular sites and additionally reduces TGFβ-related VEGF production in RPE cells.
To investigate the anti-angiogenic efficacy of these nanoparticles, the mouse model of retinopathy of prematurity was used. After a single dose treatment, the extent of neovascularization, VEGF and VEGF-R2 protein or mRNA levels were quantified using immunohistochemistry, ELISA, and quantitative RT-PCR.

Results: The quantitative determination of the neovascular area revealed that the CsA loaded nanoparticles efficiently reduced the extent of vessel proliferation, neovascular tufts and leaky vessels down to levels comparable with healthy mice. Mechanistically, the analysis of VEGF-R2 protein levels showed that the treatment significantly decreased elevated receptor levels down to healthy levels in the whole retina. For VEGF, similar results were achieved with a reduction of VEGF protein levels down to physiological levels in both, the RPE-choroid complex, and the retina, indicating successful combat of the pathological VEGF overexpression. Additionally, the interference of CsA loaded nanoparticles with the TGFβ related VEGF production was reflected by the modulation of VEGF, TGFβ, and TGFβ-R2 messenger RNA expression.

Conclusion: Taken together, our results demonstrate the enormous potential of CsA loaded nanoparticles as a novel therapeutic option for the systemic, cell-specific anti-VEGF therapy of neovascular ocular diseases, as they impressively counteract the VEGF-driven neovascularization by restoring rather than suppressing physiological VEGF and VEGF-R2 levels.

Features of early age-related macular degeneration in individuals younger than 50 years are associated with a substantial risk for progression to late disease stages – results from 18-year follow-up in the KORA study

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Purpose: Age-related macular degeneration is generally defined as a disease affecting individuals aged 50 years and older. Recently, we detected features resembling early AMD in participants of the KORA (Cooperative Health Research in the Augsburg Region) study that were markedly younger. This raised the question, whether the detected features were truly early AMD-related or artefacts mimicking AMD. We thus set out to evaluate these individuals for their risk to progress to late AMD stages in a long-term follow-up.

Methods: In the fourth independent baseline KORA survey (KORA S4), the general population of the region of Augsburg aged 25-75 years had been recruited between 1999 and 2001. Among the ~1000 participants aged 35-55 years, including persons with early AMD at age <50, a follow-up (KORA FIT) has been conducted 18 years later (individuals now aged 53 – 73 years). In both, baseline and follow-up, color fundus images were taken using non-mydriatic fundus cameras. These images were manually graded applying the Three Continent AMD Consortium Severity Scale, which distinguishes between no, mild/moderate/severe early and late AMD stages.

Results: A total of 506 participants had gradable images for at least one eye at baseline and follow-up (mean age at baseline 44.6 ± 5.5 years; 44.3% men). At baseline, 4.3% of participants (n=22) were graded as early AMD, none as late AMD. At follow up (mean follow up time 17.8 years, standard error=0.6), 9.8% were graded as early AMD (n=50) and 1.5% as late AMD (n=8). Among early AMD participants at baseline, 22.7% progressed to late AMD (n=5 of 22), compared to 0.6% among those without AMD at baseline (n=3 of 484). This yields an unadjusted Odds Ratio (OR) of 47.2 for progression to late AMD comparing individuals with baseline early AMD with those AMD-free, and an OR of 37.3 [95%-confidence interval=7.9, 175.4], P-value=$4.2*10^{-6}$, when adjusting for age.

Conclusion: Analyzing an 18-year follow-up of individuals with baseline age between 35 and 55 years, we found that persons with features resembling early AMD at baseline had a substantially increased risk of developing late AMD when compared to individuals without AMD at baseline. This does not only underscore that the identified features in these young individuals reflect truly early AMD, but also that AMD is not solely related to high age. Our results suggest that AMD definition and diagnostic target population need to be revisited.

Adeno-associated viral vector serotype 8 induces lot-specific innate immune responses in human plasmacytoid dendritic cells

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Purpose: Retinal gene therapy with adeno-associated virus (AAV) vectors is a promising approach for the treatment of inherited retinal degenerations. Nevertheless, an increasing number of pre-clinical and clinical studies show that retinal AAV-mediated gene therapy cannot only induce ocular but also systemic immune responses. Macrophages are one of the major systemic innate immune cells involved in pathogen recognition, and plasmacytoid dendritic cells (pDCs) are important sensors of viral infections. Here, we analysed innate immune responses to AAV8 in human macrophage-differentiated THP-1 cells (THP-1 cells) and primary human pDCs.

Methods: THP-1 cell and pDC cell models were established by incubating them with ligands to key pattern recognition receptors (PRRs). Subsequently, the cells were stimulated with different lots of AAV8-CMV-eGFP from different production systems and different manufacturers. Prior to this, vector genome numbers of the AAV8 vector lots were quantified by droplet digital PCR. After stimulation, inflammatory cytokines/chemokines and interferons (IFN) type I in the supernatant were analysed by sandwich ELISA and Bio-Plex.

Results: THP1 cells and pDCs responded to PRR ligands by secreting pro-inflammatory cytokines/chemokines and/or type I IFNs. Although THP-1 cells became transduced, they showed very little or no immune responses to AAV8 vectors. In contrast, pDCs were not transduced but exhibited lot-specific immune responses reflected by significant production of IP-10, MIP-1β, TNF-α and IFN-α and reactive proliferation to individual lots of AAV8. The immunogenicity of the vector batches could neither be related to the production system nor to the manufacturer.

Conclusions: AAV8 induces a cell type- and lot-specific inflammatory immune response in major innate immune cells. The lot-specific immunogenicity of AAV vectors might be related to impurities derived from the production process such as components of the cell culture medium, reagents used for AAV purification, endotoxins and host cell proteins. A thorough investigation of the actual immune-stimulatory effects of contaminants present in AAV preparations might help to reduce the immunogenicity of AAV vector preparations.

Genotyping inherited retinal disease in Northern Ireland

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Purpose: Diagnosis of an Inherited Retinal Disease (IRD) is a complex process, complicated by the phenotypic heterogeneity of this group of conditions. Historically, diagnosis was made based on clinical signs, family history, visual function and characteristic progression of these parameters over time. Advances in genotyping methods have now led to the identification of 271 causative genes, enabling molecular diagnosis in 70% of cases of IRD. Successful genotyping confirms the inheritance pattern and can indicate the visual prognosis. Molecular diagnosis is becoming increasingly important with the advent of genetic therapies. We report on the progress in genotyping the IRD population in Northern Ireland.

Methods: 397 affected patients with IRD have been recruited to the All-Ireland Retinal Degenerations Partnership Target 5000 (AIRDP) research study in Northern Ireland, UK. Participants who consented to this study had molecular testing either at a research grade level or through a CLIA–accredited laboratory. Some participants who had previous research-grade molecular findings had verification though confirmatory targeted variant testing (TVT). Participants with no previous testing underwent specific gene panels, familial variant testing (FVT) or full retinal dystrophy panel genotyping.

Results: The recruited Northern Ireland IRD patient cohort is 53% female with mean age 50 years (range 18 to 92). IRDs present in this cohort include retinitis pigmentosa, Stargardt's disease, cone rod, cone and macular dystrophies, Usher syndrome and Bardet Biedel syndrome, and the more rare choroideraemia, retinoschisis, Bestrophinopathy, fundus flavimaculatus, Sorsby's macular degeneration, central areolar choroidal dystrophy, Goldmann Favre syndrome, gyrate atrophy, hereditary peripapillary choroidal sclerosis, Leber's hereditary optic neuropathy, Leber's congenital amaurosis, and pigmented paravenous chorioretinal atrophy.
Genotyping has been performed in 265 participants, with a causative gene identified in 231 patients (87.2%). ABCA4 is the most common causative gene identified, responsible for 25% all molecularly explained IRD in Northern Ireland. USH2A is the second most common, responsible for 16%. 52 other genes were also identified, with 45 each affecting ≤5 patients.

Conclusion: An estimated 37% of Northern Ireland's IRD population have been phenotypically and genotypically defined to date, representing an opportunity for further natural history studies and interventional clinical trials. Further work is needed to determine whether the reported genetic heterogeneity is representative of global and national prevalence of IRD.

Mutant RHO^{Ile256del} clearance through the VCP/ERAD/proteasome pathway

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Purpose: Retinitis pigmentosa (RP) describes a group of hereditary retinal diseases hallmarked by photoreceptor degeneration. Mutations in the rhodopsin gene *(RHO)* are widespread, with more than 200 causative mutations, of which 30%-40% are related to autosomal dominant RP (ADRP). In Europe, the *RHO^{Ile256del}* mutation (deletion of isoleucine at position 255 or 256 in *RHO* gene) accounts for over 17% of familial ADRP cases (C. F. Inglehearn, 1991). From a clinical perspective, the severity of this mutation can be compared to *RHO^{P23H}*. In previous studies, we found that the degeneration of misfolded *RHO^{P23H}* follows the ERAD pathway and the clearance of RHO^{P23H} requires the ERAD effector valosin-containing protein (VCP), which can extract misfolded proteins from ER and escort them for proteasomal degradation. This study aims to characterize the subcellular structural and functional changes in *RHO^{Ile256del}* transfected mammalian cells, evaluate VCP inhibition's effects, and understand the pathological similarity between *RHO^{Ile256del}* and *RHO^{P23H}* transfected cells.

Methods: HEK293 and COS-7 cells were transfected with plasmids encoding *RHO^{WT}*, *RHO^{Ile256del}*, or *RHO^{P23H}*, tagged with GFP. Immunostaining was performed to reveal the localization and trafficking of transfected RHO-GFP and the co-localization between RHO-GFP with ER, endogenous VCP, ubiquitin, and PSMB5 (proteasome marker). Formation and degree of *RHO* protein aggregates were analyzed in transfected cells before and after proteasome- and VCP-inhibition.

Results: We found that RHO^{WT} mainly localizes at the plasma membrane, while mutant RHO^{P23H} and RHO^{Ile256del} accumulate in the cytosol forming cytoplasmic aggregates. The co-staining results using specific markers for RHO, ER, and VCP suggest that both RHO^{P23H} and RHO^{Ile256del} aggregates are retained within the ER, partially co-localizing with VCP. The measured average intensity of the RHO expression in the whole-cell showed a significant increase in cells expressing RHO^{P23H} vs. RHO^{WT} and RHO^{Ile256del} vs. RHO^{WT}, while there was also a significant difference in cells expressing RHO^{P23H} vs. RHO^{Ile256del}. RHO^{P23H} and RHO^{Ile256del} aggregates were ubiquitinated and partially transported to the proteasome system for degradation. VCP inhibition led to enhanced accumulation of high molecular weight (HMW) RHO-containing oligomers and aggregates.

Conclusion: Our results indicate similar clearance characteristics between *RHO^{Ile256del}* and *RHO^{P23H}* in transfected cells, including the excessive formation of misfolded RHO aggregates, retention in the ER, and, at least partially, interaction with VCP. Moreover, VCP inhibition affects the abundance of RHO aggregates. This suggests that the clearance of *RHO^{Ile256del}* also follows the VCP/ERAD/proteasome axis, raising a possibility that modulation of VCP could be considered as a potential therapy for *RHO^{Ile256del}-related* RP as well.

Evidence for a rod-to-cone lactate shuttle in the mammalian retina

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Purpose: In contrast to its early portrayal as a metabolic waste product and fatigue agent, lactate is now known to be a fuel that supports energy demand, produced continuously in aerobic condition ('Warburg effect'). The 'lactate shuttle' concept was pioneered by Brooks in 1985 who described the roles of lactate in the delivery of oxidative and gluconeogenic substrates. This notion was further refined as 'cell-to-cell lactate shuttle'. Lactate shuttling has been widely studied in skeletal muscle and in the brain, however, it is still unclear whether and where exactly this process takes place in the retina. Herein, we present data indicating the existence of a lactate shuttle between rod and cone photoreceptors.

Methods: We used immunofluorescence labelling to study the retinal expression pattern of lactate transporters (i.e. monocarboxylate transporters; MCTs) and other enzymes involved in energy metabolism. Organotypic retinal explant cultures and selective enzyme inhibition were used to alter energy metabolism and functionally validate expression patterns. ^{1}H-NMR (Nuclear Magnetic Resonance) spectroscopy was used to characterise metabolite and pathway changes after disrupting specific parts of cellular energy metabolism.

Results: We found MCT1 to be expressed on rods, while MCT2 was located on cones. Two important enzymes of the glycolytic pathway, pyruvate kinase M2 (PKM2) and lactate dehydrogenase A (LDHa), were expressed in rods. Moreover, two key components of gluconeogenesis, phosphoenolpyruvate carboxykinase 2 (PCK2) and lactate dehydrogenase B (LDHb), were found in cones. MCT1 inhibition in retinal explant culture caused extensive photoreceptor death, an accumulation of lactate in the tissue and a decreased lactate release into the surrounding medium. Curiously, cone viability was not affected by MCT1 inhibition. On the other hand, combined MCT1 and MCT2 inhibition resulted in extensive cone degeneration.

Conclusion: Our data suggests that rods use MCT1 to export lactate, while cones use MCT2 for its import. Cones may then use lactate to fuel gluconeogenesis or oxidative phosphorylation. Previous studies have shown an influence of lactate on mitochondrial biogenesis, therefore, the role of lactate in photoreceptors may go beyond its usage as a simple energy source. Additional studies, including Ca^{2+} imaging, may be used to further improve our understanding of retinal energy metabolism and lactate shuttling.

Keywords: energy metabolism, gluconeogenesis, glycolysis, Warburg effect

Investigating *ex vivo* porcine eyes as a model for testing intravitreal ocular drug delivery systems

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Typically, *in vivo* animal models (e.g. rats and rabbits) are used for testing ocular drug delivery systems (ODDS) in preclinical studies. However, the number of viable *in vitro* systems is sparse. *In vitro* and *ex vivo* systems generally provide important information of early stage ODDS in terms of expected efficacy and toxicity, they are faster and cheaper than *in vivo* systems, and they can limit the use of live animal experiments.

Here, we investigate the potential for using *ex vivo* porcine eyes obtained from the slaughterhouse for testing the vitreous retention time and biodistribution of different kinds of liposomal ODDS. Cationic, anionic, and neutrally charged liposomes with or without surface-grafted poly(ethylene glycol) (PEG) were prepared with a hydrophobic fluorophore and injected intravitreally into fresh porcine eyes. The distribution and residence time in the vitreous was assessed using an ocular fluorometer (OcuMetrics Fluoroton Master). After 1 h or 24 h post injection, the eyes were prepared for histological analysis to assess the tissue absorption of the liposomes. In parallel, the retinal toxicity of the liposomes was analyzed on retinal explant cultures from WT mice, using the TUNEL assay to quantify the amount of dying cells. Liposomes were characterized in terms of size and zeta potential to assess their surface charges.

Overall, it was found that liposomes with PEG polymers on the surface generally diffuse faster in the vitreous environment than liposomes without PEG. Cationic liposomes without PEG had the slowest diffusion. This is consistent with what is known about liposome diffusion in the vitreous. Histological sections showed retinal absorption for the PEG-coated liposomes, and the formulations did not appear to degrade in the vitreous.

The liposomes had a much shorter residence time in the vitreous compared to what is known from *in vivo* experiments. Most of the formulations were almost eliminated from the vitreous within 24 h. An elimination time of several weeks would be expected based on previous known *in vivo* rabbit data. This indicates that the *ex vivo* porcine eye system is not directly comparable to the *in vivo* situation, probably due to tissue degradation and post-mortem vitreous liquefaction. Nevertheless, the porcine *ex vivo* system enables comparative studies on the behavior of different ODDS types in the intact eye.

The effect of colony-stimulating factor 1 (CSF1) receptor inhibitor pexidartinib (PLX3397) on BV-2 microglia cells

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Purpose: Microglia are the resident retinal immune cell of the retina. Their activation contributes to various retinal neurodegenerative disorders and attracts considerable attention as a possible target for therapy. Colony-stimulating factor 1 (CSF1) is an important mediator for microglia survival and its pharmacological inhibition results in microglia death. PLX3397 is a synthetic CSF1 receptor antagonist that provides an effective depletion of retinal microglia in vivo. In this study, we aim at modulating microglia with different concentrations of PLX3397 and set a window in which these cells are not fully unresponsive but less reactive.

Methods: Murine BV-2 microglial cells were treated with eight different concentrations of PLX3397 ranging from 8 µM to 22 µM. MTT assay was applied to understand the metabolic viability in each condition. Three concentrations (low, medium, high) were selected according to MTT assays, and those were evaluated with Trypan Blue staining for cell viability. The pro-inflammatory gene expression was analyzed with RT-PCR.Three different lipopolysaccharide (LPS) concentrations were investigated with MTT assay and RT-PCR to find suitable concentrations for cell stimulation. Morphologic changes were evaluated with Phalloidin staining.

Results: PLX3397 treated microglia demonstrated a lower level of metabolic activity as concentration increased. The concentration yielding 50% MTT reduction was 14 µM. Trypan Blue staining results for 8 µM, 14 µM, and 22 µM PLX3397 stimulation showed 97.5%, 52.8%, and 6% survival rates, respectively. The three LPS concentrations 25 ng/ml, 50 ng/ml, and 100 ng/ml increased pro-inflammnatory gene expression without changing the results of cells. Cells treated with 50 ng/ml LPS and different concentrations of PLX 3397 showed lower IL1-β than vehicle treated cells. The other pro-inflammatory cytokines remained inconclusive so far and will be further investigated. Preliminary imaging of LPS stimulated BV-2 cells showed mainly an ameboid morphology for all concentrations of PLX3397.

Conclusion: As expected, microglia survival decreased as PLX3397 concentration increased in cell culture. The effective concentration of PLX3397 that modulates pro-inflammatory microglia activation remains to be determined in the forthcoming experiments.

The novel GCAP1 variant N104K-G105R associated with Cone Dystrophy shows reduced Ca^{2+}-affinity and abnormal synaptic transmission

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Purpose: The Guanylate Cyclase Activating Protein 1 (GCAP1) is a Ca^{2+}-sensor protein involved in the shutdown of the phototransduction cascade through the regulation of Guanylate Cyclase (GC). To date, up to twenty missense mutations have been associated with Cone- and/or Cone-Rod Dystrophy (COD, CORD), rare autosomal dominant diseases characterized by loss of central and/or peripheral vision, defective color perception and photophobia. Here, we characterized the first COD-associated double mutant of GCAP1 (N104K-G105R), presenting an abnormal attenuation of the b-wave in the electroretinogram (ERG).

Methods: Circular dichroism spectroscopy was employed to investigate the effects of the mutations on the conformational change associated with magnesium (Mg^{2+}) and calcium (Ca^{2+}) binding. Ca^{2+}-affinity was investigated by SDS-PAGE electrophoretic mobility and competition assay with a chromophoric chelator. Finally, the regulation of two GC isoforms was assessed by enzymatic assays.

Results: GCAP1 variant N104K-G105R displayed cation-dependent variations in both secondary and tertiary structure, thus excluding folding defects. On the other hand, the smaller gel-shift and the lack of competition with the low affinity chelator suggested that Ca^{2+}-binding was highly compromised. The novel variant showed a decrease in maximal activation of GC1 isoform compared to the WT, together with a constitutive activation under inhibiting conditions. Similar results were obtained with the GC2 isoform, although the maximal activation of GC2 by GCAP1 variants was 5-fold lower than that of GC1.

Conclusion: Our results suggest that the first GCAP1 double mutant associated with COD is characterized by a severe reduction of Ca^{2+} affinity. This results in a constitutive activation of GC1 in the outer segment of photoreceptor cells, thus in an accumulation of second messengers which ultimately leads to cell death. Moreover, the concomitant constitutively low activation of GC2 regulation, which is present also in the synaptic terminal, may alter the downstream signal transmission, thus explaining the abnormally low b-wave recorded in the patient.

Redefining the role of Ca^{2+} in hereditary photoreceptor degeneration

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Background: Mutations in retinitis pigmentosa (RP) often lead to accumulation of cGMP and over-activation of Cyclic Nucleotide Gated Channels (CNGC). Over-activated CNGC mediates Ca^{2+}-influx which has been suggested to cause rod photoreceptor degeneration. An additional source of Ca^{2+} in rods are the Voltage Gated Calcium channels (VGCC). Intriguingly, CNGC knockout was reported to improve retinal function, whereas VGCC knockout showed no improvement. Here, we further explored the role of Ca^{2+} influx through CNGC and VGCC in photoreceptor degeneration, using their specific pharmacological inhibitors L-cis- and D-cis-diltiazem, respectively.

Methods: The blocking efficacy of the compounds was tested on frog-oocytes, heterologously expressing rod and cone CNGC. The compound effects were further characterized via imaging of the light induced Ca^{2+} responses. The effect on photoreceptor degeneration was investigated on organotypic retinal explant cultures derived from *rd1*, *rd10,* and wild-type (wt) animals, using various concentrations of the compounds (25µM, 50µM, and 100µM).

Results: We found that L-cis-diltiazem could selectively inhibit rod CNGC and decrease light induced Ca^{2+} response in a concentration dependent manner. Interestingly, calpain activity was found to be unaffected by either L-cis or D-cis-diltiazem treatment in both wt and *rd1* retina. Paradoxically, calpain-2 activation was significantly increased by L-cis-diltiazem in wt. Surprisingly, no significant protective effect was found on photoreceptor survival with either L- or D-cis-diltiazem on both *rd1* and *rd10*. On the contrary, L-cis-diltiazem treatment displayed a significant negative effect on photoreceptor survival in mutant and in wt retina.

Conclusion: Our results suggest that Ca^{2+} influx via VGCC may be related to calpain activation. Importantly, CNGC inhibition does not support photoreceptor survival, therefore, contrary to what was previously thought, CNGC over-activation may not be the basis of cell death in cGMP dependent photoreceptor degeneration. This data thus provides new insights into photoreceptor degeneration pathways and may shift our focus for the development of novel therapeutic interventions away from Ca^{2+}-dependent towards cGMP-dependent mechanisms.

CRISPR/Cas9-based rescue of deep-intronic *ABCA4* variant c.5197-557G>T in patient-derived photoreceptor precursor cells

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Purpose: Stargardt disease is an autosomal recessive inherited retinal disorder caused by biallelic mutations in *ABCA4*. Deep-intronic variants (DIV), resulting in aberrant mRNA splicing, have been associated with an increasing number of cases. Upon splicing, DIV c.5197-557G>T causes a 188-bp pseudoexon insertion in the mature mRNA which is degraded via nonsense-mediated decay. Here, we aim to investigate, assess and compare three different Cas9-based strategies to rescue this splicing defect in patient-derived photoreceptor precursor cells (PPCs) by genome editing.

Methods: Three strategies employing *Streptococcus pyogenes* Cas9 were designed: single-gRNA-, double-gRNA- and double-nicking-mediated rescue. Through fragment analysis and sequencing-based methods the gDNA cut efficiency was assessed in either HEK293T cells or patient-derived fibroblasts. The ability to rescue the correct splicing was then studied with minigene assays in HEK293T cells. Patient-derived human induced pluripotent stem cells were differentiated into 2-dimensional PPC cultures with a 30-day differentiation protocol. Successful Cas9 strategies were transfected in PPCs via electroporation. Fluorescence-activated cell analysis was used to measure the transfection efficiency, and the mRNA splicing rescue was quantified with fragment analysis.

Results: After testing the gRNA cut efficiency, a total of 14 approaches were tested in minigene assays. All were able to rescue the correct splicing (min 10%±3, max 100%±0, n=3). Thirty-day-differentiated PPCs were transfected with the eight most successful approaches, in terms of gDNA cut efficiency and mRNA splicing rescue in the minigene assays. Electroporation-mediated transfection of PPCs achieved high efficiency, ranging from 68% to 93%. Heterozygous *ABCA4* c.5197-557G>T-patient-derived PPCs showed a background expression of the wild type allele of 71%±3 of total *ABCA4* transcript. Upon Cas9 delivery, the total expression of the wild type allele increased to 86%±5 (corrected for transfection efficiency, 0.1 mg/ml cycloheximide, n=2).

Conclusions: Splicing correction of c.5197-557G>T was successfully achieved in PPCs. These results show for the first time the possibility of targeting DIVs in patient-derived PPCs with CRISPR/Cas9 strategies. Finally, electroporation has been highlighted as a suitable method for *ex-vivo* delivery of said Cas9-mediated strategies into patient-derived PPCs.

Versican GAG-α domain deficiency causes rosette formation and detachment of the sensory retina in the mouse eye

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Purpose: Versican, a large chrondroitin sulphate proteoglycan is a major component of the extracellular matrix. To learn more about its specific function in the retina, we analyzed the effects of versican deficiency in mutant mice.

Methods: VCAN$^{(tm1Zim)}$ mice were investigated with a splice-variant specific gene inactivation of V0 and V2 isoforms resulting in glycosaminoglycan (GAG)-α domain deficiency of versican. We analyzed the eyes of four- and eight-week-old versicanV0/V2$^{-/-}$ and versicanV0/V2$^{+/-}$ mice and compared the results with that seen in wildtype littermates. Semithin sections were cut and investigated by light microscopy. Retinal thickness was quantified and plotted in a spider diagram. The distribution and expression of versican binding partners, like fibronectin and hyaluronan was investigated by immunohistochemistry. In addition, glial acidic fibrillary protein (GFAP) was analyzed.

Results: The loss of V0 and V2 isoforms resulted in the formation of retinal rosettes, affecting the outer nuclear layer, and photoreceptor inner and outer segments in homozygous and heterozygous versican V0/V2 deficient mice. This frequently caused detachment of the sensory retina. In contrast, analysis of the anterior chamber angle showed a wide-open angle with no obvious structural changes. The analysis of GFAP showed no changes in its expression in Müller cells, even in eyes with rosette formation and retinal detachment. The immunohistochemical analysis of versican binding partners show a dramatic reduction of fibronectin in the entire retina, but not in the anterior chamber angle of eight-week-old versican V0/V2$^{-/-}$ mice when compared to their wildtype littermates. Analysis of hyaluronan identified an extensive downregulation in the inner plexiform layer accompanied with an increase in the ganglion cell layer and the vitreous of versican V0/V2$^{-/-}$ mice.

Conclusion: Deficiency of the versican isoforms V0 and V2 causes retinal changes that indicate its important role for attachment of the sensory retina to the retinal pigment epithelium. Versican appears to play an important role in the interphotoreceptor matrix that might be affected in some forms of inherited retinal disorders.

Establishment of a blue-light induced degeneration model on retinal explants

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Purpose: Common features of many retinal diseases such as age-related macular degeneration (AMD) are neurodegenerative processes in the retina that lead to damage of the retinal pigment epithelium (RPE) and photoreceptor cells (PR) and finally to blindness. Oxidative stress is a key mediator in the pathogenesis of AMD, as well as in numerous other eye diseases. One inducer is high-energy visible light (400-450nm), mainly blue light. This photochemically stress triggers the production of reactive oxygen species (ROS) in cells and could therefore be a promising stressor for modelling the pathogenies of AMD. Not all retinal cells are assumed to be damaged by light – Müller cells (MC) are not known to be directly affected by photochemical damage. Nevertheless, MCs support PR survival and act as optical fibers, thereby playing a vital role in the reaction of the retina to light. The aim of this project was to establish a blue-light based degeneration model on porcine retinal organ cultures and thereby identifying the molecular mechanisms of blue-light damage in retinal cells.

Methods: Porcine retinal organ cultures were exposed to blue light and further cultured for different lengths of time. ROS production was measured, as well as activation of caspase 3/7 and cell viability. The degree of degeneration was analysed via immunohistology, western blot and qRT-PCR. Cell specific markers, like GFAP for MCs, RP65 for RPE and Opsin and ARR3 for PR, as well as markers for apoptosis (PARP, PUMA) and cellular stress (Hif1-α, HSP70, p53) were evaluated.

Results: A significant increase of ROS (3.02-fold, $p<0.01$) was observed 6 hours after blue-light exposure. In consistence, a significant increase in caspase 3/7 activity (3.2-fold, $p<0.001$) was measured at the same time. Cell viability was strongly reduced 6 ($p<0.01$) and 12 ($p<0.05$) hours after exposure. Via qRT-PCR measurements an increase of specific markers such as Hif-1α (2.52-fold), p53, GFAP (2.3-fold) and PUMA (2.1-fold) was detected. Correspondingly, protein expression of HSP70, PARP, phosphorylated and acetylated p53 was increased after exposure.

Conclusion: We successfully established an oxidative stress-based disease model based on blue light-induced retinal degeneration. Robust and reliable markers were identified to precisely evaluate potential novel therapeutic approaches *ex vivo*. Due to the similar morphology of the pig to the human eye, this model closely resembles the human condition.

Ablation of a prodegenerative gene confers functional protection in a mouse model of retinal degeneration

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Purpose: The heterogeneity of genetic forms of blindness represents a challenge for the development of gene-specific therapies. In principle, key pathways and mechanistic commonalities between conditions may be targeted with gene-independent therapies. One such pathway involves injury-induced axon degeneration. Here, we use a knockout mouse model to explore the therapeutic effect of the absence of a prodegenerative gene in this pathway.

Methods: Rotenone, a complex I inhibitor, was injected intravitreally to induce mitochondrial dysfunction. Optokinetic responses were measured 2 and 4 months following injection. Anti-Brn3a and anti-NF200 antibodies were used to stain retinal ganglion cells (RGCs) in wholemounted retinas and cross-sectioned optic nerves respectively. RGCs were counted using CellSens software. Area covered by axons in the optic nerve and spatial distribution of RGCs were quantified using ImageJ.

Results: The absence of the encoded protein provides functional benefit, with significantly higher optokinetic response measurements in knockout mice compared to wild-type following treatment with rotenone treatment. Moreover, this benefit was evident across age groups and sexes and was sustained over time. There was a trend towards protection of RGCs in knockouts following insult with rotenone. Moreover, the distribution of RGCs in knockouts was more even than that of wild-types. There was reduced axon staining in the optic nerves of both genotypes following rotenone treatment. In cross sections of the portion of the optic nerve proximal to the eye, there was a trend towards an increased coverage of axons in rotenone-treated knockouts relative to rotenone-treated wild-types.

Conclusion: Our data suggest a neuroprotective effect of ablation of this gene in the rotenone model. While there is a trend towards histological benefit of genetic disruption of this gene, there is significant functional benefit. As RGCs have large receptive fields and integrate signal from many photoreceptors, it may be that a more even distribution of these cells in the knockout preserves signal arising from more of the retina and may allow optokinetic response measurements to remain high in the knockout.

The diagnostic yield of whole exome sequencing in high myopia

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Purpose: High myopia (HM; ≤-6 diopters) is a heterogeneous condition, and without clear accompanying features it can be difficult to pinpoint a genetic cause. However, in high myopia, a genetic cause is more likely compared to mild myopia. The aim of this study was to evaluate the diagnostic yield of whole exome sequencing (WES) in patients with HM.

Methods: Patients with high myopia were recruited by ophthalmologists and clinical geneticists. Clinical features were categorized into isolated high myopia, high myopia with other ocular features or syndromic high myopia with systemic involvement. WES was performed using our standard gene panel including 477 genes involved in inherited eye disorders.

Results: This study included 75 patients with high myopia; the majority had isolated high myopia (76%). A genetic cause was identified in 13 patients (17%; mean age at diagnosis 13.5 (SD 17.5) years; mean refractive error -14.0 diopters (SD 6.4)). These 13 patients had mutations in retinal dystrophy genes, extracellular matrix genes and ocular developmental genes with mainly an X-linked or autosomal recessive inheritance pattern. In 17 patients (23%) a variant of unknown significance was found; further segregation or follow-up examinations are necessary.

Conclusion: The diagnostic yield of the WES vision gene panel for high myopia was 17%. In the majority of patients, a genetic cause could not be elucidated, warranting the application of other methods, such as whole genome sequencing. Therefore, we need a larger cohort of severely high myopic patients.

Hypoxia-inducible factor-2α aggravates oxidative stress-induced cell death in ARPE-19 cells

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Purpose: Oxidative stress and hypoxia have long been considered major risk factors in the pathophysiology of age-related macular degeneration (AMD). To promote prevention and to develop novel treatment strategies against AMD, it is necessary to identify the molecular pathways in which oxidative stress and hypoxia are involved. In the present study, we focused on hypoxia-inducible factors (HIF) 1α and 2α, the α-subunits of HIF-1 and HIF-2, key regulators of cellular adaptation to hypoxic conditions.

Methods: We used sodium iodate (SI), an RPE-specific oxidative stress agent, together with DMOG, a prolyl hydroxylase (PHD) inhibitor which increases HIF-α levels, to simulate AMD-related conditions in an adult RPE cell line (ARPE-19). We treated ARPE-19 cells with SI, DMOG or a combination of SI and DMOG (SI/DMOG) under 3 % O_2 in a hypoxic chamber, which is closer to the physiological oxygen fraction of RPE cells than the regularly used 21 % O_2. Treatment effects were analyzed using cell viability assays, western blot, and quantitative real-time PCR. Furthermore, siRNA was used to distinguish between HIF-1α and HIF-2α effects.

Results: Cell viability of ARPE-19 cells was significantly decreased in cells treated with SI/DMOG compared to SI or DMOG alone, suggesting that HIF accumulation exacerbates oxidative damage. Knockdown of HIF-1α and HIF-2α using siRNA revealed that HIF-2α is responsible for decreased cell viability, which was also reflected by increased HIF-2α protein levels in the SI/DMOG treatment group. We discuss our observations in terms of autophagy, because analysis of the autophagy markers LC3 and p62 showed a pattern typical for autophagy flux inhibition in the SI/DMOG treatment group.

Conclusion: We showed that HIF-2α plays a key role in oxidative stress-induced cell death in RPE cells. HIF-1 is believed to promote autophagy, thus HIF-2 is likely to play an antagonistic role by inhibiting autophagy resulting in increased cell death under oxidative stress conditions. Taken together, our data suggest that selectively blocking HIF-2α could be a potential treatment strategy to protect the aging RPE against extensive oxidative damage.

Epidemiology of Myopic Maculopathy in Germany – Results from the Gutenberg Health Study

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Purpose: To estimate the prevalence, visual function, and potential risk factors of myopic maculopathy (MMP) in Germany, and its 5-year cumulative progression/incidence.

Methods: The Gutenberg Health Study is a population-based cohort study in Mainz/Mainz-Bingen with 15,010 baseline participants aged 35 to 74 years. Ophthalmic examination included slit lamp and fundus photographs, tonometry, distant corrected visual acuity, and refraction at baseline. The five-year follow-up additionally included axial length measurements. Myopic maculopathy prevalence and 5-year progression/incidence were assessed by grading of fundus photographs according to a recent international photographic classification system (META-PM) in phakic eyes at the initial examination with spherical equivalent ≤ -6,0 D. 801 eyes of 519 participants were analysed to estimate the prevalence of MMP at baseline. 34 eyes of 27 participants with MMP, and 509 eyes of 334 participants without MMP at baseline had gradable fundus photographs at baseline and five-year follow-up to evaluate for progression/incidence, respectively. Progression was defined as any relevant enlargement of existing MMP lesion or presence of a new MMP lesion. Multivariable logistic regression analysis was used to assess risk factors for prevalence and progression of MMP.

Results: The per-person prevalence of MMP in the general population was 0.5% overall, while it was 10.3% [95%-KI: 7,9-13,3%], in highly myopic particpiants, in this Gutenberg Health Study. The following lesions were identified: diffuse chorioretinal atrophy (8,1%), patchy chorioretinal atrophy (1,3%), macular atrophy (0,5%), and plus-lesion (3,0%, n=12). Visual acuity was remarkably reduced in stage 3 and 4 (patchy and macular atrophy) compared with stage 1 and 2 (tessellations ± plus lesions, and diffuse atrophy). MMP was associated with higher myopic refractive error (p<0,001) and older age (p<0,001), while cardiovascular risk factors, gender, and socioeconomic factors were not associated.
In the five-year follow-up, 17 of 34 eyes (50%) with prior MMP presented progression, and of these, 4 deteriorated in stage. The main type of progression was enlargement of diffuse atrophy or patchy atrophy, followed by the appearance of new pathologies. IOP was associated with progression (OR=1.62 per mmHg, 95%CI: 1.51-1.59, p=0.035) although IOP levels were at a normal range. Female gender (OR=5.54, 95%CI: 0.93-32.92, p=0.060), and higher myopic refractive error (OR=1.21 per diopter, 95%CI: 0.99-1.49, p=0.063), revealed a tendency to be associated with progression.Five-year cumulative incidence of myopic maculopathy was 0.3% (95%CI: 0.02-1.92%; n=1).

Conclusions: The per person prevalence of MMP in the general population in Germany aged 35 to 74 years (restricted to highly myopic subjects) was 10.3%, while the 5-year cumulative progresssion/incidence was 50% / 0.3%, respectively. Visual acuity decreases with disease stage. The Gutenberg Health Study presents the first popoulation-based data on the prevalence and progression/ incidence of MMP from Europe.

How human microglia shape developing neurons in retinal organoids

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Microglia are resident macrophages of the central nervous system. During embryonic development, they are infiltrating the brain from the yolk sac and are actively involved in regulating neurogenesis, interneuron positioning, axonal outgrowth, and neuronal wiring. These functional observations were mostly performed in mouse models, yet, little is known whether human microglia similarly shape their early neuronal environment. Neuronal organoids differentiated from human induced pluripotent stem cells provide a unique opportunity to investigate embryonic neuronal organization and connectivity. However, they are commonly lacking microglia.

Here, we established a model system in which we assembled microglial precursor cells into retinal organoids. First, we independently generated both components and then applied microglial precursor cells at selective time points to retinal organoids. Immunostaining for the microglia marker IBA1 showed that microglia integrated into retinal structures labeled with OTX2 and RECOVERIN but the time point of microglia application significantly impacted microglia density within organoids. Commonly, microglia integration was transient and organoids lost them within a couple of days. Incorporated microglia are actively screening their environment and form phagocytic cups as determined with live cell imaging.

Overall, the integration of microglia precursor cells into neuronal tissue depends on an optimal time window. For the next steps, we are exploring how microglia presence impacts neuronal function using calcium imaging. This will provide important insights into the role of microglia in neuronal patterning and -activity during early human brain development.

Exploring inosine-5'-monophosphate dehydrogenase 1 (IMPDH1) as a novel target for the treatment of Retinitis Pigmentosa

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Purpose: Retinitis pigmentosa is a degenerative genetic disorder in which cell death can be connected to high cGMP levels in photoreceptors. This is exemplified by the *rd1* mouse model where a mutation in the *Pde6b* gene leads to decreased cGMP hydrolysis in photoreceptors. While particulate guanylyl cyclase E (PGCE) synthesizes cGMP in photoreceptors, the enzyme inosine-5'-monophosphate dehydrogenase-1 (IMPDH1) catalyses the rate-limiting step in the biosynthesis leading up to cGMP. Hence, inhibiting IMPDH1 may be a strategy for the reduction of photoreceptor cGMP levels and cell death. We explored the capacity of the registered immunosuppressive drug mycophenolate (MC) to reduce photoreceptor cGMP levels and cell death in *rd1* retinal explant cultures.

Methods: Initially, the retinal expression pattern of IMPDH1 and PGCE was assessed in wild-type and *rd1* mouse retina using immunofluorescence. Then, organotypic retinal explant cultures derived from post-natal day (P) 5 *rd1* mice were treated with MC in three different concentrations (1, 10, 87 µM). Parallel control cultures received vehicle or medium only. The retinal explants were cultured from P5 to P11 with medium changes every two days. At P11, the explants were fixed in paraformaldehyde, cryosectioned, and then stained for cell death using the TUNEL-Assay. The TUNEL-positive cells were counted and compared with controls.

Results: The IMPDH1 staining showed signals in the photoreceptor inner segments, outer plexiform layer and separate lines reaching into the retina indicative of Müller glia cells. The PGCE stainings labelled the outer segments of photoreceptors. In *rd1 in vitro* explant cultures, treated with MC, cell death appeared to be decreased in a concentration-dependent manner. While limited number of observations is still limited, this effect did attain statistical significance at the highest MC concentration used.

Conclusion: The localization of IMPDH1 expression to photoreceptor inner segments makes it a potentially druggable target for the treatment of retinitis pigmentosa. Moreover, the treatment with MC did not seem to be toxic to retinal explant cultures. However, as far as neuroprotection with MC is concerned, more data from *rd1* retinal explants will be needed for confirmation and validation.

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RNA biological characteristics at the peak of cell death in different hereditary retinal degeneration mutants

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Purpose: The present work investigated changes in the gene expression, molecular mechanisms, and pathogenesis of inherited retinal degenerations (IRDs) to identify predictive biomarkers for their varied phenotypes and to provide a better scientific basis for their diagnosis, treatment, and prevention.

Methods: Differentially expressed genes (DEGs) between retinal tissue from retinitis pigmentosa (RP) mouse models obtained during the photoreceptor cell death peak period (*Ped6b^{rd1}* at PN13, *Ped6b^{rd10}* at PN23, *Prph^{rd2}* at PN29) and retinal tissue from *C3H* wild-type mice were identified using Illumina high-throughput RNA-sequencing. Co-expression gene modules were identified using a combination of GO and KEGG enrichment analyses and gene co-expression network analysis. CircRNA-miRNA-mRNA network interactions were studied by genome-wide circRNA screening.

Results: *Ped6b^{rd1}*, *Ped6b^{rd10}*, *and Prph^{rd2}* mice had 1,926, 3,096, and 375 DEGs, respectively. Mainly, genes related to ion channels, stress, inflammatory processes, tumor necrosis factor (TNF) production, and microglial cell activation were up-regulated, while genes related to endoplasmic reticulum regulation, metabolism, and homeostasis were down-regulated. Differential expression of transcription factors and non-coding RNAs generally implicated in other human diseases was detected. CircRNA-miRNA-mRNA network analysis confirmed that these factors may be involved in photoreceptor cell death. Moreover, excessive cGMP accumulation causes photoreceptor cell death, and cGMP-related genes were generally affected by different pathogenic gene mutations.

Conclusions: We screened genes and pathways related to photoreceptor cell death. Additionally, up-stream regulatory factors, such as transcription factors and non-coding RNA and their interaction networks were analyzed. Furthermore, RNAs involved in IRDs were functionally annotated. Overall, this study lays a foundation for future studies on photoreceptor cell death mechanisms.

Keywords: retinitis pigmentosa, RNA-SEQ, biogenic analysis, cGMP-related genes, photoreceptor cell death

Transcorneal electrical stimulation evaluated by pupillary response dynamics reveals frequency-dependent activation of human retinal neurons in vivo

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Purpose: Studies on the electrical excitability of retinal neurons have shown that photoreceptors and other cell types can be selectively activated by distinct stimulation frequencies *in vitro*. The purpose of this project was to explore the possibility of cell-specific activation of retinal neurons in humans via transcorneal electrostimulation *in vivo*. Frequency-related differences in retinal responsiveness were evaluated objectively using the dynamics of the electrically evoked pupillary responses.

Methods: Eleven healthy participants and eleven patients with retinitis pigmentosa (RP) caused by mutations in either rhodopsin or PDE6A/B were recruited to examine pupillary responses and phosphenes during transcorneal electrostimulation. The currents were composed of amplitude-modulated sinusoids, containing a low-frequency envelope that was superimposed on variable carrier frequencies (4-30 Hz). During stimulation of one eye, consensual pupillary oscillations were recorded from the contralateral eye via infrared pupillography. The responsiveness was assessed by the delay of the oscillations relative to the sinusoidal envelope and its variation throughout the measurement. The preservation of photoreceptors in RP patients was assessed via pupil campimetry, revealing almost completely extinguished rod function but mostly preserved cone function. Differences in the responsiveness of normal subjects and patients were evaluated to determine if rod loss selectively manifests at specific frequencies.

Results: Pupillary oscillations matching the sinusoidal envelope were induced by electrical stimulation in both healthy subjects and patients. The calculated responsiveness of normal subjects displayed a clear local peak at 6-8 Hz. This peak was completely absent in RP patients, with a significant difference of 16 % at 8 Hz. Correspondingly, their phosphene percepts were relatively weaker in the lower frequency range.

Conclusion: The observed differences in the pupillary responses between healthy subjects and patients with outer retinal degeneration suggest that responses at lower carrier frequencies are mainly mediated by rods. In sum, this work provides more evidence that different retinal cell types can be selectively activated with specific carrier frequencies.

Does the retinal photoreceptor composition influence Müller cell heterogeneity?

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Purpose: The human macula, key for accurate vision, is exceptionally prone to neurodegenerative processes. We aim to elucidate whether a functional heterogeneity of Müller cells, the major macroglia of the retina, may explain part of this macular susceptibility. To clarify the genetic basis of this heterogeneity, we generated and analyzed high quality proteomic data from cone- and rod-rich systems from human and mice.

Methods: Müller cells, microglia, vascular cells and retinal neurons from all cone R91W;Nrl$^{-/-}$ and R91W control mice as well as from human macular and peripheral samples were isolated by immunomagnetic separation and searched for differential protein expression by use of tandem mass spectrometry. Cell type and retinal region specificity of proteins were additionally tested by evaluating their expression in published scRNAseq datasets, while particularly promising candidates were subjected to increased scrutiny. To test the role of one of these, we generated CRISPR mediated KO lines of MIO-M1 cells, which are of Müller cell origin. A culture system based on acrylamide gels of varying compliances was used to measure changes in exerted shear stress, while cytoskeletal immunostainings coupled with a bioinformatics pipeline enabled the assessment of morphological features.

Results: We found significant differences in protein expression between predominantly cone- and rod-associated Müller cells in the human as well as the murine system, strengthening our hypothesis of functional Müller cell heterogeneity in the human retina. Indeed, some proteins showed a Müller cell-specific expression pattern in both the scRNAseq and our own proteomic data making these candidates especially promising for further research. *Eppk1*, though poorly understood, is thought to play a role in cytoskeleton/intermediate filament organization, which we were able to partly corroborate by showing its mislocalization in *Gfap/Vim^{KO}* retina. Furthermore, *Eppk1* knockout in MIO-M1 cells lead to a decrease in exerted shear stress as well as a change in size, shape, and filopodia characteristics.

Conclusion: We could show that the consistently different expression profile of some proteins in human and murine Müller glia yields candidates that may reveal functionally distinct cell subpopulations. Using various cell culture based techniques, we were able to lay the groundwork for the understanding of *Eppk1* function for the biophysical properties of Müller cells that might be of special importance for macular cells. Further studies in higher order model systems might improve the understanding of why the human macula is so sensitive to disease-associated changes and open up opportunities for the generation of novel therapies for pathologies like age-related macular degeneration.

Adaptive optics retinal imaging in patients before and after gene therapy with voretigene neparvovec

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Purpose: This longitudinal follow-up study aims to monitor the pattern of the adaptive optics retinal imaging in patients with RPE65 mutation before and after gene therapy with voretigene neparvovec to reveal changes of cell patterns and possible retinal inflammatory reactions after application.

Methods: 12 eyes of 8 patients with a genetically confirmed *RPE65* mutation were treated with voretigene neparvovec. Patients were examined by the commercially available adaptive optics (AO) flood illumination retinal camera rtx1^{TM} (Imagine Eyes, Orsay, France) before the application, 2 and 4 weeks and 3, 6 and 12 months after the injection. A standardized protocol of a composition image covering approximately 13° x 13° of the central retina, was used. The observed cone mosaic pattern was correlated with the findings of the color fundus images, OCT and the autofluorescence.

Results: In 7 eyes we could acquire sufficient image quality at the baseline and follow up visits. The observation after gene-based therapy couldn't reveal any new cells or macrophages indicating an immune response or retinal inflammation. However, after the application a homogeneous, changed appearance of the cone mosaic was observable between the baseline visit and the 2 and 4 weeks follow up. Due to the retinotomy injection bleb an increase of an irregular mosaic and reduction of typical shaped cones were noticeable. After 4 weeks of postoperative recovery the cone photoreceptor mosaic has stabilized again. In one eye a postoperative central scarring was observable showing a stabilization and partially recovery after 6 months. In one eye of another patient central atrophic changes correlated with the remaining retinal imaging.

Conclusion: Using the AO retinal camera, it is possible to observe smallest changes after gene therapy even if they were not visualizable in the clinical standardized examinations. The recordings have demonstrated their value in monitoring the safety and efficacy of the therapy. In addition, the imaging helps to provide a better understanding of the processes after gene therapy.

Allele-specific regulation of hsa-miR-4513 contributes to neovascular age-related macular degeneration through modifying *CDKN2A* expression

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Purpose: MicroRNAs (miRNAs) are small post-transcriptional regulators widely discussed as promising candidates for their clinical application as biomarkers or targets for treating complex diseases such as age-related macular degeneration (AMD). To this end, hsa-miR-4513 is an excellent example as this gene harbors within its conserved heptametrical seed sequence a frequent polymorphism (rs2168518), which has previously been associated with several complex phenotypes including age-related macular degeneration (AMD) and specifically the neovascular (NV) form of the disease. So far, little is known about the biological mechanisms underlying this association. We aimed to identify allele-specific target genes of hsa-miR-4513 to clarify their contribution to NV-AMD.

Methods: We performed high-throughput RNA sequencing (RNA-Seq) in a cellular miRNA overexpression model transfected with separated hsa-miR-4513 alleles at rs2168518, namely hsa-miR-4513-G and hsa-miR-4513-A. Promising allele-specific target genes were independently verified by quantitative reverse transcription PCR (qRT-PCR) and further validated *via* protein expression. Allele-specific miRNA binding was analyzed with luciferase reporter assays. Further, publicly available databases were utilized to prioritize target genes with regard to hsa-miR-4513 related phenotypes and especially NV-AMD pathology.

Results: Overall, we identified 23 allele-specific target genes of hsa-miR-4513 and independently replicated 19 of these. Western Blot analysis and luciferase reporter assays further confirmed the allele-specific regulation of these genes by hsa-miR-4513. Remarkably, multiple of the newly identified allele-specific target genes were linked *via* text retrieval to several phenotypes previously reported to be associated with hsa-miR-4513. For NV-AMD one specific target gene is of special interest, namely *CDKN2A*, which is known to play a role in the vascular system of the eye.

Conclusion: Our study revealed multiple promising allele-specific target genes of hsa-miR-4513, which offer the unique opportunity to elucidate the association of this miRNA with neovascular disease.

Microglia modulation as target for diabetic retinopathy

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Purpose: Diabetic Retinopathy (DR) is a complication of diabetes mellitus (DM) and a major cause of irreversible blindness. The most recent evidence from human patients and mouse models show that retinal inflammation precedes the clinical and morphometric vascular changes caused by diabetes. The hyperglycemic microenvironment enhances flux of polyol and hexosamine pathways, resulting in increased production of reactive oxygen species (ROS) and the accumulation of advanced glycation or lipoxidation end products (AGEs or ALEs) which trigger inflammatory responses in the retina. Microglia cells, the resident immune sentinels of the retina, orchestrate inflammatory responses during retinal pathophysiology. However, the cellular and molecular mechanisms underlying DR pathogenesis remain elusive due to lack of animal models that can recapitulate the neural and vascular complications of DR. Therefore, in the current project, we will investigate the role of microglia-mediated neuroinflammatory responses in the pathogenesis of DR.

Planned Experiments: The present study utilizes two innovative DR mouse models, *Pdgfb*$^{i\Delta EC}$ (tamoxifen-inducible depletion of PDGF-B), and anti-PDGFRβ antibody mice to carry out detailed analysis of microglia during both early (P10) and late phases (P28) of the disease. In both models, we will analyze microglia phenotype using immunohistochemistry on retinal cryosections and flat mounts with antibodies against Iba1 & P2ry12. The pro- & anti-inflammatory gene expression profiles and protein expression will be evaluated by quantitative real time PCR (qRT-PCR) and ELISA, respectively. Further, RNAscope in situ hybridization using probes directed against vascular endothelial growth factor A (VEGF-A) and placental growth factor (PGF) will be used to determine the role of microglia in angiogenesis during DR. The mice will be treated with minocycline starting P5 and separately weaned at P21 on a special ablation animal feed containing a selective colony-stimulating factor 1 receptor (CSF1R) inhibitor, PLX3379, which transiently eliminates microglia within 1 week of oral feeding. The Optodrum and Spectral Domain-Optical coherence tomography (SD-OCT) will be used to assess visual acuity and retinal thickness, non-invasively. In vivo fluorescein angiography in each mouse will be performed to assess neovessel leakage and non-perfusion.

Conclusion: Collectively, these studies will provide detailed insight into the role of microglia in DR pathogenesis and may highlight microglia mediated inflammatory responses as an important therapeutic target for prevention and treatment of DR.

Colony stimulating factor 1 (CSF1) receptor blockade as novel tool to limit microglia reactivity in the light-damage model of retinal degeneration

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Purpose: Reactive microglia are commonly present in retinal degenerative diseases. They can secrete neurotoxic substances and contribute to photoreceptor cell death. However, as a fundamental part of the regulatory immune system they also have supportive effects. In this project, we aimed at studying the effects of complete microglia depletion in a mouse model of acute retinal degeneration using the CSF1R-antagonist PLX3397.

Methods: Cx3cr1$^{GFP/+}$ reporter mice that carried the light sensitive RPE65Leu450 variant received PLX3397 diet or control diet starting seven days prior to exposure with 15.000 lux white light to induce retinal degeneration. The effects of PLX3397 treatment were analyzed four days after light exposure. The number and morphology of retinal microglia was analyzed by *in vivo* fundus imaging and immunohistochemical stainings of retinal flat mounts. Microglia activation marker expression in whole retinal transcripts was determined by real-time RT-PCR. Optical coherence tomography (OCT) was used to measure retinal thickness.

Results: PLX3397 treatment effectively depleted microglia in healthy and light damaged retinas as shown by *in vivo* fundus imaging and Iba1-staining of retinal flat mounts. Four days after mice were exposed to bright white light, mRNA expression of the constitutive microglia marker allograft inflammatory factor (AIF-1) was strongly increased and this upregulation was absent in mice treated with PLX3397. Expression of the microglia activation marker TSPO (translocator protein (18 kDa)) was also upregulated after light exposure, and PLX3397 treatment prevented this induction. OCT revealed a thinning of the outer nuclear layer in light exposed retinas, and this thinning was also detected in light exposed retinas under conditions of microglia depletion.

Conclusion: Retinal microglia were efficiently depleted with PLX3397 under normal and light damage conditions. The absence of microglia did not change the extent of retinal degeneration after light damage. We conclude that the presence of microglia and their immunomodulation is a promising concept for the treatment of retinal degeneration diseases.

Ion channel modulators for the treatment of *cone dystrophy with supernormal rod response*? Evidence for novel interaction between K_V channels across families with therapeutic implications

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Purpose: To explore if ion-channel modulators may be repurposed as a treatment option for *'Cone dystrophy with supernormal rod response'* (CDSRR).

CDSRR is a rare progressive hereditary retinal disorder. It is caused by mutations in the *KCNV2* gene, encoding K_V8.2, a voltage-gated potassium channel (K_V) subunit. K_V8.2 subunits are incapable of forming functional channel complexes on their own, rather they require heteromerization with other members of the K_V channel superfamily. K_V8.2 thereby contributes to a channel complex that critically shapes photoreceptor biophysical properties. The exact entity of other K_V subunits contributing to this complex is not fully elaborated. While it is clear that K_V2.1 are involved, a role of K_V7 subunits is also under debate.
The existence of such K_V8.2-K_V7 complexes would be a stroke of luck from a clinical perspective, as selective K_V7 pharmacological modulators are in clinical use already and could be repurposed as treatment option for CDSRR. Moreover, their existence is of basic physiological interest as it challenges the biophysical paradigm, that only K_V2 channels can form functional heteromeric complexes across family borders.

Methods: Expression of K_V7 channel genes in photoreceptors was explored performing quantitative PCR on adult C3H mice carrying the rd1 mutation and wild-type littermates. Transcriptomic evidence was complemented on protein level by immunostaining of retinal cryosections and on functional level by pharmacological dissection of electroretinogram-type responses ("micro-ERGs") recorded from explanted wild-type mouse retinae. In addition, patch-clamp recordings and Co-Immunorecipitation were performed in cell lines to analyse if K_V7 can form functional complexes with K_V8.2.

Results: Here we show preliminary transcriptional and immunohistocemical evidence indicating that one particular member of the K_V7 family, namely K_V7.4, is selectively expressed in murine photoreceptors. Furthermore, we show that established K_V7 modulators are capable of attenuating micro-ERGs in murine retinae in a way that is indicative for a role of K_V7 in photoreceptors.
When co-expressing K_V7 with K_V8.2 in cell lines we find that K_V8.2 can be immunoprecipitated using antibodies directed against K_V7. Moreover, co-expression of K_V7 with K_V8.2 gives rise to a conductance with biophysical properties essentially distinct from those observed when expressing K_V7 alone, indicating formation of functional channels heteromers.

Conclusion: Our data provide cues for a role of K_V7 in photoreceptors. They suggest that by forming functional channel complexes with $K_V8.2$ they may contribute to the pathophysiology of CDSRR, opening opportunities for a cost-efficient development of pharmacotherapeutic options for this rare disease.

Expanding the clinical and genetic spectrum of *RAB28*-related cone-rod dystrophy: Pathogenicity of novel variants in Italian families

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Purpose: Rab-28 is a small Ras-related GTPase highly expressed in photoreceptor cells, putatively involved in membrane trafficking and phagocytosis. To date, only 6 variants have been associated with autosomal recessive Cone-Rod Dystrophies (arCORD). Here, we present the molecular characterization of 4 novel variants identified in five patients, representing the largest cohort of Rab-28 patients reported in literature.

Methods: Clinical evaluation of the patients included Fundus Autofluorescence (FAF), Optical Coherence Tomography (OCT) and Flash Electroretinogram (ERG). The autosomal dominant inheritance pattern was assessed by sequencing 11 individuals from the 5 families after the identification of Rab-28 mutations by a CORD-targeted gene panel. The structural effects of Rab-28 variants were assessed by molecular modeling and Molecular Dynamics simulations.

Results: All patients reported low vision acuity, photophobia, and color vision impairment, confirmed by flash ERG. OCT and FAF identified marked reduction of foveal thickness and central hyperfluorescent areas, suggesting RPE degeneration and photoreceptor death. Structural analysis of the novel nonsense variants suggested that the defective protein folding would result in protein degradation or nonsense-mediated mRNA decay. The missense variant T26N, however, would prevent Mg^{2+}-coordination and prevent the GDP-GTP exchange, thus locking Rab-28 in a GDP bound inactive state.

Conclusion: The molecular analysis of the novel Rab-28 variants suggested that different pathological mechanisms may be associated with arCORD clinical development and severity. Indeed, the nonsense and splicing variants are most likely implied in haploinsufficiency due to defective transcription or folding, whereas the partial loss-of-function variant T26N would prevent GTP-mediated membrane trafficking and phagocytosis.

The role of ciliary protein BBS8 in RPE phagocytosis

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Purpose: Primary cilia are microtubule-based signalling organelles, defects of which lead to ciliopathies, resulting in symptoms, such as retinal degeneration. Numerous ocular cell-types display a primary cilium, one of which is the retinal pigment epithelium (RPE). The RPE is a monolayer of polarised cells located between the neural retina and the vascular choroid. One of its most important functions is the phagocytosis of shed photoreceptor outer segments (POS), which is essential for photoreceptor functionality and integrity. Our lab previously identified the role of primary cilia during development of the RPE. Apart from the cilia-related functions, ciliary proteins have been shown to play important roles in non-ciliary processes. Here we investigated the influence of the ciliary protein BBS8 on the phagocytosis pathway in RPE cells.

Methods: We used BBS8 as a model for ciliary dysfunction, as the loss of BBS8 results in a disruption of ciliary trafficking. Using ARPE-19 cells, we knocked down *BBS8* via siRNA and conducted phagocytosis assays to measure differences in bound vs. internalised POS. Furthermore, we performed real-time qPCR on these cells to analyse potential differences in phagocytosis gene expression. These data were compared to *in vivo* data from *Bbs8*$^{-/-}$ mouse RPE. We investigated transcriptomic (QuantSeq 3'mRNA sequencing) and proteomic (mass spectrometry) data. Finally, we adopted a data mining approach to identify possible connections between BBS8 and the phagocytosis proteins.

Results: Upon loss of BBS8, significantly less POS were internalised in ARPE-19 cells. Transcriptomic analyses identified a misregulation of genes involved in POS binding and internalisation *in vivo* and *in vitro*. Furthermore, a downregulation of proteins involved in POS binding was detected in *Bbs8*$^{-/-}$ mouse RPE. Data mining identified β catenin and RAC1 as potential regulatory hubs to investigate the ciliary influence on the phagocytosis pathway.

Conclusions: We conclude that BBS8 is likely involved in POS phagocytosis in the RPE. This might suggest that ciliary proteins might not solely function in ciliary trafficking, but also in the trafficking of proteins involved in phagocytosis to the apical processes. The potential regulatory hubs identified offer an important starting point for further studies. Their associations to BBS8 and phagocytosis proteins should be examined in detail. Studying these influences on the phagocytosis pathway is vital to create a better understanding of ciliary protein involvement in ocular disorders.

Correlation between PEDF receptors and survival of retinal ganglion cells

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In diabetic retinopathy, glaucoma and other ischemic hypoxic retinopathy, the loss of retinal ganglion cells (RGCs) is one of the important characteristics of these diseases. Pigment epithelium-derived factor (PEDF) mediated and was involved in a range of signal conductions to protect neurons. There are two kinds of Pigment epithelium-derived factor receptors, patatin-like phospholipase 2 gene product/PEDF-R and laminin receptor (LR), co-expressed on RGCs and R28 retinal precursor cells. Our study found that the expression of both receptors increased significantly when cells were stimulated with complex secretions from retinal glial cells (RMC). Their co-expression were enhanced by either VEGF or hypoxic stimulation as well. After knocking down the two PEDF receptor genes, the expression levels of anti-apoptotic factors(Bcl-2, Bcl-xL) and neuroprotective factors (PEDF, VEGF and BDNF)were significantly reduced. This suggested that both of them may have an important function for maintaining neuronal survival, in particular, under hypoxia. PEDF receptors may be a new therapeutic target for retinal diseases related to retinal ganglion cell injury.

RGC axonal transport defect is an early indicator for OPA1 haploinsufficiency

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Purpose: Dominant Optic Atrophy (DOA) is an inherited optic nerve disorder which is characterized by bilateral degeneration of the optic nerve. Through the course of the disease, retinal ganglion cells (RGC) and their axons forming the optic nerve are severely impaired. Mutations in OPA1 is the leading cause of DOA and majority of the mutations results in OPA1 haploinsufficieny. Although OPA1 is a multifunctional mitochondrial protein, exact mechanism of the disease is not clear. However, mitochondrial energy metabolism and sensitivity to apoptotic stimulus are proposed to be involved. Hence, these defects should also affect axonal energy homeostasis and transport, we proposed that one of the early markers of DOA should be axonal transport defects, which most likely contributes to further disease progression. In that respect, we evaluated the effects of OPA1 haploinsufficiency on RGC axonal transport.

Methods: In order to assess the effects of OPA1 haploinsufficiency on RGC axonal transport we have used *Opa1*$^{wt/del}$ mouse model. We performed intravitrael injections of alexa flour conjugated cholera toxin subunit B (CTB) into *Opa1*$^{wt/wt}$ and *Opa1*$^{wt/del}$ mice eye between 8-12 weeks old. Conjugated-CTB (1 mg/ml) was intravitreally injected and animals were sacrificed by exsanguination and cardiac perfusion at 24 hours timepoint. Whole brains were dissected and post-fixed with 4% PFA. Preserved brains were sectioned into consecutive 100 µm coronal sections for imaging of superior colliculus (SC), lateral geniculate nucleus (LGN), suprachiasmatic nucleus (SCN), and olivary pretectal nucleus (OPN).

Results: CTB binds GM1 gangliosides and induce endosomal uptake. Intravitreal injections of conjugated-CTB resulted in RGC uptake and allowed us to visualize axonal transport. Image analysis of coronal sections showed a clear delay in axonal transport specifically at SC and LGN regions in haploinsufficient mice 1 day after conjugated-CTB injections.

Conclusion: Degeneration of RGC axons in *Opa1*$^{wt/del}$ mice is slow and the effects are mostly undetectable at early ages. Therefore, the transport delay that has been observed is independent of axonal degeneration. Here, we elucidated an early marker for OPA1 haploinsufficiency condition, which might also be a valuable therapeutic target for DOA condition.

Downregulation of *ABCA1* in iPSC-derived RPE cells impaired cholesterol efflux leading to intracellular lipid accumulation

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Purpose: Age-related macular degeneration (AMD) is a progressive disease of the macula that at advanced stages leads to blindness in the elderly population. While the neovascular form is characterized by choroidal blood vessels invading the retina, the dry form leads to atrophy of the retinal pigment epithelium (RPE) and degeneration of photoreceptors. Genes involved in lipid metabolism, including ATP-binding cassette transporter A1 *(ABCA1)* were associated with AMD through genome-wide association studies. *ABCA1* is strongly expressed in the RPE and required for lipid metabolism and export. AMD-associated genetic variants near the *ABCA1* gene were shown to modulate its expression in patient-derived lymphoblastoid cell lines, suggesting that these variants may lead to disturbed lipid transport in the human RPE.

Methods: Patient induced pluripotent stem cells (iPSC) harboring homozygous *ABCA1* genotypes for increased (rs1883025:C and rs2740488:A) or decreased (rs1883025:T and rs2740488:C) risk for AMD were differentiated into RPE. These iPSC-RPE cells were analyzed for ABCA1 gene and protein expression, their ability to phagocytize photoreceptor outer segments (POS) and the efficiency of cholesterol efflux. Experiments were conducted under normoxic and hypoxic (4% O_2) conditions to mimic the physiological conditions in the ageing eye. Furthermore, ABCA1 expression and function was also examined after Liver X receptor (LXR) agonist treatment, a regulator of cholesterol homeostasis.

Results: iPSC-derived RPE cells showed similar gene expression and morphological characteristics of RPE cells *in vivo*. Interestingly, hypoxia-treated RPE cells exhibited reduced *ABCA1* expression and dysregulation of several hypoxia-associated genes (e.g. *PDK1* and *ADM*). Diminished *ABCA1* expression was also observed in increased risk genotype-bearing iPSC-RPE cells treated with an LXR agonist compared to iPSC-RPE cells carrying the reduced risk *ABCA1* genotype. Subsequently, the lack of ABCA1 expression hampered cholesterol efflux, leading to lipid accumulation in iPSC-RPE cells.

Conclusion: In conclusion, this study points towards a functional effect of AMD-associated genetic variants in *ABCA1* on cholesterol efflux in iPSC-RPE cells and suggests that this model system may be used to study the mechanism of altered lipid metabolism in the development of AMD.

PMCA, a new player in retinal degeneration associated to Retinitis Pigmentosa

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Purpose: Retinitis Pigmentosa (RP) is a group of genetically diverse retinal dystrophies that course with progressive degeneration and loss of photoreceptors leading to blindness. The pathways involved in photoreceptor death are not completely known, yet calcium imbalance is a common trait. To get more insight into the photoreceptor degeneration process we studied the role of Plasma Membrane Calcium ATPases (PMCAs). PMCAs are Ca^{2+}/H^{+} antiporters that act as key regulators of intracellular Ca^{2+} balance. It has been described that PMCAs can be irreversible activated during programed cell death.

Methods: Two RP models were used: the *rd10* mice, that carries an autosomal recessive mutation in the *Pde6b* gene; and the P23H/P23H mice that presents an autosomal dominant mutation in rhodopsin gene. The expression levels of the *Pmca1-4* genes were analyzed by using qPCR, and the protein distribution by immunofluorescence in retinal cryosections. Photoreceptor acidification was determined by labeling of retinal explants with the fluorescence probe Lysotracker®Red DND-99. The role of PMCA in photoreceptor loss was assayed by the treatment with the PMCA inhibitor 5(6)Carboxyeosin (5(6)CE). *Rd10* retinal explants were incubated for two days with 5(6)CE and photoreceptor preservation was analyzed by measuring the outer nuclear layer (ONL) thickness. For *in vivo* studies, 5(6)CE was administrated by intravitreal injection to *rd10* mice and three days later photoreceptor preservation was analyzed likewise.

Results: The gene expression of *Pmca1, Pmca2* and *Pmca4* was significantly increased in the *rd10* retinas, while *Pmca3* expression was not changed. In P23H/P23H retinas, *Pmca1* and *Pmca4* showed a tendency of augmented expression although did not reach statistical significance. Immunofluorescence staining with a pan-antiPMCA antibody showed high PMCA levels associated to the plexiform layers and this expression pattern was maintained between WT, *rd10* and P23H/P23H retinas. Abundant lysotracker-positive photoreceptors were found in *rd10* and P23H/P23H retinas while the WT retinas displayed sparse lysotracker-positive photoreceptors. PMCA inhibition in cultured *rd10* retinal explants significantly prevented photoreceptor loss and this protective effect was confirmed *in vivo.*

Conclusion: PMCA gene expression augmented in both RP models; Acidification of photoreceptor cells by means of lysotracker labeling was found in both RP models. PMCA inhibition protected against photoreceptor loss assessed in retinal explants and *in vivo*. These results show that PMCAs may have a relevant role in the retinal degeneration associated to RP.

Effects of nicotinamide on complement activation and oxidative stress in a cellular model of age-related macular degeneration (AMD)

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Purpose: The vitamin B3 derivative nicotinamide (NA) is known for its properties in enhancing the differentiation of induced pluripotent stem cells (iPSCs) into retinal pigment epithelium (RPE) cells and is used in almost all current differentiation protocols. Recent work by Saini et al. 2017 (doi: 10.1016/j.stem.2016.12.015) suggests that NA also ameliorates an AMD-like phenotype in iPSC-RPE. Here, our aim was to investigate the effects of long- and short-term NA depletion on RPE cell integrity and functionality. Additionally, we investigated if NA supplementation might disguise an AMD-like phenotype in a patient-derived iPSC-RPE cell model system.

Methods: iPSC-derived RPE cells with a defined genetic AMD risk were grown on Transwell filters and allowed to mature for 6 weeks before analysis. NA depletion was started five, two or one week before the end of the 6-week maturation period. RPE cell layer integrity and RPE functionality were evaluated by transepithelial resistance (TEER) measurements, immunocytochemistry and a photoreceptor outer segment (POS) phagocytosis assay. Transcriptional expression of RPE markers, complement cascade genes and oxidative stress response genes was measured by qRT-PCR and changes in protein expression were validated by Western blot.

Results: Neither short- nor long-term NA depletion had an influence on TEER measurements or RPE monolayer integrity as shown by ZO-1 and BEST1 stainings. Analysis of mRNA expression revealed reduced levels of RPE marker genes *BEST1* and *RPE65* upon NA depletion, whereas the expression of complement genes *CFH* and *C3* as well as oxidative stress response genes *HMOX1* and *NQO1* were increased. Effects of NA depletion on RPE and complement marker protein expression were confirmed by Western blot analysis. Intriguingly, cells depleted of NA showed a much higher uptake of POS than cells cultivated in presence of NA. Cell lines with different genetic AMD risk scores failed to reveal differences in any of the parameters analyzed.

Conclusions: Depleting iPSC-RPE cells of NA had a pronounced effect on expression of complement cascade proteins CFH and C3 as well as oxidative stress response genes *HMOX1* and *NQO1*, which supports its potential use as therapeutic substance in AMD. It remains elusive whether the beneficial effect of NA on oxidative stress regulation and complement activation outweighs a reduced POS phagocytosis rate, as recycling of POS is one of the most vital functions of healthy RPE cells.

Optimising AAV-mediated gene delivery to Retinal Pigment Epithelial (RPE) cells

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Purpose: Stargardt disease (STGD1) is a common inherited retinal degeneration with an estimated prevalence of 1 in 10000. The disease is caused by function-reducing mutations in the *ABCA4* gene. ABCA4 is found in photoreceptors outer segments and the retinal pigment epithelium (RPE). One of its main functions is to transport vitamin A derivatives from the photoreceptor disc lumen to the cytosol. Reduced ABCA4 activity leads to build-up of bisretinoids and lipofuscin, causing toxicity, which ultimately leads to degeneration of RPE cells and vision loss.
In this study we evaluated the efficacy of ubiquitous and RPE-specific promoters to drive AAV-delivered transgene expression and determined the optimal AAV serotype for robust transduction in cell models relevant to STGD1, including primary RPE cells.

Methods: Constructs with the *EGFP* gene driven by the ubiquitous CMV or CAG promoters (AAV-CMV.EGFP, AAV-CAG.EGFP) were generated as recombinant AAV vectors of serotypes 2/2, 2/5 and 2/8. Vectors were used to examine transduction efficiencies in ARPE19 and hTERT RPE-1 cell lines, employed to model RPE, and primary porcine RPE cells. A further construct, encompassing the putative macular dystrophy vitelliform 2 (VMD2) promoter (AAV-VMD2.EGFP), was packaged into AAV2/8 to evaluate this RPE-specific promoter in primary RPE cells *in vitro*, compared to AAV2/8-CMV.EGFP. Cells were transduced for 2 days with AAV, fixed with 4% paraformaldehyde and EGFP expression was analysed both natively and by immunocytochemistry.

Results: Transduction assays with EGFP showed that AAV2/2 transduces ARPE19 and hTERT cells the most efficiently. The order of efficacy was AAV2/2 > AAV2/8 > AAV2/5. Indeed, in primary porcine RPE cells AAV2/5 was 35x less efficient than AAV2/8, which was itself ~30x lower than AAV2/2. The VMD2 promoter mediated strong EGFP expression but significantly less than CMV.

Conclusion: Our data demonstrate the most efficient AAV serotypes and optimal multiplicities of infection (MOIs) for transducing cell models relevant to STGD1. We also showed robust VMD2-mediated expression, albeit lower than CMV-mediated expression, in keeping with other tissue-specific promoters. These results will be used to design and evaluate potential therapeutics in these cell models.

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Evaluation of hyperreflective foci in the retina following subretinal delivery of adeno-associated virus in non-human primates

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Purpose: Recombinant adeno-associated virus (rAAV) have been established as a gold standard in terms of safety and efficacy in retinal gene therapy. Nevertheless, previous studies demonstrated that rAAV can induce immune responses despite the immune privilege of the eye. A potential indicator of immune cell infiltration/activation is the appearance of hyperreflective foci (HRF) in spectral domain optical coherence tomography (SD-OCT). Our aim is to evaluate whether clinical grade rAAV8 vector leads to increased appearance of HRF in non-human primates (NHPs) following subretinal gene therapy injection.

Methods: Different doses of rAAV8.hPDE6A vector (low dose: $1x10^{11}$ vector genomes (vg), medium dose: $5x10^{11}$ vg, and high dose: $1x10^{12}$ vg) were injected subretinally into the left eyes of NHPs. Right eyes received sham-injection. After 3 months of *in vivo* follow-up retinal sections were obtained and analyzed. The number of HRF on SD-OCT volume scans were counted from both eyes at 30 and 90 days.

Results: Animals from the high dose group showed more HRF than in the low ($P = 0.03$) and medium ($P = 0.01$) dose groups at 90 days. There was an increase in the mean number of HRF in rAAV8-treated eyes compared to sham-treated eyes at 90-days ($P = 0.02$). Sham treated eyes demonstrated a reduction of HRF numbers over time. In contrast, a significant increase over time was observed in the rAAV8-treated eyes of the high dose group ($P = 0.001$). The presence of infiltrating B- and T- cells and microglia activation were detected in rAAV8-treated eyes.

Conclusions: Some HRF in the retina appear to be related to the surgical trauma of subretinal injection. While HRF in sham treated retina become less frequent over time, they accumulate in rAAV8-treated eyes in a dose dependent manner. This may suggest sustained immunogenicity following subretinal injection of rAAV8 vectors at high doses.

Retinoschisin and novel interaction partners define a growing protein complex at the inner segments of the photoreceptors

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Purpose: Mutations in the *RS1* gene cause X-linked juvenile retinoschisis (XLRS), a hereditary retinal dystrophy in juvenile or adolescent males. Recently, we and others showed that the retinoschisin protein encoded by *RS1* binds to the retinal Na/K-ATPase and modulates its localization in the plasma membrane of the inner segments of photoreceptors. In this study, we aimed to identify novel interaction partners of the Na/K-ATPase-retinoschisin complex. Specifically, we have investigated extracts from porcine retina that interact with the retinoschisin / retinal Na/K-ATPase complex. This should provide new insights into the role of retinoschisin as a putative regulator of photoreceptor membrane compartmentalization.

Methods: Porcine retinal lysates were subjected to co-immunoprecipitation targeting the α3-subunit of the Na/K-ATPase (ATP1A3). Bound proteins were eluted and separated by SDS-PAGE for mass spectrometric analysis. Identified proteins were verified in co-immunoprecipitation experiments with murine retinal lysates. Localization of proteins of interest was investigated *via* immunohistochemistry in eyes from wildtype (wt) and retinoschisin-deficient (*Rs1h* knockout, $Rs1^{tm1Web}$) mice. Effect of retinoschisin on the total protein level was investigated in retinoschisin-deficient murine retinal lysates.

Results: Mass spectrometry from co-immunoprecipitates of porcine retinal lysates targeting ATP1A3 identified the voltage-gated potassium ion channels (Kv) subunits Kv2.1 and Kv8.2. Binding to the retinal Na/K-ATPase was verified. Immunohistochemical analyses in murine retinal cryosections revealed Kv localization to the inner photoreceptor segments, overlapping with the localization of retinoschisin and the retinal Na/K-ATPase. In retinae from retinoschisin-deficient mice, Kv2.1 and Kv8.2 distribution and total protein amount was greatly affected when compared to wt retinae.

Conclusions: Our data suggest that Kv subunits Kv2.1. and Kv8.2 are part of a macromolecular complex together with retinoschisin and the retinal Na/K-ATPase. Defective compartmentalization of the retinal Na/K-ATPase and its complexing partners may be an initial step in XLRS pathogenesis.

Efficient delivery of VCP siRNA via Reverse Magnetofection in Rho^{P23H} retinal explants

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Purpose: The use of synthetic RNA for RNA-based therapy and vaccination has gained increasing importance. Given the eye's anatomical seclusion, small interfering RNA (siRNA)-induced gene silencing bears great potential for targeted reduction of pathological gene expression that may allow rational treatment of chronic eye diseases in the future. However, there is an unmet need for techniques to provide safe and efficient siRNA delivery to the retina. We have used magnetic nanoparticles (MNPs) and magnetic force (Reverse Magnetofection) to deliver siRNA/MNP complexes into retinal explant tissue, targeting valosin-containing protein (VCP), a potential therapeutic target for dominant P23H mutation in rhodopsin (RHO^{P23H}) that leads to autosomal dominant Retinitis Pigmentosa (adRP).

Methods: For siRNA delivery, we used XPMag, MNPs recently developed (OZ Biosciences), optimized for safe and efficient magnetofection to retinal cell lines. We first tested MNPs to assess their efficacy in two retinal cell lines, the mouse immortalized cone photoreceptor cell line -661W- and the human immortalized retinal pigment epithelial cell line hTERT-RPE1. We established an interphase organ culture system of the postnatal retina, which allows quantitative and kinetic testing of compounds and drug delivery approaches in a chemically defined medium in the absence of serum factors. Subsequently, we established the delivery of VCP siRNA/XPMag complexes through the posterior RPE side of the retinal explants of RHOP23H rats using a magnetic force. VCP and RHO expression, photoreceptor cell survival, and microglial activation were evaluated using specific antibodies and by cell row quantification and TUNEL assay.

Results: XPMag enhanced *in vitro* VCP silencing in both cell lines by forming complexes with VCP siRNA via classic Magnetofection. Safe and efficient VCP siRNA delivery by Reverse Magnetofection was achieved into all retinal cell layers of retinal explants from the RHO^{P23H} rat. No toxicity was observed. Reverse Magnetofection does not activate gliosis or microglial activation, and reduction of VCP expression decreased photoreceptor degeneration, retinal stress, and inflammation in RHO^{P23H} retinal explants. VCP silencing through Reverse Magnetofection also restores RHO intracellular distribution in RHO^{P23H}.

Conclusions: Reverse Magnetofection offers an effective method to deliver siRNA into retinal tissue. Used in combination with retinal organotypic explants, Reverse Magnetofection can provide an efficient and reliable preclinical test platform of RNA-based therapy approaches for ocular diseases. Silencing VCP in RHO^{P23H} transgenic rat organotypic retinal cultures via Reverse Magnetofection can protect photoreceptor cell death and attenuate retinal degeneration *in vitro*.

AAV-mediated *RP2* gene replacement in fibroblast and retinal organoid models of X-linked Retinitis Pigmentosa

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Purpose: X-linked Retinitis Pigmentosa (XLRP) is a severe, early-onset inherited retinal degeneration. Mutations in *RP2* account for ~15% of XLRP cases. The ubiquitously expressed RP2 protein is thought to be involved in ciliary trafficking of lipid-modified proteins. This project aimed to characterise human-derived *RP2* null primary fibroblasts and 3D retinal organoids (ROs), and to test the efficacy of AAV-mediated *RP2* replacement in these models.

Methods: Primary fibroblast cultures were established using skin biopsies from individuals with *RP2* null mutations and unaffected controls. AAV-RP2 vectors were generated and transgene expression evaluated in cell culture and in mice. Patient-derived fibroblasts were transduced with AAV-RP2 for three days (1E5 viral genomes (vg)/cell). RP2 expression was analysed via RT-qPCR and Western blot.
Patient and control fibroblasts were reprogrammed into induced pluripotent stem cells (iPSCs) and an additional isogenic *RP2* knockout ('RP2 KO') line was generated via CRISPR/Cas9 editing. IPSCs were differentiated into mature ROs and evaluated by immunohistochemistry, RT-qPCR and RNA-seq. RP2 KO ROs were transduced with AAV-RP2 from day (D) 140-180 (1E11vg/organoid).

Results: Patient-derived fibroblasts exhibited reduced RP2 mRNA expression and lacked detectable RP2 protein, indicating nonsense-mediated decay of mutant transcript. Transduction of *RP2* null fibroblasts with AAV2/2-CAG-RP2 restored RP2 expression.
At D150, *RP2* null ROs displayed a significant increase in TUNEL-positive (apoptotic) photoreceptors and upregulated expression of pro-apoptotic genes compared to control ROs. This was followed by significant thinning of the outer nuclear layer (ONL) and a reduced percentage of rhodopsin-immunoreactive photoreceptors at D180. Treatment of RP2 KO ROs with AAV2/5-CAG-RP2 resulted in significant preservation of ONL thickness and increased rhodopsin expression.

Conclusion: RP2-deficient retinal organoids exhibited an early-onset rod degeneration phenotype not documented in previous animal models. AAV-mediated gene replacement restored RP2 expression and exerted a protective effect, increasing rod survival.

Understanding molecular mechanism of CRB1-linked retinal dystrophies using a porcine retina lysate pull-down proteomics approach

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Crumbs homologue 1 (CRB1) mutations cause inherited retinal dystrophies, including Retinitis Pigmentosa (RP) and Leber Congenital Amaurosis. They are characterized by progressive photoreceptor cell death and accumulation of pigmented deposits in the retina. Up to now no treatment is available to prevent loss of vision in these patients emphasizing the need to further understand the molecular and cellular mechanism underlying CRB1-linked retinal dystrophies.

With the aim to further elucidate a retina specific functions of CRB1 and its two close family members CRB2 and CRB3 we have investigated their retina-specific interactome. For this purpose, Strep-tag II/FLAG tagged human CRB or GFP protein (control) were overexpressed in HEK293T cells, purified by immunoprecipitation followed by a porcine retina lysate pull-down, using the purified CRB protein as a bait. Mass spectrometry was performed and CRB specific interactors relative to GFP were identified. Results show a significant enrichment of known CRB1 interacting proteins, including MPDZ, LIN7C, PALS1, EPB41L5, validating the experimental approach. Further analysis showed enrichment of proteins involved in actin cytoskeleton dynamics, autophagy, vesicle transport, phospholipid metabolism and signalling in CRB1 bait samples. Interestingly, further comparison of CRB1 with the two other family members show a major overlap of the interacting proteins between CRB1 and CRB2 but less in between CRB1 and CRB3 allowing to hypothesize specific retinal functions of each family member. Specific protein interactions link CRB family members to specific cellular pathways.

Future work will include validation of the tentative pathways by comparative analysis of iPSC-derived retinal organoids from RP patients with crb1 mutations, organoids with CRISPR/Cas9 mediated ko of CRB1- and the corresponding healthy controls.

In silico analysis of age-related macular degeneration associated loci reveals pleiotropic effects with phenotypes of the UK Biobank cohort

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Purpose: The latest genome-wide association study (GWAS) identified 34 genomic loci significantly associated with age-related macular degeneration (AMD). In the majority of cases GWAS data do not provide information about the biological mechanism underlying the association signal. Identifying GWAS signals with overlap between different diseases, however, has the potential to highlight shared pathways and pathomechanisms. To this end, this study aimed to highlight pleiotropic effects of the AMD-associated variants at the 34 known loci by using UK Biobank data, one of the largest repositories on phenotypes and omics information available to date.

Methods: Lead variants of the 34 known AMD-associated loci were analyzed in the PheWeb Browser, an online tool providing GWAS data regarding 1,403 phenotypes based on 51 – 77,977 cases and 330,366 – 408,908 controls in the UK Biobank dataset. Associations of the AMD lead variants with any of the 1,403 phenotypes were extracted with a nominal threshold of P-Value < 1 x 10-4. GWAS summary statistics for the respective traits were downloaded from the UK Biobank Summary Statistic database (Release 20180731) and colocalization analyses were performed to identify shared genetic signals between AMD and the phenotype of interest.

Results: The analysis identified five AMD-associated loci which harbor true pleiotropic effects, highlighting that the exactly same signal points to a highly significant AMD association and at the same time to an independent disease phenotype. Three of these AMD loci, known as *„ABCA1"*, *„LIPC"*, and *„APOE"*, are associated with high-density lipoprotein levels in blood plasma. The two remaining pleiotropic loci, *"ACAD10"* and *"TSPAN10"*, are associated with other eye disorders like cataract or myopia. Further, the *"APOE"* locus shared additional pleiotropic effects with the neuronal disorders Alzheimer's disease and dementia, as well as the cardiovascular phenotypes coronary atherosclerosis and ischaemic heart disease.

Conclusion: Our analysis identified five AMD-associated loci for which the underlying genetic signal contributes not only to AMD but also to other complex phenotypes. This provides an excellent starting point to explore the biological mechanisms underlying the signals of AMD association.

Lessons from a mouse model with relevance for geographic atrophy: A possible role of the learning T-cell system?

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Purpose: Age related macular degeneration (AMD) is a multifactorial disease, which involves a local, retinal process called parainflammation. It is established that cells of the innate immune system, e.g. mononuclear phagocytes, predominantly contribute to this local low-grade inflammation in the outer retina. Other types of immune cells were not so far thoroughly investigated, although a few papers delivered hints for a T-cell involvement. In this work, we try to investigate the frequencies of local T-cell subsets in the retina and analyze them comparatively to the systemic frequencies of T-cells in mouse models with relevance for AMD.

Methods: Aged (8- and 12-month-old) fraktalkine mice ($CX3CR1^{+/-, -/-}$) correspond with feature of geographic atrophy. For comparison between local and systemic effects, eyes and spleens were extracted from the mice, T-cells purified, labeled and measured using fluorescence activated cell sorting (FACS).

Results: We identified increased frequencies of activated partly antigen experienced CD4+ and CD8+ T-cell populations during aging of $CX3CR1^{-/-}$ mice. Astonishingly, we additionally found elevated T-memory frequencies in both subsets in 12-month-old homozygote mice. In the T-memory population of CD4+ and CD8+ T-cells we identified antigen experienced effector memory (TEM) populations which are increasing over time. Controls were decreasing over time or not significantly increasing indicating immune senescence by age. Compared to this local immune-phenotype systemic frequencies of memory T-cells of fraktalkine mice were lower than in controls.

Conclusion: This study shows abundancy of specialized T-cell populations at the local inflammation site of the retina in fraktalkine mice. Especially activated, antigen T-memory populations might be a trigger for the chronic low dose inflammation of AMD. Our data indicate for the first time a role of the learning immune system in a mouse model for low-grade local inflammation that leads to photoreceptor loss in aged animals. As this model displays features of human AMD, our data suggest a possible role of the learning immune system in AMD as well.

Galectin-3 as a target for retinal immunomodulation

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Purpose: Age-related macular degeneration (AMD) is a leading cause of vision loss in the western world. Chronically activated microglia is a hallmark of retinal degeneration and their therapeutic targeting may represent a potential immunomodulatory treatment option for AMD. Galectin-3 is a pro-inflammatory microglia regulator that is induced upon neuronal damage. Here, we hypothesized that modulation of galectin-3 via the small molecule inhibitor TD139 or genetic deficiency of *galectin-3* dampens mononuclear phagocyte reactivity and protects from retinal degeneration in the murine model of light-induced retinal degeneration.

Methods: BALB/cJ mice received intraperitoneal injections of 15 mg/kg TD139 or vehicle for five consecutive days, starting 1 day prior to exposure to 15,000 lux white light for 1 h. The effect of TD139 treatment on microglia reactivity was analyzed by immunohistochemical staining and *in situ* hybridization (RNAscope) of retinal sections and flat mounts. Spectral domain optical coherence tomography was performed to assess retinal thickness and thereby determine the extent of retinal degeneration. Galectin-3 knockout mice on Balb/cJ background were subjected to light damage and microglia activation as well as retinal thickness were assessed.

Results: RNAscope in situ analysis of *Aif-1* and *Lgals3* mRNA expression in BALB/cJ retinas showed migration of microglia in the outer nuclear layer (ONL) and in the subretinal space post light damage. TD139-treated animals displayed a significantly lower number of microglia in these areas. Iba-1-immunolabeling of cryosections showed many amoeboid microglia located in the ONL of light-damaged animals whereas TD139-treated mice only displayed ramified microglia in the IPL and OPL. Iba-1-stained retinal flat mounts revealed an increase of reactivate amoeboid microglia in light-damaged BALB/cJ mice, whereas the majority of microglia showed a ramified morphology in TD139-treated mice. Light-exposed BALB/cJ mice had a thinner ONL comparted to light-damaged and TD139-treated mice. The ONL of light-damaged galectin-3 KO mice was significantly preserved and microglia activation was reduced. Immunohistochemically staining also demonstrated a reduction of microglia in ONL, subretinal space and RPE.

Conclusion: Inhibition of Galectin-3 by TD139 decreased the number of chronically activated microglia and reduced retinal degeneration in the light damage paradigm. These findings were corroborated by similar findings in Galectin-3 knockout mice. Therefore, we conclude that the pharmacological inhibition of Galectin-3 may represent a concept for immunomodulation in retinal degenerative diseases including AMD.

Optimising human retinal organoid-derived photoreceptor transplantation into mouse models of retinal degeneration

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Purpose: Retinal degeneration is a leading cause of disability in the industrialized world. In retinopathies marked by photoreceptor loss, photoreceptor transplantation strategies are examined as potential treatment approaches. While transplantation settings have been optimized using murine donor cells, conditions for successful engraftment of organoid-derived human photoreceptors are still poorly understood. We thus aim to identify and define conditions that enable human photoreceptor maturation, tissue integration, and donor-host interaction upon transplantation into mouse models of retinal degeneration.

Methods: Human photoreceptors were isolated via flow cytometry of dissociated retinal organoids generated from the photoreceptor-specific Crx-mCherry-iPSC reporter line at three developmental stages, i.e. day (D)100, D200 and D300 of differentiation. Using local immune suppression, human photoreceptors were subretinally transplanted into mouse models of cone degeneration (CPFL1 mice) and cone and rod dystrophy (CPFL1;Rho-/-). After 10 weeks or 6 months, grafts were examined via immunohistochemical, ultra-structural and transcriptomic analysis.

Results: Human retinal organoid photoreceptors of all three developmental stages can successfully be transplanted and survive well in the murine retina for up to 6 months. The majority of donor cells express the pan-photoreceptor marker recoverin and the cone-specific marker arrestin 3. They show signs of maturation, e.g. the development of mitochondria-rich, inner segment-like protrusions and accumulation of synaptic vesicles. Transplanted cells present mainly in clusters which show different levels of interaction with the host retina, ranging from seemingly non-interactive cell masses remaining in the subretinal space to clusters replacing entire stretches of host ONL. Incorporation of cell clusters and existence of graft-host interactions, e.g. permeation of Müller glia processes, coincides with the presence of maturation signs. At 10 weeks after transplantation, such developments occur often in D200 but rarely in D100 and D300 grafts, suggesting 200 days to be a superior photoreceptor donor age.

Conclusion: Retinal organoid-derived human photoreceptors of varying age and developmental status have the capacity to survive and mature in the murine retina, while partly incorporating in clusters into the host ONL. Results suggest that maturation of donor photoreceptors, which is a prerequisite for functional replacement of lost photoreceptors, requires close interaction with the host tissue. Understanding the emergence of non-interactive and highly interactive grafts could have important implications for future cell replacement therapies in the eye.

The novel PKG inhibitor CN238 provides multi-level functional neuroprotection of photoreceptors and ganglion cells

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Background: Overactivation of cGMP-dependent protein kinase G (PKG) is one of the key events in the cell death mechanism that characterises hereditary photoreceptor degeneration. Previous studies have shown that inhibition of cGMP/PKG signalling can slow down photoreceptor loss in several models of retinal degenerative diseases. This makes PKG a potential target for neuroprotective strategies. In this study, we identified a novel PKG inhibitor, the cGMP analogue CN238, with strong protective effects on photoreceptors in retinal explants derived from *rd10* mice. We further investigated the effects of this compound on retinal function using multi-electrode arrays (MEAs) on *rd10* and wild-type (WT) retinal explants. The recordings revealed a stronger photoreceptor response and, surprisingly, increased retinal ganglion cell (RGCs) activity in the treated samples.

Methods: The cGMP analogue CN238, as well as the reference compounds CN003 and CN226, were tested on organotypic retinal explant cultures derived from *rd10* mice. Retinas were cultured either from P9 to P17/P19, or from P12 to P24. The protective capacities of cGMP analogues were evaluated *ex vivo* by counting the number of photoreceptor rows. At a functional level, the PKG-inhibition dependent protection of photoreceptors was evaluated with multi-electrode array (MEA) recording on treated and untreated (NT) *rd10* and wild-type (WT) retinal explants. The physical presence and viability of the RGCs was assessed on histological preparations from recorded samples via labelling with an antibody directed against RNA-binding protein with multiple splicing (RBPMS).

Results: Testing the effects of cGMP analogues on the *rd10* retina showed an increasingly stronger rescue of photoreceptors from P17 to P24, with ≈55 % and ≈46 % higher photoreceptor row counts at P24 in samples treated with CN003 and CN238, respectively. The PKG inhibitor CN238 improved photoreceptor light responsiveness when compared to NT. Moreover, RGCs in *rd10* and WT retina explants treated with the PKG inhibitors CN003 and CN238 displayed a markedly stronger and light-correlated response to stimulation, in comparison to NT or CN226. This functional preservation of RGCs was confirmed when RBPMS staining showed increased numbers of positive cells in the ganglion cell layer of CN003 or CN238 treated specimens.

Conclusion: While the lead compound CN003 confirmed its protective effects seen previously, the novel compound CN238 also showed marked photoreceptor-protective effects in *rd10* retinal explants at both viability and functional level. Furthermore, electrophysiological analysis revealed a neuroprotective effect of PKG inhibition on RGC function, despite the axotomy that characterises the retinal explant model. Together, these results revealed new insights into the properties of cGMP analogues and extend their protective capacities from degenerating photoreceptors to also RGCs, thereby significantly broadening their potential applicability for the treatment of retinal diseases.

An oxidative stress model based on iPSC-derived endothelial cells to study molecular processes in choroidal neovascularization

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Purpose: Age-related macular degeneration (AMD) is the leading cause of vision loss in developed countries and despite intensive research over the last decade its molecular pathomechanism is still not yet understood. Due to its complex nature, a unique combination of both genetic and environmental factors contribute to a person's individual risk to develop AMD. Next to the RPE recently close attention has been paid to the contribution of choroidal endothelial cells (ECs) in the initiation and progression of AMD pathology. In this study, we aimed to establish a repository of induced pluripotent stem cell (iPSC) derived ECs with a defined genetic background and known AMD risk to be used as cellular models to investigate molecular mechanisms of neovascularization. This model largely relies on an environmental trigger, specifically mimicking an oxidative stress situation.

Methods: iPSCs with a defined genetic AMD risk were differentiated into ECs using a protocol established at the Institute of Human Genetics. Structural EC properties were verified by immunocytochemistry, qRT-PCR analysis of EC marker gene expression as well as FACS analysis. Functional EC characteristics were confirmed by an *in vitro* tube formation assay, a cell migration as well as an LDL uptake assay. Oxidative stress was induced by paraquat (PQ) and the optimal PQ concentration was determined by an MTT assay after 24 hours of treatment.

Results: A largely modified differentiation protocol allowed for the generation of eight iPSC-EC lines, each four with an exceptional high or low genetic risk for AMD. All cell lines expressed EC makers CD34, CD31, CD144, PLVAP and vWF. All cell lines generated formed tubular-like structures *in vitro*, representing a key function of ECs *in vivo*. Cell lines also displayed efficient cell migration and LDL uptake, further confirming the successful and efficient differentiation of ECs from iPSCs. Treatment with 0.25 mM PQ for 24 h proved to be suitable for inducing oxidative stress without significantly reducing cell viability.

Conclusions: Having established patient-derived EC lines with known genetic AMD background and a reproducible protocol to induce cellular oxidative stress will allow us to address molecular pathways associated with AMD in a novel *in vitro* system. In future experiments, iPSC-RPE cells and iPSC-EC with a defined genetic AMD are planned to be merged in a closer to reality co-culture model.

Screening for pharmacologically active molecules for Müller Cell Dedifferentiation

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Purpose: Many blinding diseases of the retina involve neurodegeneration, e.g., ganglion cell loss in glaucoma or photoreceptor loss in retinitis pigmentosa and age-related macular degeneration. Often, the ongoing loss cannot be stopped or the disease is diagnosed at a late stage. Similarly, chemical or physical insults to the retina can lead to severe loss of retinal neurons and as a result vision loss. Therefore, there is a demanding need for restorative therapies to regain vision with patients that already have severe neuronal loss. Unlike mammalians, zebrafish Muller glia has an extraordinary regenerative capacity, which opens up the possibility of Muller glia reprogramming for compensating the neuronal loss. Since reprogramming of cells with small molecules is feasible, we performed a drug screen to elucidate the dedifferentiation capacity of mammalian Muller glia into rod photoreceptors.

Methods: In order to screen for Müller cell dedifferentiation process, we have generated a double reporter mouse line Tg(Rlbp1-GFP); Tg(Rho-Cre); ROSA26-LSL-tdTomato. Muller glia from this transgenic line that are expressing GFP were isolated and cultured *in vitro.* Upon rod fate induction, Muller glia expressed Cre (Tg(Rho-Cre)) which removed the stop codon within ROSA26-LSL-tdTomato and labeled Muller glia that has activated rhodopsin promoter. We have used this transgenic line for screening 1280 molecules that are proven to be pharmacologically active in cell signaling and neuroscience. Screen was performed on 384-well plates for 5 consecutive days at a concentration of 20 µM for each molecule.

Results: Muller glia dedifferentiation screen depended on the activation of rhodopsin promoter and thus Cre expression, which turned on tdTomato expression as a read-out. We have identified several candidate molecules, which induced tdTomato expression and highly potent molecules were classified into three pathways. Independent of rod fate induction, several molecules induced morphological changes in Muller cells and even formation of small crescent-like colonies.

Conclusion: Our approach using tdTomato as a read-out for Muller glia dedifferentiation screen was very effective. This allowed us to identify potential molecules and pathways and argued that given the right conditions, it could be possible to potentiate the limited regenerative capacity of Muller glia in mammalian retina.

Live-imaging of photoreceptor degeneration and glia pathology in human retinal organoids

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Purpose: Until today, no high resolution live-imaging studies of the morphological processes of retinal degenerative diseases have been performed in a human model system to our best knowledge. These insights would close gaps in our understanding of the cellular processes and dynamics of vision loss in neurodegenerative diseases. We recently developed a human pathology model in retinal organoids that replicates a complex phenotype with simultaneous and dynamic photoreceptor (PR) and (MG) Müller glial pathologies – which is an AMD hallmark (Völkner et al. in revision). Here, we applied this model and show that it offers access to 3D live-imaging studies, and insight into a new pathomechanism.

Methods: We use our established protocol for cone-enriched human retinal organoids from pluripotent stem cells of healthy donors. Combined application of the disease-relevant factors HBEGF and TNF are sufficient to induce a dynamic retinal pathology. Thereby, we established methods for effective real-time imaging of distinct cells by spinning disc light microscopy as well as optical coherence tomography (OCT).

Results: To study human PR and MG in living organoids, and cellular changes thereof in our pathology model, we utilized transgenic reporters for PR (CRX-mCherry, provided by O. Goureau, Paris), and a newly developed one for MG (RLBP1-eGFP). Real-time fluorescence imaging of organoids in control conditions showed minimal changes in PR and MG. Notably, in our pathology model, cell nuclei undergo radial movements within the retina, and PR are lost by cell displacement through the apical outer limiting membrane, labeled by SiR-actin live dye. This confirms our hypothesis and histology data of a new pathomechanism for retinal degeneration by PR extrusion. Clinical-like live imaging by optical coherence tomography revealed loss of photoreceptor segments, and real-time imaging shows pathologic MG cell movements, displacements, and cell divisions of RLBP1-eGFP labeled MG.

Conclusion: Live-imaging is a prerequisite to understand physiological and pathological processes in neurodegenerations. By revealing a previously unknown pathomechanism for PR degeneration, we identified a new target to develop therapeutic approaches.

Co-cultivation of RPE cells and porcine neuroretina as an *in vitro* retinal model

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Purpose: The pathological events of age-related macular degeneration are characterized by degenerative processes involving the photoreceptor cells, the retinal pigment epithelium as well as the Bruch's membrane and choroidal alterations. In order to investigate AMD pathophysiology an established porcine neuroretina cultivation was modified into a co- cultivation model combining neuroretina explants with primary porcine RPE (ppRPE) cells to imitate the *in vivo* situation and analyze the effect of a feeder layer or RPE secreted factors on the neuroretina.

Methods: Porcine neuroretina samples were co-cultivated with ppRPE cells (150,000 cells/well) for 8 days. Another group of neuroretina samples was cultivated only with conditioned media collected from ppRPE cells. Neuroretina explants cultivated alone served as controls. After 8 days, H&E staining and immunohistochemistry (n=7/group) were performed to analyze the preservation of cone (opsin) and rod (rhodopsin), microglia (Iba1, iNOS), and pre- (VGLUT) and postsynapses (PSD95). These markers as well as complement factors (factor H and C3) and cytokines (Il-6, Il-8, TNFα) were also analyzed via RT-qPCR (n=6/group).

Results: The presence of ppRPE cells led to a better preservation of rods (p=0.033) and a greater $PSD95^+$ area (p=0.0002). RT-qPCR results showed that M-opsin was significantly increased in co-cultivated samples. Also, adding ppRPE conditioned medium showed an increased $PSD95^+$ area (p=0.002). Microglia counts were comparable in all groups, while there was a significantly increased expression of complement factors and interleukins in conditioned medium samples.

Conclusion: The co-cultivation of ppRPE cells and neuroretina explants indicates a beneficial effect of a ppRPE feeder layer through soluble factors of the cells. This *ex vivo* model offer possibilities for future AMD studies, by facilitating the investigation the interaction between RPE cells and neuroretina.

Innovative drug candidates for the treatment of glaucoma

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Purpose: Glaucoma is a heterogeneous group of eye diseases, which result in damage to the optic nerve causing irreversible vision loss. Current treatments for glaucoma focus on lowering intraocular pressure (IOP). However, only one-third to half of glaucoma patients have elevated IOP at the initial stages and disease progression can still occur despite adequate IOP control. Therefore, there is an urgent need to investigate novel therapeutic concepts that complement IOP regulation. We investigated novel compounds that increase cyclic GMP (cGMP) levels in retina by releasing NO and inhibiting PDE5 simultaneously. This dual mode of action may have neuroprotective potential by decreasing IOP and increasing ocular blood flow.

Methods: Candidate compounds were tested in various neuronal and epithelial cell types of the retina. cGMP response profiles were generated for each retinal cell type following compound treatment. In addition, cell viability and phagocytosis of human RPE cells were investigated upon treatment. cGMP levels in retina were determined upon treatment of ex vivo isolated mouse retina with the compounds. For in vivo studies, TOP-V122 (1%) was applied topically using a newly developed formulation in 129S6 mice. To investigate potential physiological effects of the treatment, fundus photography, optical coherence tomography and electroretinography were applied.

Results: Following compound treatment, cGMP levels increased in rMC-1, ARPE-19 and HRPEpiC cells in both time and dose dependent manner. Treatment did not affect the cell viability and phagocytosis of human RPE cells. 3-fold increased cGMP levels were measured in ex vivo isolated mouse retina upon TOP-V122 (1µM) treatment. The effect was not dependent on endogenous NO levels in retina, but on the presence of a functional soluble guanylate cyclase. In vivo, multiple topical applications of TOP-V122 (1%) increased cGMP levels in retina compared to the control treatment of the contralateral eye by 65% (27.87 ± 5.30 pmol/mg vs. 16.92 ± 1.67 pmol/mg, n=4, P=0.0076) without affecting retinal function.

Conclusion: Novel compounds targeting PDE5 effectively increase cGMP levels in various retinal cell types and the retina. Repeated dosing did not affect retinal function and structure. The results obtained substantially contribute to understanding the potential use of PDE5 inhibitors specifically designed for retinal neuroprotection and pave the way for future research in this area.

A universal AAV-based approach to induce pathway-specific retinal pathologies in the mouse eye

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Purpose: Retinopathies display complex pathologies in patients including vascular pathologies, inflammation and fibrosis, leading ultimately to blindness. However, animal models that accurately reflect human pathologies and are easy to handle are currently missing.

Methods: Adeno-associated viruses (AAVs) are a well-characterized tool to manipulate gene expression in a tissue-specific manner and can be used to quickly generate novel disease models *in vivo*. In contrast to injection of recombinant proteins, AAV-mediated expression of genes is stable and can be used for long-term studies. Here, we injected AAVs into the mouse vitreous to express human retinopathy-associated genes that encode soluble growth factors/cytokines to mimic defined aspects of diseases.

Results: VEGF-A is a well-known vascular growth factor that is upregulated in many retinopathies such as Diabetic Retinopathy (DR) and Age-related macular degeneration (AMD). Anti-VEGF antibodies are currently the gold-standard for treatment of AMD and DR. Here, we injected AAVs to express $hVEGF_{165}$ into the mouse vitreous to mimic the vascular pathologies observed in patients. Interestingly, 3-6 weeks after injection of AAV-hVEGF in mice we observed diverse vasculopathies, including neovascularization and vascular leakage, reflecting vascular pathologies seen in patients. Next, we investigated IL-6, a pro-inflammatory cytokine that is upregulated in the vitreous of DR patients, but only little is known about the direct function of IL-6 in the disease progression. Interestingly, continuous expression of hIL-6 by AAVs led to inflammation of the mouse retina as indicated by immune cell activation and subretinal infiltration of immune cells.

Conclusion: In summary, with our AAV-based approach we induced the long-term expression of human VEGF or IL-6 in the murine eye and observed specific pathologies mimicking human diseases within few weeks. To reflect human pathologies even more closely, we next aim to combine our AAVs to induce both vascular pathologies and inflammation in the same eye. The use of AAV-mediated transduction enables the generation of novel mouse models and provides valuable insights to better understand the underlying molecular mechanisms in retinopathies.

Whole genome sequencing and *in vitro* functional analysis of 103 previously unresolved inherited retinal disease cases.

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Purpose: Inherited retinal diseases (IRDs) are a major cause of visual impairment globally. These clinically heterogeneous disorders are caused by pathogenic variants in over 270 genes. As 30-40% of cases remain genetically unexplained following preliminary sequencing measures, we aimed to ascertain a positive genetic diagnosis in a cohort of previously unresolved IRD patients using whole genome sequencing (WGS).

Methods: Following inconclusive results having employed targeted panel or whole exome sequencing, WGS was employed to identify causative variants in 103 unresolved cases. A focused in-depth interrogation of all non-coding and structural variants was implemented in genes where one pathogenic coding variant was previously identified. Functional analysis of putative splice-altering variants was performed using a midigene or minigene-based *in vitro* splice assay.

Results: Candidate disease-causing variants were identified in 32 patients. Causative coding variants were observed in *CEP78, PCARE, C21ORF2, BBS1* and *HGSNAT* among other genes. Pathogenic structural variants were detected in *PRPF31* and *RPGRIP1* as well as a CAG repeat expansion in *ATXN7*. Candidate non-canonical splice site or deep-intronic variants predicted to disrupt the splicing process *in silico* were identified in 17 monoallelic cases. 12/17 cases were resolved due to the presence of pathogenic splice defects following *in-vitro* functional analysis.

Conclusion: This study confirms that WGS is a powerful tool to identify causative variants, having previously employed less comprehensive sequencing methods as a preliminary diagnostic measure. This study also highlights the importance of the analysis of non-coding regions beyond non-canonical splice-sites. We suggest that WGS is the optimal variant detection method, enabling a genetic diagnosis in previously unresolved cases.

Mapping the daily rhythmic transcriptome in the diabetic retina

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Purpose: The eye is a rhythmic organ specifically evolved to function around the light cycle via functional circadian clocks. Diseases such as diabetes have been reported to disrupt circadian rhythms and circadian disruption emerges as an important factor in the prognosis of disease outcomes and treatment success. Herein we mapped the rhythmic transcriptome in the mouse retina to understand the extent of circadian disruption due to diabetes.

Methods: Healthy control and Ins2Akita/J diabetic mice were kept under a physiological 12h:12h light-dark cycle until 4 months of age. Deep mRNA sequencing was conducted in retinas collected every 4 hours around the day/night cycle. Computational approaches were used for detection of rhythmicity, acrophase prediction, differential rhythmic patterns, phase set enrichment analysis and upstream regulator predictions. Validation was done with invitro experiments on human retinal endothelial cells.

Results: Almost 10% of the retinal transcriptome was identified as rhythmic with a clear 12hr axis of transcriptional activity, peaking at midday and midnight. Although the 12-hour transcriptional axis is retained in the diabetic retina, it was phase advanced by approximately 1-3 hours. Downstream analysis identified oxygen sensing mechanisms and HIF1A as major predicted upstream regulators. In vitro experiments with hypoxia indicated that indeed hypoxia can phase shift the clock in human retinal endothelial cells.

Conclusions: To our knowledge this is the first study mapping the effects of diabetes in the rhythmic output in the retina. Importantly, we identified that hypoxia may be a primary driver of disrupted circadian rhythms in the diabetic retina.

The TSPO-NOX1 axis controls phagocyte-triggered pathological angiogenesis in the eye

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Purpose: Aberrant immune responses including reactive phagocytes are implicated in the etiology of age-related macular degeneration (AMD), a major cause of blindness in the elderly. Our previous work showed that TSPO is a marker for reactive retinal microglia and that the selective TSPO ligand XBD173 exerts strong anti-inflammatory and neuroprotective effects on microglia in the mouse model of laser-induced CNV. However, the biological functions of TSPO in retinal diseases remain elusive. In this study, we focus on elucidating the molecular function of TSPO in retinal immune homeostasis and angiogenesis

Methods: The mouse model of laser-induced CNV was used to mimic the exudative form of AMD. Retinal inflammation and CNV were analyzed by fundus fluorescein angiography (FFA), lectin staining and optical coherence tomography (OCT) 3, 7 and 14 days after laser coagulation. In addition, microglia morphology in laser-induced lesions was analyzed by Iba1-staining of retinal and RPE/choroidal flatmounts. To investigate cytokine levels of pro- inflammatory and pro-angiogenic markers at different timepoints after laser damage we performed ELISA assays. Isolated primary microglia were used to analyze ROS and calcium levels.

Results: Tamoxifen-induced conditional deletion of TSPO in resident microglia using $Cx3cr1^{CreERT2}$:$TSPO^{fl/fl}$ mice prevents reactivity of retinal phagocytes in the laser-induced mouse model of neovascular AMD. Concomitantly, the subsequent neoangiogenesis and vascular leakage are also prevented by microglia-specific TSPO knockout. Using different NADPH oxidase (NOX)-deficient mice, we show for the first time that TSPO is a key regulator of NOX1-dependent neurotoxic ROS production in the retina. Here, TSPO regulates the Ca^{2+} influx from the extracellular milieu into the cytosol that is required for stimulation of NOX1 activity in microglia. We also demonstrated that NOX1- derived ROS induce photoreceptor cell death in a paracrine manner.

Conclusion: TSPO is critical for the Ca^{2+} associated, NOX1-mediated production of extracellular ROS in retinal phagocytes. Targeting TSPO by gene knockout limits retinal innate immune cell responses and pathological angiogenesis. Our data define a distinct role for TSPO in retinal phagocyte-triggered neoangiogenesis and highlight the protein as a drug target for immunomodulatory and antioxidant therapies for AMD.

Anti-retinal antibodies impact retinal physiology in neuromyelitis optica spectrum disorders

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Purpose: Neuromyelitis optica spectrum disorders (NMOSD) are antibody-mediated, complement-associated inflammatory diseases of the central nervous system that primarily affect the optic nerve and the spinal cord. Approximately 80% of NMOSD patients are positive for autoantibodies against the water channel protein aquaporin-4 (AQP4). In previous studies NMOSD patients showed a progressive loss of retinal ganglion cells without opticus neuritis, which suggests an initial retinal, inflammatory process. Here, we investigate local complement and inflammatory marker expression in the retina induced by AQP4-antibodies and the systemic complement status of AQP4-antibodies seropositive NMOSD patients.

Methods: NMOSD immunoglobulins were purified using a protein A column from patient serum. Retinae were dissected from female C57BL/6J mice and cultured with purified NMOSD-IgG. After cultivation, retinae were evaluated for *c1qb, c1s, c3, cfh* and *aqp4* gene expression using qPCR. Expression of inflammatory markers was evaluated on protein level using a Multiplex Mouse Cytokine Assay and immunohistochemical staining. Patient plasma and serum samples were analysed for complement expression with a Multiplex Human Complement Panel.

Results: Alteration of the retinal homeostasis was observed after retinal NMOSD-IgG binding. Hallmarks of a shift to a pro-inflammatory, NMOSD-IgG depending environment were (i) elevated *c3* mRNA levels, (ii) increased retinal CCL3, CCL4 and TNFα secretion and (iii) intensified immunoreactivity of Müller cells. Analysis of patient plasma revealed elevated C3 and C5a and decreased C5, C4, CFB, CFD, CFP and CFI protein levels in NMOSD patients compared to healthy controls. A gender-specific decrease in CFI and C5 protein level was observed in male compared to female NMOSD patients.

Conclusion: Local changes in retinal physiology in response to NMOSD-IgG binding indicate a primary retinopathy in NMOSD and lay the basis for the next step in our project – investigating the complement involvement in retinal degeneration after NMOSD-IgG binding.

ALMS1 interactome identification and its role in ciliopathies

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Alström Syndrome is a rare autosomal-recessive disorder, that occurs in 1 to 9 out of 1 million individuals. More than 300 mutations in the ALMS1 gene are known causing cause a broad range of clinical defects most notably retinal degeneration, Type 2 Diabetes and truncal obesity. The ALMS1 gene codes for a ~0.5 MDa protein that localizes at the centrioles and the basal body of cilia. So far, the molecular function of ALMS1 and how its mutations cause or influence the severity of the Alström disease in ciliated organs remain unknown.

Based on current literature and own data, we hypothesize that ALMS1 is necessary for basal body structure and function. We found, that ALMS1interacts with gamma tubulin as well as components of the ciliary interactome related to cargo selection, cilia-related transport and signaling processes at the cilium.
In this study, superfolder GFP (sfGFP) was fused to the C-terminus of the ALMS1 gene via CRISPR/Cas9 mediated gene-editing. Protein complex analysis after pull down of the fusion protein was performed using liquid chromatography coupled to mass spectrometry (LC-MS/MS). The analysis of the resulting data suggests a role of ALMS1 in ciliary basal-body-plasma membrane docking, cilium dis/assembly and signal transduction.
Comparative phenotypic analysis of ALMS1 function in cells was performed by comparing cells with or without ALMS1. CRISPR/Cas9 mediated ALMS1 knock-out in exon 8 and 10 of retinal epithelial cells was induced and single clones selected. Investigation of ciliogenesis in ALMS1 deficient cells revealed normal looking cilia compared to the control. However, γ-tubulin was reduced in the ALMS1 ex10 KO compared to the isogenic control. This finding stresses a role of ALMS1 in basal body and cilia structure and stability. As interconnecting cilium stability appears essential to maintain proper inner to outer segment structure of photoreceptors, this at least in part, may explain, what photoreceptors are especially vulnerable to defects in ALMS1 function.

ENaC is important for proper Müller cell function and retinal integrity

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Purpose: Retinal Müller glia (RMG) are important for retinal integrity and neuronal activity by supporting neurons with nutrients, removing their metabolic end products and released transmitters, and by maintaining a constant pH, ion and water homeostasis in the tissue. The water channel aquaporin 4 (AQP4) and the inwardly rectifying potassium channels (Kir2.1 and Kir4.1) are well-known players in the transcellular water transport. The epithelial sodium channel (ENaC) has also been found to be expressed by RMG, influencing neuronal signaling. Its exact function, however, remains elusive. Here, we aim to uncover the function of ENaC in the retina in more detail.

Methods: To check for cell-specific expressions of the different ENaC subunits, we performed magnetic-activated cell sorting with subsequent qRT-PCR analysis. Immunohistochemistry of αENaC, microglia and RMG markers, AQP4 and Kir4.1 was compared between conditional glia-specific αENaC-KO and control mice and cell numbers of the different retinal layers were quantified. To examine the functional role of ENaC, we carried out volumetric measurements of RMG in retinal slices treated with benzamil, a specific blocker of ENaC.

Results: Quantitative RT-PCR revealed low levels of αENaC expression in RMG. Immunofluorescence analysis confirmed RMG-like distribution of αENaC, mainly in the plexiform layers, which was decreased in conditional αENaC-KO mice. Morphometric analysis demonstrated reduced cell numbers, including RMG, in the inner nuclear layer of αENaC-KO compared to control mice. The remaining RMG upregulated GFAP expression. In line with this gliotic response, we found a higher number of retinal microglia. Fluorescence intensity measures of the inner limiting membrane indicated that the RMG endfeet of αENaC-KO mice express less Kir4.1. Upon benzamil treatment, RMG were less able to counteract hyposmotic stress in retina slices *in vitro*.

Conclusion: Stable ion and water concentrations in the extracellular space are a prerequisite for proper retinal function. Whereas research has focused on the well-explored potassium (and accordingly water) siphoning capacities of RMG, we show here that the epithelial sodium channel ENaC is involved in RMG volume regulation as well. The loss of this capacity seems to influence retinal integrity and induce a gliotic response.

Investigations on the relationship between poly-ADP-ribose polymerase-1 (PARP-1) and calpain during hereditary retinal degeneration

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Background: Retinitis pigmentosa (RP) is a group of hereditary retinal degenerative diseases in which rod photoreceptors die due to a genetic mutation, whereas cone photoreceptors disappear secondarily, once rods are gone. Current evidence indicates that in the pathogenesis of RP, a non-apoptotic degenerative mechanism is of major importance. In this non-apoptotic cell death mechanism, mutation-induced up-regulation of cGMP on the one hand causes activation of the CNG channel, leading to Ca^{2+} influx and calpain activation. On the other hand, cGMP-dependent activation of protein kinase G (PKG) is associated with histone deacetylase (HDAC) and poly-ADP-ribose-polymerase (PARP) activation. However, it is unknown whether there is a relationship between these two pathways.

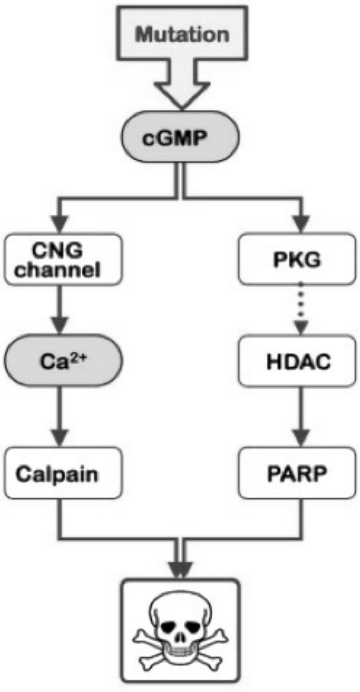

Figure 1. non-apoptotic degenerative mechanism

Methods: Here, we investigated the possible interaction by inhibiting calpain, Ca^{2+} channels, and PARP, respectively, using the well-established experimental system of organotypic retinal-degenerative explant cultures. Immunofluorescence for the activity of calpain and PARP was performed on post-natal day (P) 11 retinal degeneration 1 *(rd1)* mouse. Retina explants derived from P5 *rd1* mice were treated with calpastatin, D-cis-diltiazem, olaparib, calpastatin, and olaparib together with calpastatin, respectively, from P7 to P11. After histological workup, the effect of different treatments on cell viability was studied via the TUNEL assay.

Results: Immunofluorescent staining of calpain activity showed a significant reduction after treatment with olaparib, while calpastatin did not decrease PARP activity. Yet, the voltage-gated-Ca^{2+} (VGC) channel blocker D-cis-diltiazem significantly decreased PARP activity. Remarkably, there was no extra therapeutic effect on cell viability after the combined treatment with calpastatin and olaparib.

Conclusion: We found that the PARP inhibitor olaparib, significantly decreased calpain activity and the VGC channel inhibitor, D-cis-diltiazem, significantly decreased PARP activity. However, the combination of calpastatin and olaparib did not bring any additional effects of treatment. This indicates that 1) VGC channel activity is involved in regulating calpain and PARP activity and that 2) both enzymes are part of the same cell death pathway, and that 3) PARP activity contributes to activation of calpain (Figure 2).

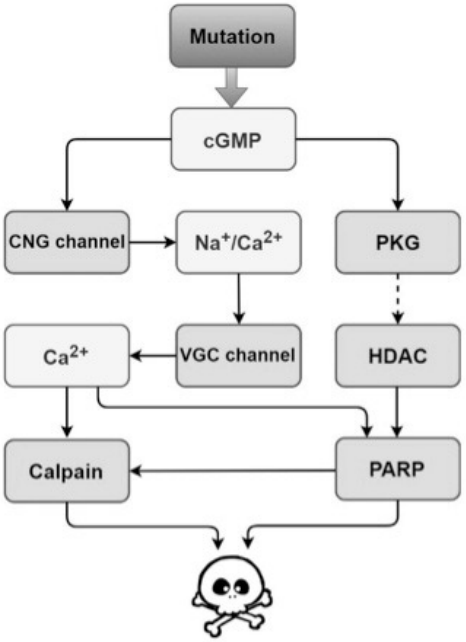

Figure 2. Pathway diagram for a non-apoptotic degenerative mechanism in photoreceptors.

Investigating photoreceptor cell death mechanisms in the new *Rho^{I256del}* mouse model for autosomal dominant retinitis pigmentosa

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Purpose: Retinitis pigmentosa (RP) is a currently untreatable hereditary retinal degeneration, in which many different gene mutations cause gradual rod photoreceptor death. The incomplete understanding of the underlying photoreceptor cell death mechanisms is a major hurdle towards effective therapy development. High intracellular cGMP is associated with the activation of calpain and poly-ADP-ribose polymerase (PARP) and, in RP animal models carrying different gene mutations, all three are often connected to photoreceptor cell death. In the present study, we investigated the possible involvement of cGMP-signaling, as well as calpain and PARP activity in a new mouse model for autosomal dominant RP caused by a mutation in the rhodopsin *(Rho)* gene.

Methods: The new *Rho^{I256del}* mouse model was established based on a disease-causing mutation found in a British family. Retinal tissue sections were obtained from both homozygous and heterozygous animals. Eight different timepoints (P11, P13, P15, P18, P20, P24, P31, P41) were analyzed, to explore the *in situ* activity of calpain and PARP. In addition, accumulation of cGMP was investigated using immunofluorescence. At least 3 different sections from 3 different animals per timepoint were analyzed.

Results: A strong activation of calpain and PARP enzymes was found in retinas from homozygous *Rho^{I256del}* mice, with a peak of calpain activity at P18. The thickness of the outer nuclear layer (ONL; *i.e.* the photoreceptor layer) decreased strongly from P11 to P24. The immunostaining for cGMP did not reveal any obvious signal in tissue sections obtained from both homozygous and heterozygous mice.

Conclusions: The activity of calpain and PARP enzymes was closely correlated to the progression of photoreceptor degeneration in the *Rho^{I256del}* retina. This may highlight both enzyme systems for future therapeutic interventions. Contrary to previous observations in other animal models, in the *Rho^{I256del}* mouse, calpain and PARP activity did not appear to be obviously connected to high cGMP in photoreceptors. However, we can, at present, not exclude a minor (below the detection limit of the immunostaining) upregulation of cGMP. Future studies in the *Rho^{I256del}* mouse retina may focus on targeting calpain and/or PARP activity for therapeutic development.

Keywords: Photoreceptor cell death; RD; cGMP; calpain; PARP

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