PRO RETINA FOUNDATION

PRO RETINA
POTSDAM-MEETING

16th PRO RETINA RESEARCH-COLLOQUIUM POTSDAM

Conference Report | April 01/02, 2022

Retinal Degeneration

Environment Matters

An Interdisciplinary Dialogue

THIS MEETING IS SUPPORTED BY

PRO RETINA FOUNDATION

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RESEARCH-COLLOQUIUM POTSDAM

CONFERENCE REPORT

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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 four objectives mentioned in the constitution are to actively support research, to give psychological and social advice for its members, to strengthen public awareness and to stand in for patient interests in politics, society and health care system.
Every member can join one of the 60 regional groups, which are spread throughout Germany. At present (2022), PRO RETINA Deutschland e. V. counts more than 6,500 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 Bonn (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 and a Working Group on Clinical Issues. 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 01, 2022

13:00 – 13:05 **Welcome remarks**
Franz Badura (Amberg, Germany)

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

14:30 – 15:00 **Keynote Lecture**
A systems biology view on age-related macula degeneration
Marius Ueffing (Tübingen, Germany)

15:00 – 15:45 **Coffee break**

15:45 –17:25	Session 2	Factors shape the efficiency of gene therapy
		Chair: Thomas Langmann
	15:45 – 16:10	Immune responses to retinal gene therapy using adeno-associated viral vectors – Implications for treatment, success and safety *Dominik Fischer (Oxford, UK)*
	16:10 – 16:35	AAV-mediated retinal pathology in mice is connected to reactive microgliosis *Anne Wolf (Cologne, Germany)*
	16:35 – 17:00	AAV vectors and CRISPRa-Cas9-based (epi)genome editing approaches for retinal gene therapy *Elvir Becirovic (Munich, Germany)*
	17:00 – 17:25	Microglia during retinal degeneration *Sandra Siegert (Kloster Neuburg, Austria)*

17:25 **Dinner**

19:00 –19:30 **Evening Lecture**
Current strategies for the inhibition of myopia
Frank Schäffel (Tübingen, Germany)

19:30 – open **Swingin' Poster Session**

PROGRAMME

Saturday, April 02, 2022

09:00 –11:05	Session 3	The role of metabolic homeostasis and mitochondria in retinal diseases
		Chair: Peter Charbel-Issa
	09:00 – 09:25	Metabolic aspects of retinal degeneration *Stelios Michalakis (Munich,Germany)*
	09:25 – 09:50	Mitochondrial Retinopathy *Johannes Birtel (Bonn, Germany/Oxford, UK)*
	09:50 – 10:15	CERKL, a retinal dystrophy gene, regulates mitochondrial function and dynamics in the mammalian retina *Gemma Marfany (Barcelona, Spain)*
	10:15 – 10:40	Serine metabolism in the retina *Marcus Fruttiger (London, UK)*
	10:40 – 11:05	Lipid metabolism in RPE *Christian Grimm (Zurich, Switzerland)*

11:05 – 11:40 Coffee break

11:40 –12:55	Session 4	Factors shaping photoreceptor environment
		Chair: Antje Grosche
	11:40 – 12:05	Incorporation, polarisation and maturation of human photoreceptors transplanted into mouse models of retinal degeneration *Marius Ader (Dresden, Germany)*
	12:05 – 12:30	Using iPSC-derived retinal models to understand and treat inherited retinal diseases *Vasiliki Kalatzis (Montpellier, France)*
	12:30 – 12:55	AMD 10q26 risk haplotype impairs subretinal monocyte elimination *Christophe Roubeix (Roche – Basel, Switzerland)*

12:55 – 13:00 Concluding remarks

13:00 Lunch and end of meeting

POSTER ABSTRACTS

Porcine cone-enriched retinal explants as a model to explore proteostasis modulation

Ana Cristina Almansa Garcia [1,2], Bowen Cao [1,2], Merve Sen [1,2], Angela Armento [1], Sylvia Bolz [1], Blanca Arango-Gonzalez [1], and Marius Ueffing [1]**

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Purpose: Degeneration of cone photoreceptors due to retinal degenerative diseases and complex maculopathies causes a major impact on the quality of life. The study of cone degeneration using murine models is complex since cones only represent a small percentage of all photoreceptors in the mouse retina. The porcine retina presents an advantage in this regard, as it possesses the cone-rich visual streak where the cone photoreceptors density reaches up to 40.000 cones/mm^2 (Hendrickson and Hicks, 2002), which is about 30% of what is reached in the center of the human fovea and about equal to cone density in the parafovea. Modulation of proteostasis through the inhibition of Valosin-Containing Protein (VCP) has proven to be protective for rods in different models of autosomal dominant retinitis pigmentosa (Arango-Gonzalez et al., 2020; Sen et al., 2021), and we have preliminary data suggesting that this protection is accomplished by a mutation independent mechanism. This study aims to assess the progress of cone degeneration in organotypic porcine retina cultures and test the effect of VCP inhibition in this model.

Materials and methods: Porcine eyes were obtained from the local slaughterhouse from six-month-old pigs. Eyes were dissected, removing the anterior part, lens, and vitreous body. The cone-enriched areas of the retina were identified by the pattern of blood vessels of the retina and dissected using 6 mm diameter punches. Cone-enriched retinal explants were transferred to transwell membranes for culture in an air-medium interphase approach and maintained in serum-free conditions for up to eight days. Retinas were treated with VCP inhibitors (ML240 or NMS-873) added in the medium every two days. General retina morphology, cell death, and cone-photoreceptor survival were assessed using immunostaining, TUNEL assay, and ONL cell row quantification.

Results: From day 0 to day 8 in culture, progressive degeneration of the porcine explants can be observed, with a considerable increase in TUNEL positive cells at day 6 and a degeneration of cones and cone outer segments starting at day 7 – 8. Interestingly, after VCP inhibition, we observed an increased cell survival reflected in a reduction in the number of TUNEL positive cells. Furthermore, there was also an improvement in the morphology of the photoreceptor outer segments and the opsin expression.

Conclusions: Our preliminary results suggest that VCP inhibition can delay cone photoreceptor degeneration in cone-rich areas of porcine retinal explants. These results encourage us to explore the potential of VCP inhibition in more complex "AMD-like" models as well as for rod-cone dystrophies.

This study was supported by the ProRetina Foundation, the Foundation Fighting Blindness FFB (Grant PPA-0717-0719-RAD), the Kerstan Foundation, and the Maloch Stiftung.

VCP inhibition restores phototransduction, impaired non-apoptotic cell death, and affects protein and energy homeostasis in the Rhodopsin P23H retina

Blanca Arango-Gonzalez [1], Merve Sen [1,2], Bowen Cao [1,2], Ana Almansa Garcia [1,2], Angela Armento [1], Sylvia Bolz [1], Marius Ueffing [1]

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Purpose: The ATPase Valosin-containing protein (VCP) exerts critical functions in a broad range of cellular processes, including the unfolding and retrotranslocation of ubiquitinated misfolded ER proteins to the proteasome for degradation. Counterintuitively to its role in protein quality control, pharmacological inhibition (Arango-Gonzalez et al., 2020; Sen et al., 2021a; Sen et al., 2021c) or RNAi based silencing of VCP expression (Sen et al., 2021b) slows down photoreceptors (PR) degeneration caused by the autosomal dominant Rho^{P23H} misfolding mutation. With the aim to understand the molecular mechanisms of this activity, we investigated the effect of VCP inhibition (VCPi) on several cellular processes in Rho^{P23H} transgenic rat retinae.

Methods: Retinal organ cultures prepared from heterozygous Rho^{P23H} transgenic rats were treated with the VCP inhibitor ML240 as previously described (Arango-Gonzalez et al., 2020). Cultures were examined by conventional histological techniques, immunohistochemistry, electron microscopy (EM), and immunoblotting (WB) using specific antibodies against proteins involved in the photocascade, cGMP, AIF as well as ER-stress markers. We also studied *in situ* markers of non-apoptotic cell death.

Results: Immunostaining for proteins involved in phototransduction showed a restoration of light-dependent physiological distributions of RHO, transducin, and arrestin in RHO^{P23H} treated with ML240. The cGMP staining showed a reduced number of positive cells and decreased staining intensity in the outer segments of the treated retinae. The number of cells stained for calpain activity or by an antibody reacting with poly ADP-Ribose (PAR), a product generated by PARP-related enzymes, both markers for caspase-independent cell death was also significantly reduced. WB analysis of ERAD and UPR markers revealed up-regulation of PDI, Grp94, and PSMB5, and down-regulation of ubiquitin, BiP, and CHOP, demonstrating an attenuating effect of VCPi on the ER stress signaling. We also found a reduction of AIF levels as well as an improvement of the mitochondrial after VCPi.

Conclusion: VCP inhibition attenuates several pathophysiological consequences of the RHO^{P23H} misfolding mutation. VCPi reduces the number of dying PR, acting specifically on the non-apoptotic cell death pathway. Moreover, it restores the light-dependent and light adaptive distribution of phototransduction proteins, essential for vision. Furthermore, our results show that VCPi rebalances ER-stress-related response patterns. Finally, its inhibition improves mitochondrial morphology and density, which may be a consequence of a lesser energy demand after VCPi. Our results

provide a resource for understanding the yet enigmatic function of VCP in PR neuroprotection and place VCPi as a promising therapeutic option to treat retinal degeneration.

This study was supported by the Foundation Fighting Blindness FFB (Grant PPA-0717-0719-RAD), the Kerstan Foundation, the Maloch Stiftung, and the ProRetina Foundation.

Complement Factor H (CFH) Y402H polymorphism alters the phenotype of iPSC-RPE cells and leads to retinal degeneration in a novel co-culture model for Age-related Macular Degeneration (AMD)

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Purpose: Age related macular degeneration (AMD) is a progressive and degenerative disease of the macula and the 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 have shown that FH loss in RPE cells disturbs cell homeostasis by altering additional functions and that those damaged RPE cells can cause retinal degeneration. In this study we investigated the impact of the AMD risk polymorphism Y402H on iPSC-RPE cells and their effects on the retina in a novel co-culture system.

Methods: Following differentiation of iPSC cells into mature RPE cells, we investigated the phenotype of iPSC-RPE cells carrying *CFH* 402Y (low risk) or 402H (high risk). To assess the activation levels of relevant signaling pathways, cells were collected for protein lysis, followed by Western Blot. Changes in gene expression were monitored via qPCR analysis. We established a co-culture model comprised of iPSC-RPE cells and porcine retinal explants, obtained from the visual streak of the porcine retina, rich in cone photoreceptors (PR). Cultures were maintained for 2 days, then fixed and sectioned for imaging.

Results: iPSC-RPE cells carrying *CFH* 402H showed increased activation levels of mTOR pathway, NF-kB pathway and ubiquitin-proteasome system, compared to iPSC-RPE cells 402Y. Retinas cultured with iPSC-RPE cells carrying *CFH* 402H showed signs of retinal degeneration compared to retinas cultured with iPSC-RPE-402Y cells. In detail, we observed a reduction in the number of cones, while outer nuclear layer (ONL) thickness and number of PR cells in the ONL were not altered.

Conclusions: Our data support the hypothesis that RPE-derived FH plays a wider role in retinal homeostasis in addition to its known complement-regulatory function. RPE cells carrying *CFH* 402H are unable to properly support the neuroretina, ultimately leading to photoreceptor loss. These findings may help elucidate the function of FH in the retina and our newly developed co-culture system may provide a suitable model to test medical interventions.

Novel peptides isolated from peptide phage display libraries shows significant binding specificity to Factor H Related protein 3 (FHR3)

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Background: Dysregulation of the complement system has long been implicated in eye diseases like age-related macular degeneration (AMD). The human Factor H (FH) family proteins have been recently described to play an important role in this dysregulation. With an exception to FH, the functions of the other highly homologous FH-related (FHR) proteins remain still elusive. Hence, to decipher the functions the SciFiMed consortium (https://www.scifimed.eu/) was made. One of the primary goals is finding various novel peptide binders to each of the FH family proteins. Here we report new FHR-3 binders in order to elucidate their FHR-3 function and hence its role in AMD.

Methods: Ph.D.™-7 (linear) and Ph.D.™-C7C (cyclic) phage display peptide library kits were used to screen for FHR-3 binders. Three rounds of bio-panning were performed before the phages eluted, titrated and checked for binding specificity by polyclonal phage ELISA. An FHR protein multiplex assay was developed inhouse. This assay was further used to validate the phage elutes from each library. Single colonies were isolated and DNA was sequenced. These positive phage clones were again validated in monoclonal phage ELISA and multiplex ELISA.

Results: Two FHR-3 positive phage clones from the Ph.D.™-7 library were identified with linear structured sequences. They showed high specificity to FHR-3 in direct phage ELISA and the multiplex assay. Also, they had no cross reactivity to the other FH-family proteins. The phage elutes from Ph.D.™-C7C (cyclic) also showed positive binding to FHR-3 and no cross-reactivity. Currently, the sequencing results of the phage clones from Ph.D.™-C7C library is awaited.

Conclusion: The phage clones and subsequent peptides identified in our study shows specific binding to the FHR-3.Hence has the potential to throw significant light on its function though further validation and more intensive peptide search is planned in later stages of the study.

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: Anaphylatoxin receptor knockout (KO) mice *(C3ar1/C5ar1)* were exposed to 10,000 lux white light for 30 minutes. 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: Iba1-staining of retinal flat mounts and sections showed migration of amoeboid-shaped microglia in in the subretinal space (SR) and outer nuclear layer (ONL) four days post light damage without differences in experimental groups. Expression levels of complement proteins *C1qa, C3, Cfb,* and *Cfh* as well as pro-inflammatory markers *iNos* and *Tspo* were elevated after light exposure, with a tendency to a more regulated alternative complement pathway in C3aR1 KO animals. SD-OCT analysis showed no rescue of retinal degeneration by C5aR1 depletion. However, a partial rescue of retinal thickness was shown in C3aR1 KO mice, which was mirrored by significant less membrane attack complex (MAC) occurrence in the outer retina.

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

Establishment of a complement modulating gene addition therapy targeted to Müller cells

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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. Here, we employ a Müller cell-specific expression system to produce two truncated versions of the secreted complement regulator Complement Factor H (miniCFH) in the retina. This measure was designed to counteract excessive complement activation and avoid degeneration of retinal tissue due to para-inflammation. The Müller cell-specific AAV variant ShH10 and the GFAP promoter that is active during Müller cell gliosis ensured the expression of the regulator in Müller cells robustly surviving tissue damage. This approach aims to limit expression of the exogenous protein to retinal distress.

Methods: Expression and secretion of the constructs were tested *in vitro* in HEK293 cells. Western blot, ELISA, heparin and C3 binding assays were used to analyze the traits of the regulatory miniCFH constructs. Ex-vivo transduction of retinal explants was used to ensure appropriate expression of the complement regulators. Müller cell-specific gene delivery was facilitated by intravitreal injection of AAVs generated with the ShH10 capsid. In vivo expression and efficacy were evaluated by immunohistochemical stainings.

Results: We demonstrated *in vitro* that the miniCFH variants bind C3 and have the ability to adhere to the surface of host cells to exert their protective functions. Expression of the construct was restricted to Müller cells and was also higher in gliotic Müller cells than in control cells, consistent with our intention to link therapeutic protein expression to damaged retina. Furthermore, we confirmed that the regulatory proteins are secreted *in vitro* and do not accumulate in the cytosol *in vivo,* but can be detected on/in non-EGFP-positive cells, indicating secretion.

Conclusion: We successfully established a Müller cells-specific, gliosis-coupled expression system of complement regulatory constructs and in-depth characterized its functionality *in vitro* and *in vivo.* The efficacy of these constructs to limit retinal degeneration to a necessary minimum is currently tested in a murine ischemia/reperfusion model.

Modelling of NMOSD pathology in human retinal organoids utilizing patient-derived Aquaporin-4 autoantibodies

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Purpose: Patients with neuromyelitis optica spectrum disorders (NMOSD) show Aquaporin 4 (AQP4) autoantibodies in about 70 % of all cases. In neuronal tissues, this water channel is predominantly expressed by glial cells including Müller glia. Initial stages of disease appear similar to multiple sclerosis, however in NMOSD the retina shows more severe degeneration and is currently discussed to be primarily affected. In NMOSD patients, the binding of AQP4 autoantibodies is thought to activate the complement cascade resulting in the formation of the membrane attack complex causing loss of retinal cells. In a recent study of cultured Müller glia, the binding of AQP4 antibodies resulted in an internalization of the target causing failures of water and calcium homeostasis. By the treatment of human iPSC-derived retinal organoids with patient-derived AQP4 autoantibodies, we are now aiming to establish a novel disease model recapitulating NMOSD pathology in a complex *in vitro* nerve tissue system with the focus on complement activation.

Methods: Two different lines of human iPSCs were cultured and differentiated into retinal organoids. Differentiation was monitored using immunohistology for Müller glia at various stages after retinal induction. The IgG-fractions from serum of NMOSD patients and healthy controls were purified. The quality was accessed using AQP4 ELISA and Western blot. Isolated fractions were pooled and used for immunohistochemistry on fixed organoid sections to highlight affinity. *In vitro* binding was tested by incubation of antibody pools with mature organoids (>D150) for 3 consecutive days. After these preliminary tests, organoids were treated for 1 hour, 3 & 14 days in culture. Currently samples are analyzed via immunohistochemistry, Western blot, qPCR and multiplex ELISA to detect different aspects of the complement activation.

Results: In our pilot experiments, we could show that both iPSC-lines produce organoids featuring key aspects of the human retina such as marker expression for mature Müller glia and the laminar structure of the retina. Both, on fixed tissue and in vital organoids, patient-derived antibodies displayed immunoreactivity to cells localized specifically in the inner nuclear layer. Moreover, we could demonstrate the expression and secretion of complement components key to drive the activation of this proinflammatory cascade.

Conclusions: We established a pipeline for a novel disease paradigm that will allow us to investigate into the early phases of NMOSD caused by autoimmunoreactive AQP4-IgG. With this, we hope that we can shed light on the consequences of AQP4 antibody binding to Müller glia modulating their function and survival, as well as on mechanisms driving the activation of the local complement cascade in this complex three-dimensional human retinal culture system with high translational potential.

The dominant-negative mutant *Rho*^{lle256del} mutation causes protein aggregates and interacts with the VCP-dependent ERAD pathway

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Purpose: Dominant rhodopsin *(Rho)* mutations account for approximately 25 % autosomal dominant retinitis pigmentosa (adRP) and result in blindness over time. However, the molecular mechanism on how these dominant mutations exert their pathological behavior and whether they share a common degeneration mechanism has long been elusive. We demonstrated that the autosomal dominant *Rho*^{P23H} forms cytoplasmic aggregates that are subsequently degraded by valosin-containing protein (VCP) dependent Endoplasmatic Reticulum (ER) associated degradation (ERAD) pathway. ERAD is a quality control mechanism of cells to clear misfolded and dysfunctional protein species by proteasomal degradation. Here we investigated the pathophysiological properties of the mutant *Rho*^{lle256del} protein, resulting from a dominant *Rho*-adRP mutation that accounts for over 17 % of familial adRP in Europe.

Methods: Applying immunofluorescence-based protein detection in cells, we analyzed the behavior of the *Rho*^{lle256del} protein with respect to protein aggregation and degradation. We co-stained rhodopsin (RHO) and specific ERAD markers (Calnexin, VCP, ubiquitin, and PSMB5) in transiently transfected HEK293 and COS-7 cells. To test whether the degradation of RHO follows the ERAD pathway, VCP and proteasome inhibitors were administrated to the transfected cells, and RHO expression was evaluated using western blotting. Finally, to determine the dominant-negative effect's role, cells were co-transfected with mutant *(Rho*^{P23H} and *Rho*^{lle256del}*)* and wild-type rhodopsin *(Rho*^{WT}*)* mimicking the heterozygous condition.

Results: Similar to *Rho*^{P23H}, mutant *Rho*^{lle256del} protein forms cytoplasmic aggregates mainly retained within the ER and partially co-localized with VCP, ubiquitin, and the proteasome system. VCP and proteasome inhibition enhanced the accumulation of high molecular weight RHO-containing oligomers and aggregates. In co-transfected cells, the RHO^{WT} was partially captured and trapped by the mutant RHO^{P23H}, or RHO^{lle256del} aggregates. As such, RHOWT cannot reach its physiological destination in the membrane.

Conclusions: Our results show that dominant *Rho*^{lle256del} and *Rho*^{P23H} mutations share the same 'VCP-dependent ERAD' degradation pathway. Furthermore, they indicate that the dominant-negative effect is in part responsible for the adRP pathology. These findings bear implications for the mechanisms on how dominant mutations that result in protein aggregation affect cell survival.

This work is funded by the Kerstan Foundation, the CFC, and the Foundation Fighting Blindness. FFB (Grant PPA-0717-0719-RAD).

Fuel for vision: Lactate flow from rods to cones in the mammalian retina

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Background: The retina is one of the most metabolically active tissues in the body. Its high energy demand is satisfied to a large extent by 'aerobic glycolysis', *i.e.* the conversion of glucose to pyruvate and then to lactate under aerobic conditions, instead of direct mitochondrial oxidation. Lactate is can serve as a fuel to support energy demand and lactate shuttling has been studied widely in skeletal muscle and brain. However, it is still unclear whether and where exactly this takes place in the retina. Herein, we present data indicating the existence of a lactate shuttle between rod and cone photoreceptors.

Methods: Immunofluorescence was employed to study the retinal expression pattern of lactate transporters (*i.e.* monocarboxylate transporters; MCTs) and other key enzymes involved in energy metabolism. Organotypic retinal explant cultures and selective inhibitors were then used to alter energy metabolism and functionally validate expression patterns. NMR spectroscopy-based metabolomics was used to further study the metabolite concentration alterations by disrupting specific parts of 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, while two key components of gluconeogenesis, phosphoenolpyruvate carboxykinase 2 (PCK2) and lactate dehydrogenase B (LDHb) were found in cones. When retinal explant cultures were treated with an MCT1 inhibitor (AZD3965), this caused extensive photoreceptor death, an accumulation of lactate within the tissue and a decreased lactate release into the surrounding medium. Curiously, cone viability was not affected by MCT1 inhibition. A similar effect was observed after combined MCT1 and MCT2 inhibition (AR-C155858), except that this also produced extensive cone degeneration. Both rod and cone degeneration were also found after PKM2 inhibition (with Shikonin), which led to a strong reduction in lactate production.

Conclusion: The present 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 showed an influence of lactate on mitochondrial biogenesis, and, therefore, the role of lactate in photoreceptors may go beyond its use as a simple energy source. Addi-

tional studies, including Ca^{2+} imaging, may further improve our understanding of retinal energy metabolism and lactate shuttling.

Keywords: energy metabolism, gluconeogenesis, glycolysis, Warburg effect

The role of surface charge in the active liposomal delivery of neuroprotective compounds to photoreceptors

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Delivery of small molecular drugs to photoreceptors after intravitreal injection is often difficult, since the drug concentration in the vitreous drops substantially after only a few hours. Liposomes are lipid-based nanoparticle systems and can be used as ocular drug delivery systems with the aim of increasing the retention of small molecular drugs in the vitreous of the eye. In addition, after intravitreal injection, liposomes are able to diffuse to the retina and can be taken up by retinal cells, potentially increasing the availability of the drugs to photoreceptors. The extend of this uptake depends on the surface charge, with cationic liposomes generally inducing more cell uptake than anionic or neutral ones. This may provide a potential for cationic liposomes to actively transport drug molecules to photoreceptors.

Three types of liposomes (cationic, cationic with surface-grafted poly(ethylene glycol) (PEG), and anionic with PEG) were prepared with a fluorescent liposome tracer and tested in three types of systems: 1) HEK293T cell cultures to determine the difference in cell uptake between the liposomes. 2) *ex vivo* porcine eyes for bio-distribution studies within the eye. 3) Organotypic retinal explant cultures for uptake at the retinal level. Finally, the anionic and cationic liposomes with PEG were loaded with the drug Rp-8-pCPT-PET-cGMPS (CN04), which has been shown to reduce photoreceptor degeneration in mouse models of retinal degeneration. The efficacy of drug-loaded liposomes was tested in explants derived from the *rd1* mouse model.

In HEK293T cells, cationic liposomes with or without PEG displayed improved cell uptake than anionic ones. However, in *ex vivo* porcine eyes, only PEG-coated liposomes could reach the retina within 24 h due to the restriction of the vitreous. Cationic PEG-coated liposomes had an overall higher uptake in the retina. In the explant culture system, similar to the HEK293T cell cultures, only cationic liposomes showed a high uptake. These seemed to accumulate in the inner retina where the signal was about 20-fold stronger than that from anionic liposomes. After the explants were treated with encapsulated CN04, only cationic liposomes induced a significant ($p \leq 0.01$) reduction of photoreceptor cell death.

The results demonstrate that cationic liposomes with PEG might offer an improvement over anionic liposomes in the amount of drug delivered to photoreceptors, as they exhibited more accumulation in the retina, both in the porcine eye model and in organotypic retinal explant cultures. This accumulation likely leads to more drugs being transported to photoreceptors.

Recruitment of Mast cells and Bruch's membrane remodelling is a common early AMD precursor in both 1q32 and 10q26 disease-risk allele patient groups

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Purpose: To identify and compare the protein content of human Bruch's membrane (BrM) from human donor eyes stratified for genetic risk of age-related macular degeneration (AMD).

Methods: Human BrM was enriched from donor eyes without AMD but who were carrying homozygous genetic risk at either: the 1q32 allele only (Chr1, n=8); the 10q26 allele only (Chr10, n=8); or who were homozygous protective at both alleles (n=8). We used untargeted mass spectrometry-based quantitative proteomics to compare the protein content of each donor risk group compared to the protective controls. One eye from each donor was used for proteomic studies with the contralateral eye processed for histological analysis.

Results: Both Chr1 and Chr10 risk groups shared commonalities when compared to the low-risk group, particularly the increased accumulation of mast-cell specific proteases (*i.e.* tryptase, chymase and carboxypeptidase A3). Histological analyses of submacular tissue from contralateral eyes confirmed increased choroidal mast cell accumulation with genetic risk, as well as evidence of their active degranulation and corresponding collagen denaturation within BrM.

Conclusion: Increased mast cell inner choroidal infiltration, degranulation and subsequent BrM remodelling are early events in AMD pathogenesis and represents a unifying mechanistic link between Chr1 and Chr10 mediated AMD.

Rescue of *ABCA4* splicing defects by single gRNA enhanced-deletion CRISPR/Cas9 innovative genome editing approach

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Purpose: Stargardt disease is an autosomal recessive inherited retinal disorder caused by biallelic mutations in *ABCA4*. Isolated and clustered deep-intronic variants (DIV) resulting in mRNA miss-splicing are an important cause leading to pseudoexon formation, disrupting the normal *ABCA4* reading frame. Here, we aim to establish an innovative genome editing approach empowering a newly bioengineered enhanced-deletion Cas9 variant (EDCas9), being able to promote deletions at the target site. Single gRNA molecules coupled to EDCas9 (gRNA/EDCas9) are targeted towards sequences involved in mediating splicing defects (e.g. DIVs and cryptic splice sites), thereby inducing profound sequence perturbation, ultimately resulting in splicing rescue.

Methods: SpCas9 variant was engineered to introduce deletions at the targeted sequences by fusion to an exonuclease partner. By targeting the DIV itself, the rescue of c.597-557G > T-induced aberrant splicing was assessed in minigene assay and patient-derived photoreceptor precursor cells (PPCs). Meanwhile, rescue of splicing defects mediated by the DIVs clustered in intron 36 composed of: c.5196+1013A > G, c.5196+1056A > G, the novel c.5196+1134C>G, c.5196+1137G>A and c.5196+1216C>A were preliminarily evaluated by minigene assay. Single gRNA/EDCas9 combinations were targeted to cryptic splice sites shared in common among variants, thereby resulting in a single genome editing strategy applicable to several DIVs.

Results: Minigene assay assessment of the splicing rescue for the isolated c.5197-557G>T DIV showed substantially higher rescue of correct transcripts induced by gRNA/EDCas9 compared to gRNA/wild-type Cas9 (wtCas9) – coupled to the same gRNAs. Further evaluation of two single gRNAs in PPCs resulted in splicing rescue of > 75 % and < 45 % mediated by EDCas9 and wtCas9, respectively, indicating > 66 % rescue enhancement when implementing EDCas9. Similarly, relative percentage rescue of the clustered DIVs, tested by minigene assay, provided substantial and consistent increase of correct transcripts mediated by gRNA/EDCas9.

Conclusions: The novel single gRNA/EDCas9 genome editing approach outperforms gRNA/wtCas9 in rescuing an isolated and clustered *ABCA4* DIVs. The implementation of gRNA/EDCas9 enables to address splicing defects by using single gRNAs, thereby overcoming the need of using paired gRNAs to generate a large intronic deletion which may result in genomic instability, unwanted recombination events and increased chance to induce P53-mediated genotoxicity due to the formation of multiple double-stranded breaks.

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CD44 signaling in Müller glia cells – A key factor in retinal degeneration?

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Purpose: Retinitis pigmentosa (RP) is a group of inherited retinal disorders that result in blindness due to a progressive loss of photoreceptors. Gene therapy, which offers great hope for a cure, is usually focused on correcting the genetic mutation. However, so far 3000 mutations in more than 70 different genes have been found to cause RP, making the development of a suitable gene therapy challenging. An alternative gene-independent approach is to identify dysregulated proteins in RP, that can potentially serve as a common therapeutical target. We performed a proteome analysis on retinas from an RP mouse model and found CD44, a cell-surface glycoprotein, to be significantly upregulated in mutant mice. While the role of CD44 is well established in cancer progression and chronic inflammation, its function in retinal pathophysiology is unknown. In this study, we investigate the role of CD44 in retinal degeneration, focusing on its involvement in the pro-inflammatory response and metabolism.

Methods: We investigated the proteome of a *Pde6b*-deficient mouse model *(Pde6b$^{STOP/STOP}$)* at different time points of disease progression. We use immunohistochemistry and biochemical tools (qRT-PCR and western blot) to validate CD44 expression in *Pde6b$^{STOP/STOP}$* and CD44-knockout (KO) mice. In addition, we developed a CrisprCas9 approach to overexpress CD44 in wild-type animals.

Results: In line with the proteomics data WB, qPCR and IHC validated the observed upregulated CD44 expression in *Pde6b$^{STOP/STOP}$* mice. IHC analysis showed that CD44 is mainly expressed by the apical microvilli of Müller glia cells (MGCs). Importantly, CD44 was also upregulated in other RP mouse models. CD44-KO retinas have no detectable expression of CD44 and a normal retinal structure. Interestingly, the pro-inflammatory cytokine interleukine-1β (IL-1β) was upregulated in *Pde6b$^{STOP/STOP}$* mice, while it was decreased in CD44 KO mice. On the other hand, CD44 is known to interact with and suppress PKM2 activity, a key enzyme in glycolysis. WB and qRT-PCR showed that PKM2 expression was downregulated in *Pde6b$^{STOP/STOP}$* mice. To better understand the role of CD44 in the pro-inflammatory response and metabolism, we crossed *CD44*-knockout mice with *Pde6b$^{STOP/STOP}$* mice.

Conclusion: CD44 is expressed in MGCs and dramatically elevated in diseased retina. We hypothesize that CD44 is an upstream driver of inflammation and makes photoreceptor cells less robust and resistant to stress by stimulating the pro-inflammatory response. This suggests that MGCs contribute to the disease progression of RP and that the downregulation of CD44 might increase photoreceptor survival in RP retinas.

Dual-active gene therapy targeting RPE and photoreceptors rescues mouse models of chronic hypoxia

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Purpose: The combination of reduced choroidal blood flow, increased Bruch's membrane (BM) thickness, and drusen formation leads to reduced oxygenation of the outer retina in the ageing eye and contributes to AMD pathology. This implicates a cellular response to chronic hypoxia in disease development and progression. Hypoxia-inducible factors (HIFs) are the major regulators of the cellular response to reduced oxygenation. Whereas HIF1α controls the hypoxic response in photoreceptors, HIF2α was identified as the main transcription factor in the RPE. Because alterations in oxygenation affect both RPE and photoreceptors in AMD, we developed a dual-active AAV gene therapy that targets both cell types in order to specifically knockdown the respective *HIF* transcription factors.

Method: *Hif1a-* and *Hif2a-* shRNAs were designed, validated *in-vitro,* and incorporated into miR-E backbones. The transfer vector consisted of a GRK1/EGFP/shHif1a-miR_VMD2/mCherry/shHif2a-miR expression cassette designed to provide specific downregulation of *Hif1*α in photoreceptors and *Hif2*α in RPE, respectively. This vector was packed into an adeno-associated virus serotype 8 (AAV8-shHifs), and a corresponding control virus was generated with two scrambled shRNAs (AAV8-shCtrl). Viruses were subretinally injected into two chronic hypoxia mouse models - $Rod^{\Delta Vhl}$ and $RPE^{\Delta Vegfa}$. In $Rod^{\Delta Vhl}$ animals, deletion of von Hippel Lindau (VHL) in rod photoreceptors leads to HIF1α-dependent retinal degeneration during ageing. In $RPE^{\Delta Vegfa}$ mice, the RPE-specific knockout of *Vegfa* leads to choriocapillaris vasoconstriction, resulting in RPE/BM hypertrophy and photoreceptor layer thinning. Fundoscopy/OCT and immunostaining were performed to evaluate transduction efficiency and inflammatory signs. Morphological assessment as well as OCT volume scans were conducted to evaluate the outer nuclear layer (ONL) thickness across the retina. Retinal function was evaluated by ERG.

Results: Selected shRNAs efficiently downregulated the expression of *Hif1*α (75 %) and *Hif2*α (70 %) *in vitro*. Subretinal injections of the AAV8-shHifs and AAV8-shCtrl into wildtype mice resulted in a widespread transduction of photoreceptors and RPE. Expression of mCherry and EGFP was restricted to the RPE and photoreceptor cells, respectively, with some EGFP signal appearing in the RPE, likely due to phagocytosis of photoreceptor outer segments by the RPE. No toxic effects or inflammation were observed 6 months after injection, as revealed by morphology and immunostainings. $Rod^{\Delta Vhl}$ mice treated with AAV8-shHif showed significant protection as evidenced by increased ONL thickness compared to AAV8-shCtrl injected animals. However, ERG assessment did not show functional improvements. AAV8-shHif treatment of the $RPE^{\Delta Vegfa}$ hindered RPE/BM hypertrophy and consequently resulted in a significant increase in ONL thickness.

Conclusion: Simultaneous targeting of different cell types, in our case photoreceptors and RPE, and driving cell type-specific expression of therapeutic genes is fundamental for treating multifactorial diseases like AMD. Here we demonstrate the protective effects of the simultaneous downregulation of *Hif1*α in photoreceptors and *Hif2*α in RPE cells by a single AAV. This treatment significantly improved photoreceptor survival in two different chronic hypoxia models.

Cellular and molecular mechanisms of retinal degeneration in the *Nr2e3$^{rd7/rd7}$* mouse model of enhanced S-cone sensitivity syndrome

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The photoreceptor-specific nuclear receptor Nr2e3 is not expressed in *Nr2e3$^{rd7/rd7}$* mice, a mouse model of the recessively inherited retinal degeneration enhanced S-cone sensitivity syndrome (ESCS). We characterized in detail C57BL/6J *Nr2e3$^{rd7/rd7}$* mice *in vivo* by fundus photography, optical coherence tomography and fluorescein angiography and, *post mortem,* by histology and immunohistochemistry. White retinal spots and so-called 'rosettes' first appear at postnatal day (P) 12 in the dorsal retina and reach maximal expansion at P21. The highest density in 'rosettes' is observed within a region located between 100 and 350 µM from the optic nerve head. 'Rosettes' disappear between 9 to 12 months. Non-apoptotic cell death markers are detected during the slow photoreceptor degeneration, at a rate of an approximately 3 % reduction of outer nuclear layer thickness per month, as observed from 7 to 31 months of age. *In vivo* analysis of *Nr2e3$^{rd7/rd7}$ Cx3cr1$^{gfp/+}$* retinas identified microglial cells within 'rosettes' from P21 on. Subretinal macrophages were observed *in vivo* and by confocal microscopy earliest in 12-months-old *Nr2e3$^{rd7/rd7}$* retinas. At P21, S-opsin expression and the number of S-opsin expressing dorsal cones was increased. The dorso-ventral M-cone gradient was present in *Nr2e3$^{rd7/rd7}$* retinas, but M-opsin expression and M-opsin expressing cones was decreased. Retinal vasculature was normal. We discuss the mechanisms observed in the murine retina with respect to possible disease mechanisms and therapeutic approaches in patients.

The dark side of blue light – Enhanced oxidative stress levels lead to apoptosis in porcine eyes

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Purpose: Awareness towards short-wave blue light (BL, 400-500nm) exposure is rising due to enhanced use of BL enriched LEDs. Furthermore, BL damage on retinal cells is increasing with age, as antioxidant defence weakens. BL has a high photochemical energy leading to enhanced production of reactive oxygen species (ROS). BL is discussed to be responsible for the development of dry eye, cataract and age-related macular degeneration (AMD). To establish a light induced degeneration model for oxidative stress induction and retinal degeneration, we exposed porcine retinal organ cultures to BL. Based on our recently studies, we now further evaluated Müller Cell (MC) activation and apoptosis.

Methods: Porcine retinal organ cultures were cultured in inserts, exposed to BL and further cultured for 24 or 48h. The degree of degeneration was analysed via immunohistology, western blot and qRT-PCR. Cell specific markers, like GFAP for activated MCs, and Opsin/Rhodopsin for PR, as well as markers for apoptosis (p63, p53, TNF-a, NfκB, Bax) and cellular stress (HSP70) were evaluated. Induction of cell death of BL exposed whole eyes was examined via TUNEL-staining.

Results: BL exposure increased mRNA expression of cell specific markers such as *GFAP,* as well as apoptotic markers *(TNFα, p53, NfκB)*. In contrast, gene expression of *Rhodopsin* was strongly reduced. Protein expression of oxidative stress significantly induced HSP70, the p53-inducer p63 as well as the p53 downstream target Bax. Immunohistochemistry displayed increased acetylated p53-expression in BL exposed retinal explants, as well as significantly more GFAP-expression. Quantification of the TUNEL+ cells of BL-exposed whole eyes revealed a significant increase of dead cells in the photoreceptor layer as well as in the entire retina compared to control whole eyes.

Conclusion: BL may play a key role in several ocular diseases, such as AMD, in which an increase of oxidative stress resembles a key mediator. In our studies we demonstrated enhanced expression of apoptotic proteins in BL exposed porcine retinal explants, as well as increased cell death, activation of MCs and reduced *Rhodopsin* levels. It can be concluded that BL is suitable to investigate retinal diseases based on an induction of oxidative stress. This model can therefore be further used to develop therapeutic options to inhibit retinal cell death induced by oxidative stress.

Polarized monolayer formation after transplantation in a RPE degeneration mouse model depends on the developmental stage of human iPSC-derived RPE donor cells

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Purpose: A potential treatment approach for blinding diseases characterized by RPE loss is the transplantation of in vitro generated pluripotent stem cell-derived RPE cells. RPE suspension as well as sheet transplantations have already entered the clinical stage in first trials, however, characteristics of suitable donor RPE cells are currently not well defined. Here, we therefore analyzed the influence of RPE passage age in culture and donor cell number on graft morphology and photoreceptor rescue after transplantation into the RPE -depleted mouse eye.

Methods: Endogenous RPE in C57Bl/6J mice was depleted via tail vein injection of 30 mg/kg $NaIO_3$. One week later, human iPSC-derived RPE were transplanted subretinally, using 50,000 or 100,000 cells at maturation stages P1 or P2, with vitreally applied immune suppression. Upon collection, eyes were processed for EM and immunohistochemistry and RPE coverage of the retina assessed.

Results: While both P1- and P2-RPE show extensive monolayer formation, pigmentation and correct asymmetric localization of marker proteins in vitro, P1-RPE exhibited superior post transplantation outcomes. After three weeks in vivo, P1-RPE covered approximately double the retinal area than P2-RPE (~ 30 % versus 15 %, of whole retina, respectively). More importantly, the majority of P1-RPE was present as a well polarized monolayer attached to Bruchs membrane, with basal nuclei and Collagen IV deposition, and apically localized melanin pigment, ATPase and F-Actin. In contrast, P2-RPE occurred mainly in the form of disorganized clusters lacking apico-basal polarity. In addition, P2- but not P1-RPE induced a strong immune reaction in the host. Further, comparison of transplants with 50,000 vs. 100,000 P1-RPE cells showed improved monolayer formation for lower cell numbers (~ 90 % of RPE area with 50,000 cells vs 75 % with 100,000) without reduction of retinal area RPE coverage. Only areas with correctly formed RPE monolayers were associated with rescue of $NaIO_3$-associated ONL aberrations and a largely normal, organized ONL morphology.

Conclusion: The passage age/developmental stage of human iPSC-derived RPE greatly influences its capacity to form polarized monolayers and rescue of photoreceptors in the $NaIO_3$-induced mouse model of RPE loss. Our findings underline the importance of stringent definition of RPE cells suitable for use in in vivo transplantation approaches.

The influence of PLVAP deficiency on choriocapillaris integrity

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Purpose: In the pathogenesis of geographic atrophy (GA) it is still controversially discussed in which order retinal pigment epithelium (RPE) and choriocapillaris (CC) degenerates. We follow up on the hypothesis that primary impairment of the CC causes secondary changes in the RPE that will eventually cause photoreceptor degeneration as seen in GA.

Methods: We used CAGG-Cre-ER/*Plvap*$^{fl/fl}$ mice and their *Plvap*$^{fl/fl}$ littermates of C57BL/6 background and induced a deficiency of the plasmalemma vesicle-associated protein (PLVAP) at an age of 4 weeks via tamoxifen administration (5mg/ml 3x/d for 5 days). PLVAP forms diaphragms bridging the fenestrated endothelium of capillaries like the CC, thus its loss presumably results in CC impairment. The animals were analyzed at an age of 8 or 12 weeks. The PLVAP protein synthesis was evaluated by Western Blot analysis at the age of 8 weeks. To gain specific information about the distribution of the PLVAP loss and CC morphology, we performed a CC flat mount preparation technique including the mechanical removal of the RPE, and a staining against PLVAP and the endothelial cell marker cluster of differentiation 31 at the age of 12 weeks. For future experiments, we wanted to enable the simultaneous observation of RPE and CC. Therefore, we established a protocol for CC/RPE flat mount staining with an intact RPE, where only the pigmentation was removed by bleaching with 10% hydrogen peroxide. The bleached RPE/CC flat mounts were stained against PLVAP and the tight junction protein Zonula occludens-1.

Results: The Western Blot data verified that PLVAP protein synthesis was significantly reduced to 42 % in CAGG-Cre-ER/*Plvap*$^{fl/fl}$ mice in comparison to control littermates. The CC flat mounts revealed a PLVAP loss, which was not equally distributed but restricted to scattered areas in the CC. We succeeded to establish a flat mount preparation technique, which allows the simultaneous observation of CC and overlying RPE.

Conclusion: With our animal model we were able to induce a conditional PLVAP deficiency. The deficiency was focused on scattered areas of the CC, in which the fenestration and thus the function is presumably impaired. In future experiments we will use the established RPE/CC flat mount technique to investigate, whether the RPE overlying areas of PLVAP loss shows signs of degeneration.

Efficacy of a complement modulating gene addition therapy in the retinal ischemia/reperfusion model

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Purpose: Degenerative retinal diseases may have a variety of different causes, but come along with a similar inflammatory reaction, including activation of the complement cascade as part of the innate immune system. Inappropriate regulation of the complement system can cause further tissue damage. We aim to investigate if the expression of a complement inhibitor in the retina can decelerate degeneration in an ischemia mouse model by rebalancing overshooting complement activation. We consider Complement Factor H (CFH), the main inhibitory complement regulator, as a promising candidate to dampen excessive complement activation. Delivered into the eye by the injection of an adeno-associated viral (AAV) vector, a shortened, functionally optimized version of CFH (miniCFH) was expressed directly in the immune privileged retina, specifically addressing Müller cells for exogenous protein expression.

Methods: In this retinal ischemia model the intraocular pressure is raised above the systolic arterial blood pressure to cease ocular blood circulation. Post ischemic eyes are injected with AAVs and are analysed 3 days and 14 days post ischemia (dpi). We use morphometric analysis of the outer and inner nuclear layer and ganglion cell layer in combination with results from cell death detection assays to evaluate cell survival. Complement protein distribution is assessed by immunohistochemistry. Retinal complement activation was demonstrated by Western blot.

Results: We found that CFH protein expression was reduced in ischemic eyes compared with healthy controls, confirming it as a promising target for gene addition therapy. In addition, ischemic untreated eyes exhibit higher levels of C3 cleavage products than control eyes. Importantly less C3 activation products were detected in miniCFH-treated eyes, indicating that complement activity has successfully been constrained. Analysis of retinal integrity and cell death via TUNEL staining did not display major protective effects on cell survival for the 3-day period after ischemia. Further histologic evaluation after 14 dpi will reveal possible effects of miniCFH on the ischemic retina relative to uninjured control eyes. The distribution of complement components will be visualized and interpreted by immunohistochemical staining.

Conclusion: Having demonstrated that glial expression of miniCFH reduces complement activity in the damaged retina, we continue to evaluate the applicability of this novel gene addition therapy approach, which can potentially be used to treat a variety of different retinal diseases associated with complement overactivation, regardless of the underlying genotype.

Establishing a screening pipeline to identify novel WNT inhibitors for use in the RPE

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Purpose: Although many modern diseases are well researched, for a large proportion there are no suitable treatments available, necessitating the discovery of new drugs. A common method for the identification of novel compounds and molecules are high-throughput screens, which enables the analysis of compound libraries. Here, we developed a screening pipeline based on a fungal compound library to identify novel inhibitors of the WNT signaling pathway. This pathway is involved in developmental processes, whereby dysregulation can lead to different disorders including various eye diseases such as retinal degeneration, age-related macular degeneration, and diabetic retinopathy. In particular, we have identified that WNT inhibition improves maturation of iPSC-derived RPE. Current inhibitors are unspecific, not highly effective and little is known about their precise function or mode of action. This highlights the importance of finding new screening methods to identify more effective WNT inhibitors specifically for use in the RPE.

Methods: Using a fungal compound library, we established a screening pipeline to identify novel inhibitors of the WNT signaling pathway in RPE cells. We performed Luciferase- and MTT assays to pick out possible inhibitors using TCF/LEF HEK293T reporter cells. Promising compounds were subsequently purified and further validated using RT-qPCR. To investigate possible cell-specific effects, we applied successful compounds to RPE-J cells and analyzed expression of WNT components and morphology.

Results: Four of the investigated compounds showed strong WNT inhibitory activity with minimal toxicity in the initial-and confirmational Luciferase- and MTT assay. After further validation screenings, one of the four identified substances resulted in downregulation of gene expression of all tested WNT targets in HEK293T, - and RPE-J cells.

Conclusion: In summary, we have established a screening pipeline that has enabled us to identify a new promising WNT inhibitory drug from a fungal library. In the long term, this substance could be used as a medication for diseases caused by hyperactivation of the WNT signaling pathway. Since we specifically screened for its application in RPE, a potential utilization would be improved maturation of iPSC-RPE, and the treatment of ocular diseases such retinal degeneration, age-related macular degeneration, and diabetic retinopathy.

Evaluation of nitric oxide synthase activity in retinal ganglion cells of organotypic explant culture

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Background: Retinal ganglion cell (RGC) death normally occurs in organotypic explant culture in response to optic nerve axotomy. RGC death has previously been connected to excessive nitric oxide (NO) signalling. A previous study showed that inhibition of cGMP-dependent protein kinase G (PKG) in retinal explants derived from wild-type (WT) mice rescued viability and function of RGCs after 12 days of culture. Here, we assessed the viability of RGCs in WT mouse retinal explants at different culture time points. To understand whether the degeneration of RGCs after optic nerve axotomy could be correlated with an increase in NO/cGMP/PKG signalling, we also examined the distribution of nitric oxide synthase (NOS) -positive cells.

Methods: Organotypic retinal explant cultures derived from post-natal (P) day 5 WT mice were cultured until P6, P7, or P11. RGC viability was assessed via labelling with an antibody directed against RNA-binding protein with multiple splicing (RBPMS), a specific marker for one population of RGC. The viability of NOS-positive cells was assessed using NADPH-diaphorase assay.

Results: Analysis of NOS-positive cells showed that NOS activity was mainly localized in the ganglion cell layer (GCL). In addition, the percentage of NOS-positive cells per 1 mm^2 decreased by 65 % from P6 to P7 and by 88 % from P7 to P11. A similar trend was observed for the numbers of RBPMS-positive cells, with a decrease of 83 % from P6 to P7 and 100 % from P7 to P11 in the percentage of positive cells per 1mm^2. The general loss of NOS and RBPMS positive cells thus followed a similar trend. Moreover, at P11 there were no RBPMS positive cells left.

Conclusion: NOS-positive cells were dramatically reduced in the first 48 hours after explanation, a reduction that appeared to occur in tandem with that of RBPMS positive cells. This correlation makes a causal relationship between NOS activity and RGC death seem likely. Potentially, RGC degeneration in retinal explants following optic nerve transection could be triggered by NOS activation in the first two days of culture and may be related to NO/cGMP/PKG signalling.

Dynamin-1 and -3 are essential for the structure and function of mouse rod photoreceptor synaptic terminals

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Purpose: Photoreceptors are highly polarized retinal neurons that absorb light and generate an electrical signal through the process of phototransduction. Light-dependent changes in the membrane potential drive glutamate release from the synaptic terminal onto the second order neurons, bipolar cells (BCs) and horizontal cells (HCs). The photoreceptor's synaptic ribbon facilitates a high rate of exocytosis (vesicle fusion), which is complemented by endocytosis (vesicle retrieval). Endocytosis commonly requires the GTPase dynamin, whose two isoforms, Dnm1 and Dnm3, are expressed specifically in neurons. Here, we evaluated the impact of deleting Dnm1 and 3 on rod photoreceptor structure and function.

Methods: *Dnm1- and Dnm3*-floxed mice were bred with transgenic *iCre75* mice to generate rod-specific single *($^{rod}Dnm1^{-/-}$ and $^{rod}Dnm3^{-/-}$)* and double knockouts *($^{rod}Dnm1^{-/-}/3^{-/-}$)*. ERG recordings were used to test photoreceptor function and signal transmission to BCs. Photoreceptor structure was evaluated by immunohistochemistry, confocal microscopy and electron microscopy.

Results: Immunostaining confirmed the conditional deletion of Dnm1 and 3 from rods. In $^{rod}Dnm1^{-/-}/3^{-/-}$ mice, scotopic ERG a-waves were normal at P90, consistent with normal outer nuclear layer thickness and rod outer segment length; whereas scotopic b-waves were significantly impaired, consistent with a thinning of the outer plexiform layer. Immunostaining of photoreceptor synaptic terminals with ribbon synapse-related proteins (RIBEYE and bassoon) and synaptic vesicle-related proteins (VGLUT1 and complexin-3) showed degeneration of rod terminals. Consistent results were obtained by electron microscopy. In contrast, cone pedicles and the cone-driven ERG in $^{rod}Dnm1^{-/-}/3^{-/-}$ mice remained intact. At P90, $^{rod}Dnm1^{-/-}/3^{-/-}$ retinas showed a reduced number of HCs (anti-calbindin) and an increase in neurite sprouting from rod BCs (anti-PKCα). No abnormalities of any sort were observed in single knockouts of either Dnm1 or 3, suggesting that their function in rods is redundant.

Conclusions: Deletion of Dnm1 and 3 from rods causes functional impairment and degeneration of the synaptic terminal. This degeneration is accompanied by structural defects in postsynaptic neurons and loss of light-driven signals in rod BCs. Normal function of rod synapses requires dynamin-dependent endocytosis.

Anti-VEGF – XBD173 combination therapy as new therapeutic concept for neovascular age-related macular degeneration

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Age related macular degeneration (AMD) is a multifactorial disease and a leading cause of visual loss in the elderly western civilisation. Early AMD is characterized by drusen deposits which can cause a disruption of the Bruch's membrane, this enables blood vessels to grow through. This choroidal neovascularisation (CNV) leads to fluid exudation, which causes detachment of the retinal pigment epithelium from the choroid and therefore a rapid deterioration of the patients' vision.
The treatment of neovascular AMD is currently only possible with VEGF inhibitors such as Aflibercept. Additionally, up to 36 % of patient become resistant to anti-VEGF therapy and the frequent intravitreal injections are an uncomfortable routine for the patients.

Another promising therapy option is the modulation of the immune response that occurs during AMD progression. The degrading processes within the retina lead to an activation of microglia within the retina. One potential target for modulation is the translocator protein (TSPO) which is part of microglial outer mitochondrial membrane and upregulated upon inflammation. The TSPO ligand XBD173 was recently shown to decrease microglia reactivation and inhibit the NOX1 specific ROS production in the already well-established laser model of wet AMD.

In this project, we hypothesise that the combination of Aflibercept with XBD173 inhibits not only neovascularisation but also microgliosis and has a beneficial effect compared to a treatment with only one of the substances in the laser model of CNV. The progression will be monitored using non-invasive optical coherence tomography and fluorescein angiography, immunohistochemical staining of retinal and pigment epithelial flatmounts, western blots, qPCR and RNA sequencing.

Characterization of two pathological Cav1.4 L-type calcium channel variants

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Purpose: Cav1.4 L-type calcium channels are predominantly expressed at the photoreceptor terminals and in bipolar cells, mediating neurotransmitter release. Mutations in its gene, *CACNA1F,* can cause congenital stationary night-blindness type 2 (CSNB2), which is reliably diagnosed through electroretinography as patients' symptoms are diverse and can manifest in variable levels of night-blindness but also photophobia or low visual acuity. This project aims to elucidate the biophysical properties of two variants, Cav1.4-R964G and Cav1.4-R1288L (reference sequence: JF701915.1), both S4 charge-neutralizing mutants that were found in patients diagnosed with CSNB2.

Methods: For electrophysiological recordings, Cav1.4 variants were produced in Flp-In-293 cells, which stably expressed β3 and α2δ1 auxiliary subunits, whereas tsA-201 cells were transiently co-transfected to generate the channel mutants and their ancillary subunits for western blot analyses. Additionally, we combined homology modelling with molecular dynamics simulations to understand the functional effects at atomistic detail.

Results: Cav1.4-R964G and Cav1.4-R1288L were less expressed in the membrane fraction *in vitro* in comparison to their wild type reference channel, indicated by western blot analyses. This finding is in accordance with whole-cell patch-clamp recordings, which revealed a reduction of current density compared to wild type channels, with Cav1.4-R964G having 23 % and Cav1.4-R1288L 12.4 % of the wild type current density. Consistent with a loss of gating charges, both substitutions lead to a significantly increased slope of voltage-dependent activation while Cav1.4-R1288L also shifts the voltage-dependence of activation towards more positive potentials. In agreement with these observations, we find that Cav1.4-R1288L destabilizes the activated state, while Cav1.4-R964G diminishes interactions in intermediate resting states with the inner negative cluster.

Conclusions: The detected changes in expression level and biophysical properties for the Cav1.4-R964G and Cav1.4-R1288L likely account for differences in signal transmission in retinal cells, thus, leading to CSNB2.

Hypoxia-inducible factors aggravate ferroptotic cell death in RPE cells under oxidative stress conditions by promoting the Fenton reaction

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Purpose: Oxidative stress and hypoxia in the retinal pigment epithelium (RPE) have long been considered major risk factors in the pathophysiology of age-related macular degeneration (AMD). However, no convincing model system was available which allowed systematic investigation of these two risk factors in a high-throughput approach. For this purpose, we developed an AMD cell culture model which involved oxidative stress and stabilization of hypoxia-inducible factors (HIFs), key regulators of cellular adaptation to hypoxic conditions.

Methods: We treated a human RPE cell line (ARPE-19) with sodium iodate (SI), an oxidative stress agent, together with dimethyloxalylglycine (DMOG) which leads to chronic HIF stabilization. Treatment effects were characterized by cell viability assays, FACS, inhibitors of cell death pathways, a lipid peroxidation assay, a superoxide dismutase (SOD) assay, siRNA knockdown, western blot, and quantitative real-time PCR.

Results: HIF stabilization by DMOG aggravated cell death induced by SI. Iron-dependent ferroptosis was identified as the main cell death mechanism. Moreover, SOD activity and expression of iron transporters were upregulated in cells treated with SI+DMOG associated with an increase of intracellular H_2O_2 and iron, respectively. H_2O_2 and iron are substrates of the Fenton reaction which leads to the generation of hydroxyl radicals that trigger lipid peroxidation, resulting in ferroptosis. In line with this, SOD2 knockdown reduced cell death and chelation of ferrous iron by 2,2′-Bipyridyl completely rescued cells.

Conclusions: Using our newly developed AMD model, we could show for the first time that HIF stabilization under oxidative stress conditions promotes the Fenton reaction in RPE cells, resulting in ferroptotic cell death. Thus, our study provides a novel link between hypoxia, oxidative stress and iron metabolism in AMD pathophysiology. Since iron accumulation and altered iron metabolism are characteristic features of AMD, our cell culture model is suitable for testing new treatment options against AMD in a high-throughput approach.

Profiling of mitochondrial metabolism in healthy and gliotic Müller cells

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Purpose: Müller cells stabilize ion, volume, and metabolic homeostasis in the retina, for example, by delivering nutrients that can then be metabolized by neurons. Müller cells themselves are thought to engage in primarily glycolytic metabolism. Nevertheless, a substantial number of mitochondria are found in Müller cells, containing large amounts of the translocator protein 18 kDa (TSPO). TSPO is localized in the outer mitochondrial membrane and its function remains controversial. It is thought to be involved in cholesterol shuttling as well as neurosteroid production and that it positively modulates mitochondrial energy metabolism. Here, we aim to understand whether and how TSPO expression affects mitochondrial function of homeostatic and gliotic Müller cells in the murine retinal ischemia/reperfusion model.

Methods: A clue to mitochondrial fitness is their very negative membrane potential of approximately -150 mV, which can be assessed by the JC 1 assay. The eyes of WT and Müller cell-specific TSPO knockout (KO) mice were subjected to transient ischemia. Retinas were isolated, dissociated, and individual Müller cells were measured under normal and hypoosmotic conditions. To this end, the intensity of red fluorescence emitted by JC-1 aggregates in healthy mitochondria with normal membrane potential is divided by the fluorescence of the single molecule JC-1 dye that occur at higher amounts when the mitochondrial potential breaks down. This ratio can thus serve as a parameter that allows conclusions about the mitochondrial health of the cell investigated. Currently, we are also establishing a FLIM imaging pipeline to analyze the effects of glia-specific TSPO KO on the NAD(P)H level of Müller cells to get insight into putative changes of their energy metabolism depending on the experimental condition.

Results: For the JC-1 assay, we developed a pipeline that allowed us to rapidly and reproducibly obtain and analyze data from single acutely isolated Müller cells. We observed a decreased ratio of red to green JC-1 fluorescence intensity, indicating a decrease in mitochondrial potential upon hypoosmotic stress and glutamine exposure. We hypothesize that the red-to-green ratio of JC-1 decreases more rapidly and to a lower level in Müller glia from ischemic eyes than in their counterparts from non-ischemic eyes.

Conclusion: We have successfully established the JC-1 assay to study mitochondrial health in Müller cells under genetic, pharmacological or pathological stress conditions. We will compare these results with data collected using FLIM-based NAD(P)H imaging, currently established in the laboratory, and the Seahorse assay to study energy metabolism in Müller cells. We speculate that glial TSPO KO leads to changes in the overall energy state of Müller cells, which could result in impaired mitochondrial capacity and thus decreased glial homeostasis function.

Aptamer-based therapy of retinopathies via activation of the BDNF/TrκB-signaling pathway

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Purpose: Degenerative processes of the retina, as they occur in AMD or *Retinitis pigmentosa,* result in the death of photoreceptors leading to blindness. Due to the multifactorial causes and the various genes involved, specific targeted therapies are often a challenge in these diseases. Therefore, symptomatic treatment by prevention of cell death through neuroprotective mechanisms is a good option. In this study an aptamer, which can activate the TrκB-receptor was evaluated as novel therapeutic option. Aptamers, are small DNA/RNA or peptide based strands which specifically bind to their target molecules and thus exert their effects via inhibition or activation of the associated pathways.

Method: Firstly, different concentrations of the aptamer (10 nM, 100 nM, 200 nM and 500 nM) were tested on primary porcine Müller cells (MCs) and porcine retinal explants. The expression of the specific cellular markers and markers of the TrκB-signaling cascade (pAkt, CNTF, bFGF, BDNF) were examined after 24 h and 48 h. Two retinal models of oxidative stress induction, one with $CoCl_2$ and another one with blue light (BL), were used to investigate the neuroprotective effect of the aptamer. MCs or retinal explants were first exposed to the stressors ($CoCl_2$ for 48 h; BL for 1.5 h) and then treated with the aptamer. Cell viability, cell death assays as well as WB, qRT-PCR and ELISAs were performed to proof the activation of the neuroprotective TrκB-signaling cascade. As reference samples treated with BDNF (1 ng/mL, 10 ng/mL, and 100 ng/mL) were used.

Results: Already 10nM of the aptamer led to a delayed but stronger activation of the TrκB-receptor after 48 h. A significant increase was also noted in the downstream targets of the TrκB-receptor and cellular markers in samples treated with the aptamer (100 and 200 nM). The amount of phosphorylated Akt increased 3-fold after 24 h with 100 nM and 6-fold after 48 h with 10 nM of the aptamer. While the effect of BDNF treatment was similarly strong, it disappeared completely after 48 h. In the degeneration models, the increase in oxidative stress-induced markers such as Casp3/7, Bax/Bcl-2, iNOS, TNFα was counteracted by aptamer treatment.

Conclusion: Our data proved the functionality of the aptamer on retinal cells and retinal explants, as the downstream targets of the TrκB-receptor were activated. In the oxidative stress models on MCs and retinal explants, cell death could be counteracted, and the survival of the cells improved.

Funding: DFG

Retina Suisse patient database: Towards the OPHTA-module of the Swiss registry of rare diseases

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The Retina Suisse Patient Database project was started in Spring 2018 as a collaboration between the patient organization Retina Suisse and the Universitätsklinik für Augenheilkunde of the Inselspital, Bern University Hospital. The aim is to establish a registry grouping all patients living in Switzerland affected by inherited retinal diseases (IRDs) and to expand it to other rare eye diseases.

First, we modified existing informed consents for genetic analysis in order to incorporate the possibility to be registered in the Retina Suisse Patient Database. With this informed consent clinical, genetic and personal data can be gathered. These informed consents have been prepared in all national languages and in English, and are used in all University Eye Clinics, State Eye Clinics and Private Practices all over Switzerland. Second, established the competences to request for reimbursement of genetic analyses by the Swiss health insurances. Third, we expanded the existing in-house molecular diagnostic pipeline to genetic eye diseases. Importantly, this databank could be established thanks to a large collaboration between university eye clinics, state eye clinics and private practices located all over Switzerland.

We will present in detail the number of patients registered over the past years, the success rate of reimbursements obtained for genetic analyses from the health insurance companies and the amount of genetic analyses performed.

Finally, we will present our current efforts to transfer the existing Retina Suisse Database into the Swiss Registry of Rare Diseases, a project of the Swiss Confederation coordinated by the KOSEK (KOordinationsstelle für SEltene Krankheiten) and located at the Institute for social and preventive Medicine of the University of Bern. This will allow for a safe long-term storage of clinical and molecular diagnosis of patients affected by rare eye diseases.

Interaction of gliotic and fibrotic response during scar formation in retina

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Background/Context/Introduction: Tissue repair is a protective response following injury, but repeated or prolonged injury can lead to fibrosis, a pathological condition of excessive scarring. To understand the dynamic mechanisms underlying fibrosis, it is important to identify the principles of regenerative processes involved in tissue repair. In this study, we present a time course of retinal wound healing, including the accumulation of scar-forming extracellular matrix and potentially involved cell types.

Material and methods: C57BL/6J mice underwent laser photocoagulation to induce fibrosis without damaging Bruch's membrane. The volume of the lesion and ensuing fibrosis was quantified with OCT measurements. Confocal microscopy of paraffin sections stained with different fibrotic [IL-1β, fibronectin, collagen type 1,3,4,5 and alpha-smooth muscle actin (αSMA)] and gliotic markers (GFAP, S100beta, nestin) prepared every week after laser injury (d7, d14, d21, d28, d35, d42, d49). Additionally, Western Blot detection for α-SMA was performed. A RT2 Profiler PCR Array (QIAGEN) was used to screen the genetic expression of 84 extracellular matrix (ECM) and cell adhesion-involved genes.

Results: From day 1 to day 49 after laser injury the subretinal scar in the OCT images increased. IHC stainings revealed that glial cells were involved and the expression of IL-1β was observed. However, this signal was also be observed in the end feet of Müller cells at day 21. Furthermore, the expression of GFAP-positive, activated glial cells did not decrease over time, but nestin expression could be observed from day 35 especially in the scar forming area. Such gliotic scarring prevented regeneration. However, the expression of the different collagens in the lesion area increased reciprocally, whereas the fibronectin content decreased.

Conclusions: In our work, we have illustrated the time course of fibrosis and summarised the most important features of the development of a gliotic scar. Thereby, glial cells give a multipotent signal, which potentially includes inflammatory features called hot fibrosis.
Furthermore, a "shut down" of ECM involved genes occurs at day 21 whereas an overexpression is observed at day 49.

Does retinal photoreceptor composition influence Müller cell heterogeneity?

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Purpose: The human macula is exceptionally prone to neurodegenerative processes. We aim to elucidate whether a functional heterogeneity of Müller cells may explain part of this macular susceptibility. We generated and analyzed high quality proteomic data from cone- and rod-rich systems from human and mice and identified molecular pathways to test their potential involvement in heterogeneous glial subpopulations shaping the specific macular morphology and function.

Methods: Müller cells, microglia, vascular cells and retinal neurons from all cone R91W;Nrl-/- and R91W control mice as well as human macular and peripheral samples were isolated 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. Traction force microscopy was used to measure changes in exerted shear stress, while cytoskeletal immunostainings coupled with a bioinformatics pipeline enabled the assessment of morphological features. Additionally, we analysed the cellular and secretory protein composition by MS/MS and applied nanoparticle tracking to quantify extracellular vesicles.

Results: We found significant differences in protein expression between predominantly cone- and rod-associated Müller cells in both human and murine systems, strengthening our hypothesis of functional heterogeneity of Müller cells in relation to the functional requirements of the cells they interact with in different retinal subregions. Indeed, various protein pathways showed a Müller cell-specific differential expression pattern in both the scRNAseq and our own proteomic data making these especially promising for further research. From a list of proteins relevant for cell-matrix interaction being upregulated in macular Müller cells, we chose epiplakin *(Eppk1)* for further evaluation. Though poorly understood, *Eppk1* is thought to play a role in cytoskeleton/intermediate filament organization, which we were able 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. We also found lower expression of CD9, a marker for extracellular vesicles concomitant with fewer secreted particles in the mutants. Throughout the analyzed datasets from primary and immortalized human Müller cells, we found differences in the regulation of extracellular matrix proteins.

Conclusion: We were able to show consistently different expression profiles of cone- and rod-associated Müller cells that converged on pathways representing extracellular matrix organization and cell adhesion and identified *Eppk1* as an exciting central molecular player in this context – a protein that is known to confer mechanical stability to keratinocytes in the skin. Using various approaches, we pioneer 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 glia. Further studies investigating the role of *Eppk1* in human 3D retinal tissue models might improve our understanding of why the human macula is so sensitive to disease-associated changes.

Trained immunity in retinal degeneration

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Purpose: Age-related macular degeneration (AMD) and Retinitis Pigmentosa (RP) are the most common retinal degenerative diseases, leading to severe visual impairment. Currently, there is no effective treatment strategy for dry AMD and RP. Indeed, activated microglia and pro-inflammatory environments were found to be closely associated with the formation of drusen in retinal degenerative disorders. Therefore, the adaptive characteristics of inate immunity, or trained immunity, may induce the activation of resident microglia, causing the death of nearby photoreceptors. Furthermore, environmental factors, such as obesity, which is one of the most important risk factors for AMD, trigger inate immune reprogramming. However, contribution of the trained immunity in the etiology of multifactorial and monogenic retinal degenerative disorders remains to be investigated. In this study, we explore the role of trained immunity in the development of maladaptive inflammatory responses in the mouse retina.

Methods: In the first part of the project, we would like to determine if innate immune memory can be imprinted in the retina. To test this hypothesis, BALB/c mice will be either fed a high-fat diet or injected with peripheral or local microbial stimulus (β-glucan). After the waiting periods, these animals will be subjected to the light damage paradigm as a priming stimulus. We also test the effect of microbial stimulus on generating an enhanced inflammatory response to subsequent genetically induced retinal damage in our Fam161a gene trap ($Fam161a^{GT/GT}$) mouse model.

In the second part of the project, we will perform a proof-of-concept study and determine whether blocking immunological memory in retinal microglia using cell-specific knockout of Tak1 ($TaK1^{MGKO}$) can positively influence disease outcome in the two mouse models of retinal pathology. By crossing $TaK1^{MGKO}$ with $Fam161a^{GT/GT}$, we would also like to use conditional Tak1 knockout in conjunction with the inherited retinal degeneration model.

At the end of the experiments, microgliosis and retinal degeneration will be assessed. To explore the gene expression pattern and epigenetic basis of trained immunity, sorted microglia will be used to define chromatin modifications using different sequencing methods.

Thus, by understanding molecular mechanisms mediating pathologically active microglia in the retina, our study opens new avenues in immunomodulatory therapies for retinal degeneration.

Minocycline and CSF1R inhibitor attenuate retinal inflammation and vascular abnormalities in the pericyte-inhibition mouse model

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Purpose: Diabetic retinopathy (DR) is a complication of diabetes that causes vision loss. Although DR is associated with retinal microvasculature complications, neuroinflammation is manifested early in disease. Microglia, the resident phagocytes of the retina actively regulate inflammation to maintain homeostasis and prevent tissue damage. Physiological stress due to hyperglycemia initiates an overt activation of microglia that drive tissue pathology. Despite having several animal models of diabetes, they fail to recapitulate the vascular and neural complications of the human disease. Herein, we have utilized a pericyte-depletion mouse model to study the molecular and cellular components of DR pathogenesis.

Methods: C57BL/6J mouse pups received a single s.c dose of 30 µg of anti-PDGFRβ mAb (clone APB5) at P1 while the control group received 30 µg of IgG. For one treatment arm, mice received i.p injections of minocycline (45 mg/kg) once daily from P5 – P9 or P27. The other treatment included weaning mice at P21 with PLX3397 diet for 1 week. The retinal vascular and neural changes at P10 and P28 were determined immunohistochemically. The expression of marker genes was quantified with qRT-PCR and visual acuity was assessed in live mice using an optodrum. The retinal thickness and vessel leakage were determined using SD-OCT and fluorescein angiography, respectively.

Results: Inhibition of PDGFB/PDGFRβ signaling had variable effects on the mouse retina. In the vasculature, APB5 produced an altered vasculature at P10 and P28. The APB5 activated retinal microglia and induced loss of photoreceptors in the outer nuclear layer (ONL). Key biomarkers of microglia reactivity such as *Ccl2, Il-β, Lgals3, Tnf-α, Tspo* and angiogenic factors *Pigf, Icam-1* were upregulated in the diseased retina at P10 and P28. Further, reactive gliosis, vessel leakage, and reduced retinal thickness were observed in the adult mice. Treatment with minocycline attenuated the expression of cytokines and angiogenic factors, reduced vascular leakage and preserved retinal thickness. Oral feeding with PLX3397 diet for 1 week preserved retinal vasculature. The control injection of IgG produced no significant changes in the retina.

Conclusions: Minocycline and a CSF1R inhibitor, PLX3397, partially attenuate retinal inflammation and vascular abnormalities in mice with ocular phenotype of DR.

Restoration of retinal function in the USH1C$^{p.R31*}$ pig by AAV-based gene therapy in a pilot study

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Purpose: Usher syndrome (USH) is the major cause of inherited deaf-blindness in human. While rehabilitative treatments are available for the hearing loss applying hearing aids or cochlear implants there is no therapy for the ophthalmic component of USH to date. A major reason is the lack of an adequate animal model. To overcome this, we have recently generated a knock-in pig model for USH1C by introducing the human *USH1C* exon 2 bearing the p.R31* disease-causing nonsense mutation (Grotz et al. 2022, EMBO Mol Med). The USH1C pig pronounced profound hearing loss from birth on, vestibular areflexia, and prepubertal onset of retinal dysfunction, phenotypes as seen in USH1C patients. Here, we utilize the USH1C porcine model for a gene therapy pilot study applying AAV based-gene therapy.

Methods: Established AAV8, AAV9 or a recombinant Anc80 vectors, expressing eGFP under control of a CMV promoter were subretinal injected in eyes of age matched wild type pigs. GFP expression was immunohistochemically analyzed 5 weeks post injection. In a 3-years-old USH1C pig the Anc80.CMV.harmonin_a1 vector was sub-retinally injected into one eye while the other eye was sham-treated with PBS. After 12 month, *USH1C/*harmonin expression and localization in retinae were analyzed by qPCR, Western botting, and immunohistochemistry, respectively. In addition, ERGs were measured. One additional 3.5-years-old USH1C pigs was treated accordingly.

Results: Fluorescence microscopic analysis of eye cup preparations demonstrated that all tree capsids transduced ocular cells at the injection site. Further, immune-histochemical analysis revealed that all three vectors transduced RPE cells and photoreceptor cells efficiently. AAV9 and Anc80 additionally transduced Müller glia cells. No relevant off-target expression of eGFP was noted for the tested AAVs. Analyses of the USH1C pig revealed recovery of *USH1C/*harmonin expression in the Anc80.CMV.harmonin_a1 treated retina region when compared to untreated regions and the sham-treated eye. In addition, amplitudes of the a- and b-waves were significantly increased in the scotopic and photopic Ganz Feld ERG, compared to the sham-treated eye. ERGs of the second treated animal showed a similar increase even after 3 month post treatment.

Conclusion: The USH1C pig model is a suitable disease model for the evaluation preclinical therapeutic strategies. Given the expression of *USH1C/*harmonin in photoreceptors and Müller glia cells (Nagel-Wolfrum et al. 2021), AAV9 and Anc80 are suitable vectors for USH1C gene therapy.

We confirmed that the *USH1C/*harmonin_a1 isoform is a therapeutic relevant isoform for USH1C gene therapy. The success of exploratory gene therapy approach in older USH1C pigs also indicates a large window of therapeutic opportunity for USH1 patients.

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Müller cell specific TSPO function as a beneficial element for preservation of retinal neurons after transient retinal ischemia

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Purpose: Translocator protein (TSPO) is a mitochondrial membrane protein that is in discussion to be involved in steroid hormone metabolism. In the retina, TSPO is predominantly expressed by Müller glia, microglia, endothelial and pigment epithelial cells. Treatment of damaged retinae with agonistic TSPO-ligands (e.g. XBD173) indicate a promising therapeutic potential. Described effects include a reduction of neuronal degeneration, anti-inflammatory effects and dampened microglial reactivity. However, the mechanism by which TSPO agonists mediate the neuroprotective effects remains largely unknown and is discussed controversially. Given that TSPO seems to be involved in the mitochondrial cholesterol shuttling that is critical for steroid hormone synthesis, we speculate that the beneficial actions of TSPO agonists work via an increased production of neurosteroids by Müller glia in the damaged retina.
To investigate our hypothesis, we use a combined approach of Müller glia-specific conditional TSPO knockout (KO) using Glast-CreERT2 and systemic application of a novel TSPO ligand GRT16085N (GRT16) in a murine model of retinal ischemia.

Methods: First, we established the recombination protocol for the newly generated Glast-Cre^{ERT2} TSPO$^{fl/fl}$ line. Utilizing this Müller cell-specific TSPO KO line or modulation of TSPO by GRT16 treatment, we compared the effects at 14 days post lesion (dpl). To this end, we performed morphometric measurements of retinal layers, immunohistochemistry for neuronal subpopulations, microglia and Müller cell gliosis. To highlight the functional integrity after lesion, we performed electroretinography measurements. Finally, tandem mass spectrometry for retinal tissues was established to uncover the influence of TSPO-modulation on steroid hormone synthesis.

Results: Our recent data indicate a neuroprotective effect of GRT16 after retinal ischemia at 14 dpl on cells of the inner nuclear layer and ganglion cells. Furthermore, Müller cell gliosis appears to be reduced by GFAP intensity measurements. On the contrary, the Müller glia-specific TSPO KO caused a decreased number of ganglion cells, while microglia numbers and GFAP intensity remained equal to numbers detected in ischemic eyes of control animals. Finally, initial steroid hormone measurements show major metabolites to be present in native retinal tissue.

Conclusion: GRT16 appears to improve neuronal survival after lesioning, similar to XBD173. Knockout of TSPO specifically in Müller cells worsened neuronal survival. Overall, our observations

support the hypothesis that the neuroprotective effects of TSPO modulation depend in part on effects on glial TSPO. To further confirm our theory, analyses of microglial morphology, ERG, and steroid hormone analysis are in progress.

Adaptive optics ophthalmoscopy in retinitis pigmentosa: Typical patterns

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Introduction: Due to advances in optics, electronics and computation, morphological changes of the photoreceptor mosaic can be made visible by adaptive optics ophthalmoscopy (AOO). Retinitis pigmentosa (RP) is a degenerative retinal disease that affects primarily the rod photoreceptors followed by the loss of cone receptors. This paper aims to present typical findings in RP with the adaptive optics flood illumination retinal camera rtx1.

Methods: 174 patients with syndromic or non-syndromic RP were examined with the commercially available adaptive optics flood illumination retinal camera rtx1 and spectral domain optical coherence tomography (OCT) and fundus autofluorescence (FAF) imaging at the Center for Ophthalmology, University of Tuebingen. AOO patterns were studied in the context of multimodal retinal imaging.

Results: Five different patterns in RP could be observed: 1) unspecific atrophy, 2) central visibility of cones, 3) "puffy" cones, 4) the "cheetah" pattern, and 5) atrophic pigment clumping. These patterns in AOO correlated with findings on OCT and FAF imaging. We hypothesize that these patterns represent specific stages of photoreceptor degeneration in RP.

Conclusion: AOO provides an additional dimension to high-resolution retinal imaging in RP, enabling to determine patterns of retinal degeneration. Future evaluation of cone photoreceptor mosaic using AOO imaging is warranted to determine changes on an even more microscopical level, e.g. photoreceptor integrity.

Digital progress hubs health - MiHUBx – A digital ecosystem for strengthening medical research, diagnostics and therapy in Saxony: An introduction to use case "Ophthalmology meets diabetology"

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Goal/Purpose: As part of the Medical Informatics Initiative (MII), the German Federal Ministry of Education and Research (BMBF) is funding six new collaborative projects, the "Digital Progress Hubs Health". These pursue the goal of improving the availability of data and collaboration between the various areas of healthcare - from inpatient and outpatient treatment to rehabilitation and aftercare. Thus, the MII will also include the outpatient sector in the future.
In the Medical Informatics Hub in Saxony (MiHUBx), Dresden University of Technology, Dresden University Hospital, Chemnitz University of Technology, Chemnitz Hospital and Mittweida University of Applied Sciences have been pooling their expertise since 01.09.2021 to bring research and care closer together. The goal of MiHUBx is the development of a cross-sector and service-based infrastructure to enable networking and efficient collaboration of a wide variety of actors and initiatives in health research and care in Saxony.

Methods: In three use cases, based on health care data, (1) methods of AI are applied to unlock new knowledge, (2) statistical models are used to make predictions that have a direct impact on patient care, and (3) communication channels are established to enable faster, intersectoral data exchange. This will create solutions in different application areas to reduce the gap between research and care. For the detection and treatment of diabetic eye diseases, in Use Case 1 "Ophthalmology meets Diabetology" a decision support system, including networked data from the early phase of diabetes to late phases of diabetes-related eye diseases such as diabetic macular edema, will be developed and evaluated. To this end, computer science but also (ophthalmic) medical professionals work closely together and design demonstrators in a participatory manner.

Results: In our paper we show the first state of the art on time series visualizations of (networked) ophthalmic data for acquired retinal diseases in the form of a dashboard. Furthermore, we present the development of algorithms for decision support systems on an AI-based mockup for OCT biomarker classification.

Summary: This should enable early detection, close monitoring, and sustained treatment of severe disease progression in particular. Data for future studies will be obtained, which should lead to new insights for improved treatment options. In this way, the workload and time required for care can be significantly reduced. The development of increasing age and the increase in diabetic eye diseases with a growing shortage of ophthalmologists, especially in rural areas, can also be adequately counteracted.

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 retinal flat mounts. Microglia activation marker expression in whole retinal transcripts was determined by qRT-PCR. OCT was used to measure retinal thickness; TUNEL, and cone staining were used to quantify the extend of retinal degeneration and photoreceptor cell death.

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 AIF-1 was strongly increased and this upregulation was absent in mice treated with PLX3397. Expression of the microglia activation marker TSPO 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. However, PLX3397 treatment could not prevent photoreceptor cell death, and even increased the amount of TUNEL positive cells in the outer nuclear layer.

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. Furthermore, the photoreceptor debris were not phagocytosed and remained in the retina. We conclude that the presence of microglia and their immunomodulation is a promising concept for the treatment of retinal degeneration diseases.

Dissection of the role of IFT140 in cellular signaling and its effect on retinal degeneration

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Purpose: IFT140 is a part of the IFT complex A (IFT-A), a crucial component of the ciliary machinery which facilitates the retrograde transport of cargo e.g. in photoreceptor cells. Mutations in IFT140 itself can cause a vast spectrum of diseases, including early onset, severe retinal dystrophy, leading to blindness at a young age. Within this study we attempt to dissect the role of IFT140 mutations by detecting disease-induced alterations in the protein interaction pattern of IFT140 and to thereby identify potential targets for therapeutic intervention in pathways regulating tissue homeostasis. We have compiled a list of missense mutations in IFT140 found in patients suffering from ciliopathies with retinal dystrophies.

Methods: To analyse the IFT140 protein interactome and its changes induced by IFT140-associated mutations we collected a list of 24, previously published missense mutations in IFT140 from patients suffering from ciliopathies. We generated HEK293 stable lines expressing IFT140 wildtype or ciliopathy-associated IFT140 mutants with a N-terminal Strep/FLAG-tag followed by cell lysis and immunoprecipitation. Subsequently label-free quantitative LC-MS/MS analysis of the purified interactomes was performed, comparing wildtype and mutant conditions to each other as well as to a negative control. Through the analysis of 6 replicates each and stringent statistical filters, we were able to gain robust and reproducible dataset.

Results: For some mutations we could observe an impairment of the IFT-A complex stability, while for most mutations, the interactome was not affected. We therefore assume that at least a subset of missense mutations might disrupt the stability of the IFT-A complex, changing its composition and potentially hampering its molecular function. Additionally, components important for cilia specific Sonic Hedgehog signaling (Shh), most notably TULP3, were identified as potential interactors of IFT140, which are affected by mutations. Primary cilia are essential for functional Hedgehog signaling and defects have been implicated in retinal dystrophies.

Conclusion: Our data suggests an involvement of IFT140 in Shh via disruption of the Interaction between the IFT-A complex and the protein TULP3. As Hedgehog signaling can be pharmacologically targeted, we hope to generate a rationale for treating such forms of blindness in the future.

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The role of the primary cilium 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 RPE development as well as playing a role in phagocytosis. Here we investigated the influence of a dysfunctional vs. ablated primary cilium on the phagocytosis pathway in RPE cells in an attempt to identify the underlying pathogenic mechanism.

Methods: As loss of IFT20 results in complete ablation of the primary cilium, IFT20 knockdown was chosen to model ciliary absence. BBS6 was chosen as a model to analyse ciliary dysfunction. Using the RPE-J cell line, a cell line used to investigate phagocytosis in vitro, we knocked down *Ift20* or *Bbs6* via siRNA and examined the expression of key phagocytosis related genes via real-time qPCR and protein expression via western blot. In addition, we also determined phagocytic ability.

Results: The knockdown efficiency of RPE-J Ift20 and Bbs6 KD cells was verified to ensure depletion of the target gene. Upon loss of a functional primary cilium, significant differences in the expression of phagocytosis genes were detected. These differences were partially recapitulated on the protein level which might account for differences in phagocytosis function.

Conclusions: Recent studies have shown that the primary cilium can influence RPE phagocytosis. With the presented data, we started analysing the underlying molecular mechanisms involved. We found specific targets, which might act downstream of ciliary function. A better understanding of these pathways is vital to uncover targetable pathogenic mechanisms underlying cilia-associated in ocular disorders.

A novel dual AAV-vector approach for gene therapy of Usher syndrome 1B

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Purpose: Usher syndrome (USH) is the most common cause for inherited deafblindness. Currently, there is no therapy that can halt or improve retinal degeneration in USH patients. Mutations in the unconventional molecular motor protein *MYO7A* (USH1B) are the predominant cause of USH1, the most severe USH subtype. However, *MYO7A* (6.7 kb) exceeds the packaging capacity of recombinant adeno-associated-viral (rAAV) vectors (4.7 kb), the gold standard vectors for gene therapy. To overcome this constraint, dual AAV vectors containing two split fragments of the gene of interest or lentiviral strategies have been developed in the past, but all have significant limitations. These include the low reconstitution efficiency of currently used dual rAAVs or the low transduction efficiency of retinal cells when lentiviral vectors are applied. There is thus an unmet medical need to develop a highly efficient approach for expression of large genes in the retina. Here, we present a novel dual AAV vector strategy based on reconstitution of the split fragments at the mRNA level. This strategy was applied to reconstitute *MYO7A in vitro* and *in vivo.*

Methods: The coding sequence of human *MYO7A* was split into two non-overlapping fragments, equipped with elements required for transcript expression and subsequent reconstitution via mRNA *trans*-splicing, and packaged into AAV2/8(Y733F) capsids. Reconstitution was evaluated in vitro in 661W and HEK293 cells co-transfected with the dual plasmid vectors at transcript level using qRT-PCR and sequencing. For in vivo analysis, C57BL/6J mice were injected subretinally with rAAVs at p30 and retinal eyecups were isolated 4 weeks post injection for subsequent western blotting and Immunohistochemistry experiments. For quantification of protein expression, semi-quantitative western blotting was performed.

Results: Reconstitution of *MYO7A* was confirmed *in vitro* at mRNA level. We achieved around 56 % *MYO7A* expression relative to the endogenous *Myo7a in vivo.* Additionally, the histological data indicates a correct localization of reconstituted *MYO7A* in rod photoreceptor outer segments.

Conclusions: Our mRNA *trans*-splicing dual rAAV vectors yield high levels of reconstituted *MYO7A* in the murine retina, suggesting a high therapeutic value. To further evaluate the therapeutic potential of this strategy, we will apply it to a conditional *Myo7a* knockout-mouse-model and to human retinal organoids.

AAV-PHP.eB targets various retinal cell types depending on delivery route in mice

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Purpose: Various serotypes of recombinant adeno-associated virus (AAV) transduce the retina with high efficacy. Tropism and safety of AAV vectors are of prime interest for gene delivery to the retina. In the present study, we characterise the transduction profile of AAV-PHP.eB, a serotype developed from AAV9 using *in-vivo* directed evolution. One of the main features of AAV-PHP.eB is the ability to cross the blood-brain barrier and transduce the CNS with high efficiency.

Methods: A CMV-EGFP expression cassette in AAV-PHP.eB capsid was delivered via systemic, intravitreal and subretinal routes in adult mice. The same expression cassette was also administered intravitreally and subretinally utilising benchmark control serotypes, i.e. AAV2/2 and AAV2/8, respectively. Subsequently, EGFP expression was analysed in the transduced retinas.

Results: Systemic and intravitreal delivery of AAV-PHP.eB transduced retinal ganglion and horizontal cells efficiently with systemic delivery providing pan-retinal coverage of the retina. Subretinal delivery targeted photoreceptor and retinal pigment epithelium cells with remarkable efficacy. The number of EGFP transduced cells and EGFP mRNA levels were similar when the retinas were transduced intravitreally utilising 7.5E + 08 vg of AAV-PHP.eB or the control AAV2/2 capsid. In contrast, EGFP fluorescence intensity and mRNA levels were 50 – 70 times higher in photoreceptors, when subretinal injections with 3.0E + 07 vg/eye of AAV-PHP.eB were compared to the control AAV2/8 serotype.

Conclusion: Our results highlight pan-retinal transduction of ganglion cells (via systemic route) and robust transduction of photoreceptor cells (via subretinal injection) as the most attractive features of AAV-PHP.eB in gene delivery to the mouse retina.

The potential of photoreceptor-specific miRNAs in health and disease

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In Retinitis pigmentosa (RP), rod photoreceptors degenerate and at late stages, cone photoreceptors in the central part of the retina are becoming insensitive to light. Cone outer segments start to degenerate, leading to complete blindness. A subset of miRNAs, known to be master regulators of gene expression, have been shown to be particularly important for cone outer segment maintenance and function. This includes the photoreceptor specific miRNA cluster miR-182/96/183 as well as miR-124. Since miRNA AAV-mediated gene transfer to embryonic-stem cell derived retinal organoids led to the outgrowth of short outer segments *in vitro,* we aim to inject AAV vectors carrying the genetic information of the aforementioned miRNAs into the subretinal space of two different RP mouse models. Cone photoreceptor specific promotors restrict miRNA expression in cone photoreceptors. Thereby, we are striving for the development of a neuroprotective as well as a neuroregenerative approach, to reinforce the ability to maintain high-acuity vision in retinal degenerative diseases. We also apply the AAVs to human induced pluripotent stem cells (hiPSCs)-derived 3D retinal organoids and 2D photoreceptor cultures. Here, we aim to accelerate cone photoreceptor outer segment maturation whilst growing retinal organoids. In 2D retinal cultures, our goal is boost cone photoreceptor maturation. We have already succeeded in generating cone precursor-like cells by forward programming of hiPSCs using three transcription factors. The extensive use of these miRNAs *in vivo* and *in vitro* will pave the way to mechanistically dissect and exploit photoreceptor-specific miRNAs for cone photoreceptor development, maintenance, and function.

Retinoschisin and novel interaction partners of the Na/K-ATPase define a growing protein complex at the inner segments of photoreceptor cells

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Purpose: X-linked juvenile retinoschisis (XLRS) is a hereditary retinal dystrophy manifesting in juvenile or adolescent males and is caused by mutations in the *RS1* gene on Xp22.13. The protein encoded by *RS1* has been shown to be part of a plasma membrane associated macromolecular complex through binding to the retinal Na/K-ATPase. To gain a deeper understanding of the composition and function of this complex, we aimed to identify additional interaction partners of the Na/K-ATPase-retinoschisin complex and to further elucidate the role of retinoschisin as a putative regulator of photoreceptor membrane compartmentalization.

Methods: Co-immunoprecipitation targeting the α3-subunit of the Na/K-ATPase (ATP1A3) was performed in porcine retinal lysates and bound proteins were eluted, separated by SDS-PAGE and subjected to mass spectrometric analysis. Newly identified protein species were verified in co-immunoprecipitation experiments of murine retinae. A possible influence of retinoschisin-deficiency on proteins of interest was investigated by looking at their localization in eyes of wildtype (wt) and retinoschisin-deficient (*Rs1h* knockout, Rs1tm1Web) mice *via* immunohistochemistry as well as their total mRNA and protein expression in murine retinal lysates. Kv channel regulated ion flow was analyzed by patch-clamp analysis of Y-79 cells, incubated with or without purified retinoschisin.

Results: Mass spectrometry identified the voltage-gated potassium ion channel (Kv) subunits Kv2.1 and Kv8.2 as new interaction partners of the retinal Na/K-ATPase-retinoschisin complex. Immunohistochemical stainings in murine retinal cryosections revealed Kv localization to the inner photoreceptor segments, overlaying both retinoschisin and retinal Na/K-ATPase localization. In retinae from retinoschisin-deficient mice, Kv2.1 and Kv8.2 revealed a pathological spatial distribution, an effect which has previously also been seen for the retinal Na/K-ATPase. While Kv2.1 and Kv8.2 total protein amount is greatly reduced in retinoschisin-deficient retinae, overall protein quantity of the retinal Na/K-ATPase remains unaffected. Of note, retinoschisin-deficiency appears to have no effect on Kv channel regulated potassium ion flow.

Conclusion: Our analyses identified the Kv channel subunits Kv2.1 and Kv8.2 as novel constituents of a growing macromolecular complex at the photoreceptor inner segments. Defective compartmentalization of this complex in early stages of retinal developmental may be a critical step in XLRS pathogenesis possibly rendering a simple gene replacement therapy of the retinoschisin protein little effective.

Comparison of full-field stimulus threshold measurements in RP-patients in miosis and mydriasis

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Fullfield stimulus threshold (FST) testing is a psychophysical test, established in the rod functional diagnostics of inherited retinal diseases (IRD). The chromatic FST has become an important read-out in clinical trials and approved treatments representing a sensitive marker of rod functional rescue. The current protocol recommends widening of the pupils For FST testing, leading to increased photophobia.

Purpose: To investigate the difference between FST measurements in miosis and mydriasis in healthy eyes and RP-Patients.

Methods: 20 healthy control patients as well as 20 retinitis pigmentosa (RP) patients were tested. From the RP groups, 10 patients were selected with only remaining central visual field of approximately 10 – 20° while the other 10 patients were selected with additional peripheral areas. One pupil of each proband was dilated, the other eye of the same patient was measured in physiological width (miosis). The order of testing in miosis vs. mydriasis was randomized. The FST testing was conducted using Diagnosys Espion E2/E3 (Diagnosys LLC, Cambridge, UK) using white, blue and red stimuli. Patients with asymmetrical findings between left and right eye or with relevant findings in the macula such as macular edema or macular hole were not considered in order to allow the comparison between eyes. The statistical analysis was conducted using a paired t-test.

Results: In total 40 subjects were tested. The median age of the control group was 36 years and the median age of the RP-patients was 45. The best corrected visual acuity (BCVA) was between 0.0 and -0.1 logMAR in the control groups and very variable between "hand sign" and 0.0 logMar in the RP-patients.
The FST results in healthy probands of the control group showed no statistical significance between miosis and mydriasis while testing with blue or red stimulus. However, there was a statistically significant difference (p=0,017) between miosis and mydriasis while measuring with white stimulus.
The FST results for white, blue and red stimuli in the whole group of RP-patients showed significant differences between mydriasis and miosis. The measurements in miosis showed in average a higher threshold than the measurements in mydriasis (by 3,28 dB for blue colour, 2,23 dB for white and 1,46 dB for red stimulus) (Fig.1). In group analysis for the RP-patients with constricted visual field, with white and red stimulus did not show a statistical significance between miosis and mydriasis. However, the FST measurements with blue stimulus was significantly different (p=0.05) between miosis and mydriasis, showing a higher threshold in miosis (by 4,15 dB%). In the group

analysis for RP-patients with better preserved visual field, FST results showed statistically significant differences between mydriasis and miosis for white (p=0,002), red (p=0,04) and blue (p=0,024) stimuli.

Discussion: FST is an important evaluation of rod function. Our data show that in healthy individuals if tested with red or blue stimulus, there is no statistically significant difference between testing in miosis and mydriasis, unlike FST measurements with white stimuli. However, this small difference is not of clinical significance.
However, we could prove that FST testing in mydriasis is important in RP-patients with all three colors (blue, white, red), except the more progressed group of RP-patients with only concentric restrictions of the visual field if using blue stimulus. This might be due to the highest specificity of blue light for rods.
While it seems to be acceptable to conduct FST measurements in miosis on healthy eyes, it is not generally recommendable for RP-patients. This is especially important as disease progression seems to have a different effect to FST values depending on pupil dilation.

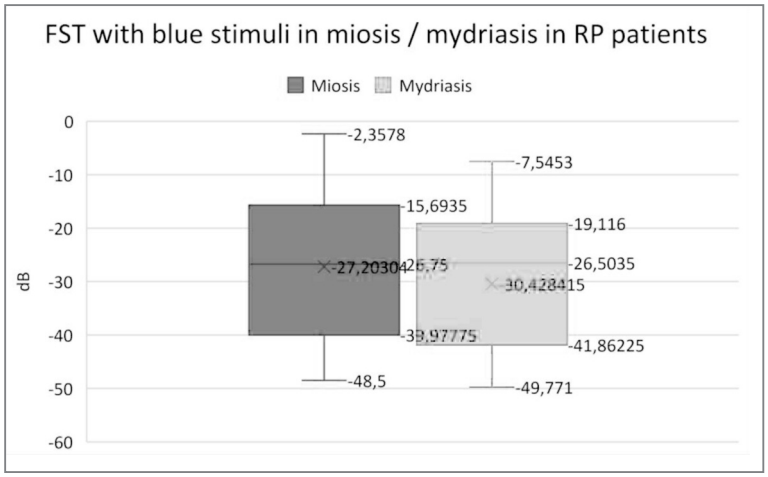

Figure 1:
The FST measurements with blue stimuli in miosis and mydriasis in comparison.

RPGR$_{ORF15}$ sequencing in NGS-based panel diagnostics

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Retinitis pigmentosa (RP) refers to a group of progressive retinal dystrophies that initially affect the peripheral retina and can finally lead to blindness. With a prevalence of 1:4,000, RP represents the most prevalent form of inherited retinal dystrophies (IRD). All Mendelian traits can be observed, including X-linked RP with usually severe disease expression in hemizygous males. Among the genes that cause X-linked RP, mutations in *RPGR* are responsible for the majority of cases. The *ORF15* isoform (NM_001034853) of *RPGR* is primarily expressed in the retina and comprises a large C-terminal exon with a hard-to-sequence tandem-repetitive central region that represents a known mutational hotspot.

Given the high prevalence of *ORF15* mutations in RP patients and recent successful clinical studies of gene therapy treatment in patients with *RPGR*-related RP (RP3), our aim was to implement robust *RPGR*$_{ORF15}$ analysis in a routine IRD diagnostics workflow with a minimum of additional effort. We have developed a protocol for NGS-based IRD diagnostics that applies additional transcript specific lockdown probes for *RPGR*$_{ORF15}$ enrichment in combination with a bioinformatics quality-based sequence read trimming step to ensure sufficient sequencing coverage and quality.

We verified our method by means of 20 positive controls, achieving 100 % sensitivity and accuracy. Applied to our cohort of patients with clinically diagnosed RP (years 2020 and 2021), we identified causative *RPGR* mutations in 38 cases (6 % of patients). In 27 cases, mutations were located in *RPGR*$_{ORF15}$ (71 % of *RPGR*-positive cases), 9 of which represented yet unreported (truncating) variants, altogether illustrating the significance of efficient *RPGR*$_{ORF15}$ diagnostics.

To die or not to die – Does loss of bassoon make cone photoreceptors more vulnerable for neurodegeneration?

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The presynaptic protein bassoon (BSN) is an important component of the active zone of chemical synapses, where it contributes to synapse assembly and function. Recent studies show a novel function of BSN as an inhibitor of presynaptic autophagy and proteasomal degradation in brain neurons.

In the retina of two BSN-deficient mouse lines, *Bsn*$^{\Delta Ex4/5}$ and *Bsn*^{gt}, we found degeneration of cone photoreceptors and retinal remodeling. This raises the question of whether BSN is important for the survival of these highly active sensory neurons by controlling homeostasis pathways. Interestingly, we did not find such a retinal phenotype in a third BSN-deficient mouse line (*Bsn*^{ko}). Since our recent results show that proteasomal degradation is disrupted in the retinae of both *Bsn*^{gt} and *Bsn*^{ko} mice, disruption of protein degradation alone is not sufficient for cone photoreceptor degeneration. We hypothesize that an additional trigger is required.

The trigger for cone photoreceptor degeneration could be the presence of a residual BSN fragment, as found in the *Bsn*$^{\Delta Ex4/5}$ mouse line. This hypothesis is supported by the finding that we can induce a retinal phenotype in *Bsn*^{ko} mice by crossbreeding them with *Bsn*^{gt} mice. Interestingly, the presence of a wild-type Bsn allele is sufficient to prevent the degeneration triggered by the *Bsn*^{gt}-transgene. In our study, we want to find out whether Bsngt mice express a residual BSN fragment and whether its presence interferes with homeostasis processes, leading to cone photoreceptor cell death.

So far, our data suggest that BSN, in addition to its synaptic function, plays a role in processes of cellular homeostasis and survival of cone photoreceptors in the retina.

Oxidative stress induced by $NaIO_3$ causes apoptotic damage to ARPE-19 cells

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Purpose: Oxidative stress, a characteristic of AMD, can be triggered through damage to the retinal pigment epithelium (RPE). This effect in turn causes retinal damage. Therefore, the effects of oxidative stress on a human RPE cell line (ARPE-19) were investigated in this study.

Methods: To induce oxidative stress, 300 µM $NaIO_3$ were applied to ARPE-19 cells for 3 h. Untreated ARPE-19 cells served as controls. After a 4-day cultivation period, RT-qPCRs were performed (n = 5 / group). All cell samples were analyzed for levels of specific genes relevant to autophagy *(MTOR, SQSTM1, BNIP3)* and apoptosis *(FASLG, CASP3, CASP9)*. Additionally, different concentrations of $NaIO_3$ (0.5 mM, 1 mM, 5 mM, and 10 mM) were compared using the same setup and analyzed by RT-qPCRs (n = 10/group). Immunohistochemistry was performed to visualize apoptotic effects (e. g., cleaved caspase 3) and an assay for cell proliferation was performed (BrdU).

Results: Application of 300 µM $NaIO_3$ resulted in a higher *CASP3* mRNA expression (rel. exp.: 1.44; p = 0.018). Furthermore, *FASLG* expression was significantly increased in the $NaIO_3$ group (rel. exp.: 4.17; p = 0.026). However, no significant differences were observed with regard to autophagy markers. Both apoptotic and autophagic effects were seen in the $NaIO_3$ concentration series. Enhanced expression of *FASLG* (rel. exp.: 3.77; p = 0.008), *MTOR* (rel. exp.: 3.20; p = 0.007), CASP9 (p = 0.048), HIF1α (p = 0.046) and NFkB (p = 0.016) was detected at 0.5 mM $NaIO_3$. The presence of 5 mM $NaIO_3$ also resulted in significantly higher expression of *FASLG* (rel. exp.: 3.12; p = 0.037) and *NFkB* (rel. exp.: 1.70; p = 0.031). Higher expression of *SQSTM1* (rel. exp.: 2.12; p = 0.021) was observed for 10 mM $NaIO_3$. Cleaved caspase 3 staining visualized the apoptotic effect mediated by $NaIO_3$.

Conclusion: Based on the RT-qPCR analyses and staining, oxidative stress induced by $NaIO_3$ primarily has an apoptotic effect on ARPE-19 cells, but higher concentrations of the stressor also induce autophagy. Further studies at protein level should provide more insight into the effects of this stressor.

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Assessing the variability of iPSC-derived RPE cells with defined genetic risk for age-related macular degeneration (AMD)

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Background: In recent years, induced pluripotent stem cell derived retinal pigment epithelial cells (iPSC-RPE) have become a widely used cell culture model to investigate the molecular mechanisms of a variety of retinal diseases with primary pathology allocated to this cell type. Still, most studies suffer from the fact that patient-derived cell lines are cumbersome in handling, leading to studies frequently being conducted with a low number of cell lines. Consequently, previous studies using iPSC-RPE cells to study AMD-related pathomechanisms showed inconsistent results as inter-cell-line-variability was rarely considered. Here, we addressed this issue by testing 22 clones from 20 iPSC-RPE cell lines with a defined genetic risk for AMD and performed an in-depth characterization, both under baseline culture conditions as well as upon oxidative stress induction by sodium iodate (SI) and nicotinamide (NA) depletion.

Materials and Methods: RPE cells were characterized by transepithelial electrical resistance (TEER) measurements, immunocytochemical analysis with tight ZO-1 BEST1 and CTNB markers, as well as mRNA expression analysis of *BEST1, C3, CFH, HMOX1, NQO1* and *RPE65*, with BEST1 and RPE65 being confirmed on protein level. Mitochondrial integrity and function were analyzed with the Agilent Seahorse XF Analyzer.

Results: During a six week maturation period on transwell filters, all 22 cell lines formed a tight monolayer, as visualized by BEST1 and ZO-1 stainings and increasing TEER values. *RPE65* and *BEST1* mRNA and protein expression indicated RPE specificity of the cell lines. NA depletion resulted in decreased *BEST1* and *RPE65* mRNA expression, while *C3* and *CFH* were significantly upregulated. Oxidative stress induction by SI treatment resulted in a marked increase of *HMOX1* and *NQO1* mRNA expression. All data obtained were analyzed with regard to the overall differences between the cell lines and differences between HR and LR AMD cell lines. None of the comparisons revealed statistical significance, mainly due to a considerable high inter-cell-line variability. Only Seahorse measurements of mitochondrial activity and integrity showed an increasing trend in all measurements regarding HR versus LR cell lines, which however failed to reach statistical significance.

Conclusion: The relative and absolute values obtained for the different parameters analyzed were rather variable between cell lines rendering to datasets statistically not significant after adjustments for multiple testing. Overall, however, there was a reproducibility of measurements within tolerable limits. Still, the variability should be kept in mind and may result in false conclusion when small numbers of iPSC-derived cell lines are used in a study.

The RPE-specific complement system correlates with EMT during disease development

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Purpose: In the eye, fibrotic diseases like proliferative vitreoretinopathy and age-related macular degeneration are associated with a de-maturation of the retinal pigment epithelium (RPE). The underlying pathomechanisms are regulated by the epithelial-mesenchymal transition (EMT). The RPE shows an intracellular expression of components of the complement system which is involved in the development of retinopathies. Here, we describe a cell-based maturation model to compare immature and mature RPE cells and elucidate the role of the RPE-specific complement system in EMT.

Methods: Immature and mature ARPE-19 and induced pluripotent stem cell (iPSC)-derived RPE cells were cultivated using distinct media and time periods. Cellular physiology was determined based on transepithelial resistance and cell capacity. Complement components were detected using quantitative gene expression analysis, immunocytochemistry, multiplex immunoassays and Western blot. EMT markers were associated on transcript and protein levels. The apical and basal cytokine secretion profile was correlated.

Results: Our data unveiled a RPE-unspecific mesenchymal phenotype of immature RPE cells. This was accompanied by an upregulation of EMT markers. Screening of 65 cytokines revealed the upregulation of an EMT-associated cytokine secretion in immature RPE cells. In contrast to this, the mature RPE cells displayed an epithelial RPE-specific morphology and functionality.
Furthermore, we described distinct complement profiles for immature and mature RPE cells. The cellular C3 activation status was correlated with the EMT status. In immature RPE cells increased EMT markers and C3a (active C3 fragment) generation were linked by a mutual reinforcement. In the mature state an increased C3 turnover and immediate iC3b (inactive C3 fragment) generation by complement factors I and H promoted RPE maturation. These results were partially validated in iPSC-derived RPE cells (work ongoing).

Conclusion: In conclusion, the analysis of the RPE cell maturation model resulted in the identification of a pathological environment in immature RPE cells that is accompanied by a cell-based complement activation and inflammation. Contrary to this, mature RPE cells, similar to the physiological *in vivo*-RPE, depicted a dampened complement activity. Furthermore, this project lays the groundwork for a more precise elucidation of the relationship of the cellular RPE-specific complement system and its role in EMT-associated retinal diseases. These findings underline the importance of a full maturation of *in vitro*-RPE models that is unfortunately often overlooked.

Development and quantification of photoreceptor outer segment formation

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Purpose: Photoreceptor cell transplantation aims to replace or support degenerated photoreceptors of defective eyes by introducing healthy donor cells. To achieve functionality after transplantation the formation of proper photoreceptor outer segments (OSs) is important. These membranous structures transfer incident light into a biochemical signal which is the crucial first step in the process of vision. Despite this, OSs have not been studied in detail in the context of transplantations. This project aims to establish quantification methods for OS formation and quality, particularly at an ultrastructural level.

Methods: OS formation was examined during development of C57Bl/6JRj wild type mice to establish quality and quantification measurements. Eyecups of pups between postnatal ages P4 to P20 were analyzed via light and electron microscopy including OS-specific immunohistochemistry. Electron microscopic images were used to establish an innovative ultrastructural quantification measurement for the alignment of OS membrane stacks. Images were analyzed using a custom written MATLAB code that extracts the orientations of membranes either from the image gradient or from the Fourier transform of sliding image patches. Then the coherency or alignment between these orientations is calculated both locally (within a given radius) and globally (across individual OSs).

Results: Immunohistochemical analyses revealed that both structural and functional markers are expressed at similar timepoints. The expression patterns changed most between P4 and P12. Major changes in the number and ultrastructure of the OSs occurred simultaneously. Additionally, a method for quantifying the orientation of membrane stacks within OSs has been developed. Coherencies ranged between 0 and 1 with mean local coherency as observable for partly stacked membranes and global coherency as observable for alignment of membranes across the entire OS.

Conclusion: A set of immunohistochemical markers which can be used for characterizing the structure and potential function of OSs has been identified. More to the point, the quality of OSs can be quantified on an ultrastructural level. These measurements provide the basis for analyzing the effects on OS formation after alterations of donor cells or different host environments with the aim to promote functionality of photoreceptor cells in a transplantation setting.

Soluble terminal complement complex and its components increases intracellular Ca^{2+} concentration in ARPE-19 cells

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The problem: The group of Robert Mullins demonstrated a lifetime long accumulation of terminal complement complex (TCC) in the choroid of age-related macular degeneration retinas (Mullins et al., 2014). Previous work showed that normal human serum (NHS) as complement source incites a strong and reproducible Ca^{2+} response by ryanodine-receptor dependent activation of the L-type channel in ARPE- 19 cell (Genewsky et al., 2014) that was absent after heat-inactivation. Further analyses demonstrated that anaphylatoxin generate Ca^{2+} signals are independent of L-type channels (Busch et al., 2017). This raised the question which complement component generated the NHS reaction. As C7 depletion changes the Ca^{2+} response to NHS, we hypothesize that TCC is the main contributor. Depleted serum helped to decipher the action of each TCC components.

Results: We found in C6 depleted serum highly variable waveforms, C7 depleted serum resulted in short Ca^{2+} peaks, while C9 depleted reacted biphasically. Ca^{2+} signaling from depleted sera revealed a TCC-specific effect and the variability in Ca^{2+} waveforms depended on TCC components.
In general, blocking sarcoplasmic Ca^{2+}-ATPase (SERCA) with thapsigargin, L-type channel with isradipine or ryanodine receptor with dantrolene, abolished Ca^{2+} peaks. In addition, specific conditions included activation of extrusion mechanisms: (i) blocking L-type channels before C7-depleted serum is added, (ii) blocking Ca^{2+}-ATPase before exposing the cells to C6-depleted serum. These results demonstrate that the Ca^{2+} signal depends on endogenously expressed ion channels but not on a lytic formation. Furthermore, the results show a co-dependence on TCC components. Thus, we aimed to investigate the effect and role of the soluble TCC by analyzing changes in Ca^{2+} signaling.
Exposure of ARPE-19 cells to soluble TCC resulted in an instant, but persistent Ca^{2+} influx into the cells. This signal was completely blocked by Ca^{2+} inhibitors: thapsigargin, isradipine and partially blocked by dantrolene. Endorsing our findings, endogenously expressed ion channels in the cell membrane mediate the response of soluble TCC. Furthermore, immunohistochemistry supported the observation as soluble TCC remained on the cell surface to elicit a response, rather than being internalized by endocytosis.

Conclusions: In conclusion, TCC has a regulatory impact on the cell activity by an, so far, unknown Ca^{2+} dependent signaling pathway. The results suggest that C6 is the major mediator of this pathway but requires C7 and C9 to generate a stable and reproducible Ca^{2+} signal. Furthermore, this pathway uses endogenously expressed Ca^{2+} signaling proteins: L-type channel, sarcoplasmatic Ca^{2+} stores and ryanodine receptor coupling. These results indicate a new function of the TCC and a need to refine the concept of sublytic-TCC.

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Retinal neuroprotection by controlled release of a VCP inhibitor from self-assembled nanoparticles

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Purpose: Mutations in rhodopsin *(RHO)* lead to its misfolding resulting in autosomal dominant retinitis pigmentosa (adRP). Pharmacological inhibition of the ATP-driven chaperone valosin-containing protein (VCP), a molecular checkpoint for protein quality control, slows down retinal degeneration in animal models of adRP. However, poor water-solubility of VCP inhibitors poses a challenge to their clinical translation. In order to enable the delivery of VCP inhibitors to the retina, we have developed and investigated two formulations for the VCP inhibitor ML240 in organotypic cultures of RHO^{P23H} rats, a model known to mimic human adRP.

Methods: We tested two different formulations to encapsulate the VCP inhibitor, ML240, namely carrier obtained by self-assembling of methoxy-poly (ethylene glycol)$_{5kDa}$–cholane (mPEG-cholane) or methoxy-poly (ethylene glycol)$_{5kDa}$–cholesterol (mPEG-cholesterol) polymers. ML240 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 $RHOP^{23H}$ transgenic rats treated with both nanoparticles with encapsulated drug and compared to the corresponding controls. Photoreceptor cell survival and structural integrity were evaluated by cell row quantification and TUNEL assay. RHO distribution and microglia activation were assessed by immunofluorescence using specific antibodies. The possible side effects of ML240-loaded nanoparticles were examined by fundus camera and OCT in wild-type adult rat eyes before and two weeks after intravitreal (IVT) injection.

Results: Both formulations increased the water-solubility of ML240 by two orders of magnitude and prolonged the drug released over ten days. Application of ML240 using either of the two delivery systems in retinal organotypic cultures resulted in a similar increase of cell viability compared to free drug alone, however, at a lower dose. In addition, encapsulation of ML240 in mPEG-cholane showed superior photoreceptor protection, normalized RHO localization, and alleviated inflammatory microglial responses in an in vitro rat model of retinal degeneration. Long-term IVT administration showed good tolerability of the formulations without any visible fundus alterations such as conjunctival bleeding, induction or worsening of cataracts, retinal detachment, or other retinal morphological alterations.

Conclusions: As a perspective beyond drug-based VCP inhibition, our study may be considered proof of concept that a colloidal self-assembling system can serve as an advanced delivery system for treating retinal degeneration. Polymer-based nanoparticles can provide increased solubility, slow-release, and prolonged action of a drug combined with excellent ocular tolerability. Beyond the scope of our study, and as the retina is part of the central nervous system (CNS), we also suggest this technology for further studies to facilitate drug delivery to the CNS.

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Characterizing the functional effect of CNGA3 missense variants associated with achromatopsia using an aequorin-based bioassay

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Purpose: Achromatopsia (ACHM) is a rare congenital retinal disorder caused by impaired cone photoreceptor function. Depending on geographic origin, 40 – 80 % of ACHM cases carry variants in *CNGA3*, which encodes the A3 subunit of the cyclic nucleotide-gated (CNG) channel, a key component of the cone phototransduction cascade. 150/204 currently known CNGA3 missense variants are functionally uncharacterized from which a large number of variants is of uncertain pathogenicity (VUS) hampering genetic diagnostics and consequently confirmation of clinical diagnosis. Therefore, we aim to establish an expert-based assessment of yet functionally uncharacterized CNGA3 missense variants combining *in silico* genetic validation and functional evaluation.

Methods: To assess functionality of mutant homomeric CNG channels, we used a luminescent bioassay based on the calcium-sensitive photoprotein aequorin. Wildtype or mutant homomeric CNGA3 channels were heterologously expressed in HEK293 cells stably expressing aequorin. Following activation of CNG channels by the membrane permeable ligand 8-Br-cGMP, the corresponding CNG channel–meditated Ca^{2+} influx was reported via aequorin-mediated luminescence. The luminescence signal is proportional to the amount of Ca^{2+} and therefore correlates with CNG channel functionality. Since variants might completely abolish CNG channel function, dot blot analysis of whole cell lysates was used to verify CNGA3 protein expression.

Results: To evaluate the capability of the assay to assess mutant CNG channel function, we validated our assay by functionally re-testing 12 previously extensively studied CNGA3 missense variants, hereby confirming their loss of function or wildtype-like behavior in our assay. Currently, we have successfully examined 103/150 yet functionally uncharacterized missense alterations. Of these, 48 mutant channels did not show any function, 24 variant channels displayed luminescence signals indicative for altered but residual channel function, and 31 mutant channels exhibited wildtype-like behavior. Using our approach, we confirmed the preceding *in silico* classification for 5 (likely) benign variants as well as for 52 (likely) pathogenic variants. 20 VUS could be re-classified as (likely) pathogenic based on functional evaluation and 26 VUS gave rise to channels showing wild-type-like luminescence signals and thus could be re-classified as (likely) benign.

Conclusion: Our medium-through-put, aequorin-based calcium influx assay represents an essential tool to characterize the impact of CNGA3 missense variants on the function of homomeric CNGA3 channels finalizing the re-classification of VUS.

Understanding molecular mechanisms underlying CRB1-linked retinal dystrophies

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Purpose: Crumbs homologue 1 (CRB1) is one of the major genes linked to autosomal recessive Retinitis Pigmentosa (RP) and the more severe form Leber Congenital Amaurosis (LCA). Up to now, no treatment is available to prevent photoreceptor loss in these patients, emphasizing the necessity to further elucidate the molecular mechanisms and potential disease modifiers.

Methods and Results: With the aim to further assess the retina specific functions of CRB1, we investigated its retina-specific interactome using a porcine retina pull-down proteomic approach followed by mass spectrometry analysis. Results not only show a significant enrichment of known CRB1 interacting proteins, validating the experimental approach, but also novel potential interactors involved in actin cytoskeleton dynamics, vesicular transport, and signalling. Next, we compared the interactome of CRB1 WT and CRB1 C948Y, which is the most frequent mutation reported in patients and located in the extracellular domain of the transmembrane protein. Preliminary data show that the majority of potential interactors identified are maintained in the CRB1 mutant. Further, we performed co-immunoprecipitation using Flag and HA tagged CRB overexpression constructs followed by western blot analysis. Preliminary data indicate, that CRB1 and CRB2 homo- and hetero-dimerize potentially through their extracellular domains. This may increase CRB complex stability within the cell membrane of photoreceptors and Müller glia cells. To study the CRB1 C948Y mutation within its human endogenous context, iPSC-derived retinal organoids were generated from two patients carrying a homozygous CRB1 C948Y mutation and suffering from RP. Analysis using Western Blot and Immunofluorescence show a reduction of CRB1 expression in both C948Y patients compared to a healthy control indicating that the mutation alters protein production or stability.

Conclusion: Conclusively, we provide potential novel interactors of CRB1 in the retina, which provide the basis for future validation of CRB1 function. We show that the CRB1 C948Y mutation, while maintaining the majority of CRB1 interaction partners, leads to reduced levels of CRB1 in patient retinal organoids which may affect integrity and stability of the outer limiting membrane. Future work will include validation of the tentative pathways in patient-derived organoids and investigation on the consequence of the C948Y mutation on the dimerization of the CRB proteins.

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Genetic targeting or pharmacological inhibition of galectin-3 dampens microglia reactivity and protects from retinal degeneration

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Purpose: Dysfunctional humoral and cellular innate immunity are key components in the development and progression of age-related macular degeneration (AMD). Chronically activated microglia and their disturbed regulatory system contribute to retinal degeneration. Galectin-3, a b-galactose binding protein, is a potent driver of macrophage and microglia activation and has been implicated in neuroinflammation, including neurodegenerative diseases. Here, we hypothesized that modulation of galectin-3 via TD139 or genetic deficiency of galectin-3 dampens mononuclear phagocyte reactivity and protects from retinal degeneration.

Methods: Galectin-3 expression in AMD patients were analyzed by immunohistochemical stainings. Galectin-3 knockout and BALB/cJ mice were exposed to white bright light with an intensity of 15,000 lux for 1h. BALB/cJ mice received intraperitoneal injections of 15mg/kg TD139 or vehicle for five consecutive days, starting 1 day prior to light exposure. The effect of galectin-3 deficiency or inhibition on microglia was analyzed by immunohisochemical staining and *in situ* hybridization of sections and flat mounts. Pro-inflammatory mediators were measured by qRT-PCR. Optical coherence tomography was performed to assess retinal thickness and to determine the extent of retinal degeneration.

Results: Immunohistochemical stainings of human AMD samples demonstrate that increased galectin-3 is confined to activated microglia present in dry AMD lesion areas. Iba-1-stained cryosections showed many amoeboid microglia located in the ONL of light-exposed mice, whereas TD139-treated and galectin-3-deficient mice displayed attenuated number of microglia in the OPL. Retinal flat mounts revealed an increase of reactivate amoeboid microglia in light-exposed mice, whereas the majority of microglia showed a more ramified morphology in TD139-treated or galectin-3 deficient mice. *Aif-1* and *Lgals-3* mRNA expression in BALB/cJ retinas demonstrated migration of microglia in retinas post light exposure. TD139-treated mice showed a significantly lower number of microglia in these areas. TD139-treated or galectin-3-deficient mice displayed a thicker retinal thickness comparted to vehicle or WT group post light exposure.

Conclusion: Galectin-3 is strongly upregulated in reactive retinal microglia of AMD patients and in two related mouse models of light-induced retinal degeneration. Specific targeting of galectin-3 by genetic knockout or using TD139 blocks microglia reactivity and protects from retinal damage in different models of light-induced retinal degeneration. This defines galectin-3 as potent driver of retinal degeneration and highlight the protein as a drug target for ocular immunomodulatory therapies.

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

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Background: Over-activation of cGMP-dependent protein kinase G (PKG) is a key event in hereditary photoreceptor degeneration. Here, we identified a new PKG inhibitor, the cGMP analogue CN238, with robust protective effects on photoreceptors in retinal explants derived from *rd10* mice. We further studied the impact of this compound on retinal function by means of multi-electrode arrays (MEAs) on *rd10* retinal explants. Moreover, we used kinase activity profiling to assess which kinases and protein targets could potentially mediate PKG-dependent effects.

Methods: 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 effects of cGMP analogues were evaluated *ex vivo* by counting the number of photoreceptor rows. The PKG-inhibition dependent protection of photoreceptors was further evaluated with MEA recording on treated and non-treated (NT) *rd10* retinal explants. RGC viability was assessed on histological preparations from recorded samples via labelling with RNA-binding protein with multiple splicing (RBPMS) antibody. Kinase activity was determined on lysates of *rd10* retinal explants NT or CN238-treated using STK PamChip®. CN238-mediated regulation Kv1.6 was investigated using Ca^{2+}-imaging on *rd10* acute retinal explants exposed to either CN238, the PKG activator CN056, or Margatoxin, a potent inhibitor of Kv1.6.

Results: Analysis of the effects of PKG inhibition on the *rd10* retina showed ≈55 % and ≈46 % increased photoreceptor survival at P24 in samples treated with CN003 and CN238, respectively. The PKG inhibitor CN238 preserved the light reactivity of photoreceptors and RGCs compared to NT or CN226. The preservation of RGCs was confirmed by RBPMS staining, which showed an increase in the number of RGCs in samples treated with CN003 or CN238. Analysis of kinase activity revealed a reduction in phosphorylation of the voltage-dependent potassium channel Kv1.6 by≈45% in *rd10* retinal explants treated with CN238, suggesting Kv1.6 as a possible target for PKG. Finally, Ca^{2+}-imaging on acute *rd10* retinal explants showed a delay in RGC repolarization after exposure to either CN238 or Margatoxin, compared to those exposed to CN056 or control.

Conclusion: The compounds CN003 and CN238 showed protective effects for *rd10* photoreceptors in terms of viability and function. Furthermore, electrophysiological analysis revealed a neuroprotective effect of PKG inhibition on RGC function, despite the axotomy that characterises the retinal explant model. Moreover, the differential phosphorylation of Kv1.6 in treated and NT explants and Ca^{2+} imaging experiments hints at a possible role of Kv1.6 in the cell death pathway and an inhibitory action of CN238 on Kv1.6. Together, these findings revealed new insights on the

properties of cGMP analogues and extended their protective properties from degenerating photoreceptors to RGCs, thus significantly expanding their potential applicability for the treatment of retinal diseases.

Preclinical human model system for retinal cell replacement therapy: Human photoreceptor transplants self-integrate into human retina models

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Purpose: Cell transplantation therapy is a promising approach to restore loss of neurons and visual function in retinal diseases. While several studies have shown that mouse and human cell transplants may integrate into rodent and non-human primate retina in vivo, cell integration and restoration are not yet efficient. It remains unclear if this depends on the animal model and pathology, and if transplants even could integrate in humans. To facilitate translation, we aimed to develop a preclinical human cell therapy research system, and to test whether human transplants might integrate into human retina models.

Methods: To achieve this, we used our previously established human retinal organoid system from pluripotent stem cells. We generated 200 day old organoids with and without photoreceptor-specific CRX-mCHERRY reporter to enrich donor cells by FACS for transplantation and as host retina models, respectively. Cell transplantation was analyzed by live imaging, viral labeling, and histology.

Results: We sought to develop preclinical research approaches mimicking in vivo-like retinal cell transplantation therapy: To model intraepithelial transplantation, single photoreceptors isolated from organoids were precisely transplanted into healthy retina organoids by microinjection. To model subretinal transplantation, 30'000 photoreceptors were placed in subretinal-like contact with healthy retina organoids in a U-shaped 96-well cell culture well. Histological analyses were performed after 6 weeks. We observed survival of intraepithelial transplants. Strikingly, we frequently observed that subretinal transplants spontaneously self-integrated as large cell clusters into organoid hosts. Integrated photoreceptors were localized at the host photoreceptors layer level and in other retinal layers. Transplants expressed markers and showed characteristic morphological features indicative of integrated photoreceptors.

Conclusion: Using organoid technology, we established a human-to-human cell transplant therapy research system, and showed that photoreceptor transplants become self-integrated into human retina models. This preclinical human system might assist mechanistic studies and optimization of cell replacement therapy for all retinal cell types and diseases. Our data advance cell transplantation therapy further towards clinical translation, and sets the stage for preclinical validation studies in adult and primary human donor retina ex vivo.

Impact of drusen inducting media on co-cultured retinae

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Purpose: A clinical hallmark of age-related macular degeneration is extracellular deposit of debris, so called drusen that accumulate below the retinal pigment epithelium (RPE). In the present study, three different possible drusen induction media were applied into a co-culture model of porcine neuroretina and primary RPE (ppRPE) cells. The aim of this study was to mimic AMD pathology in vitro and to investigate whether the induction of drusen, via different conditioned media, influences the cultured neuroretina explants.

Methods: Primary RPE cells were isolated and cultured until confluency. Then potentially drusen inducing media were applied for 8 days: supplemented with BSA (0.42 % w/vol), or homogenized porcine retina (HPR), or rod outer segments (ROS). One group was co-cultivated with ppRPE without any prior drusen induction. These four groups were co-cultivated with porcine neuroretina explants for another 8 days. Neuroretina cultivated alone served as controls. H&E staining and immunohistochemistry (n = 7/group) were performed on the neuroretina samples at the end of the study to analyse cones (opsin) and rods (rhodopsin). In addition, microglia *(Iba1, iNOS),* complement factors (*CFH* and *C3*) and pro-inflammatory cytokines *(Il-6, Il-8, TNFα)* were also analysed via RT-qPCR in neuroretina samples (n = 6/group).

Results: RT-qPCR results revealed a significant downregulation of *GFAP* in all groups compared to controls ($p < 0.05$). Remarkably, a significant downregulation of *CFH* was detected in ppRPE co-culture and the HPR group in comparison to controls. Moreover, an evident upregulation of pro-inflammatory cytokines *Il-6*, and *TNFα* was observed.

Conclusion: Due to our application of possible drusen induction media into co-culture we were able to see an alteration in the expression of markers representing macroglia, the complement system, and cones. The co-cultivation of ppRPE cells and neuroretina explants supplemented with HRP and ROS, to gain drusen like debris, indicates a degenerative effect on neuroretina samples. Nevertheless, the detailed impact on neuroretina samples and RPE needs to be investigated in more detail.

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Transplantation of human retinal organoid-derived photoreceptors into the pig retina

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Purpose: Retinal diseases caused by the death of photoreceptors (PRs) represent a major cause for blindness in developed societies. Therapeutic interventions aiming to replace damaged or lossed PRs with pluripotent stem cell-derived donor cells has made significant progress in recent years. In this study, we investigated human PRs after transplantation in a large animal model, the pig, using clinical-relevant surgical procedures.

Methods: Human photoreceptors were selected by FACS from 210-day-old retinal organoids generated from a CRX-mCherry iPSC-reporter line. 50µl PR suspension were transplanted subretinally into right eyes of 10 wild-type pigs (Landschwein, age: 3 months). Intraocular access was performed under general anesthesia by pars plana vitrectomy. Two experimental groups of 5 animals each were formed and experimental eyes were removed after 2 or 8 weeks (w), respectively. Cryostat sections were used for histological processing, immunostaining and microscopic analysis.

Results: With the surgical method established here, PR suspensions could be safely introduced subretinally into the pig eye. In 9 out of 10 eyes, human PRs were detected in the subretinal space. These cells were located in the inferior part of the bleb in form of a sickle, most likely due to gravity forces. Survival of transplanted human PRs was shown by reporter (mCherry) and human-specific marker (Ku80, Arr3) expression after 2w and 8w. Overall, a cell survival of 2.75 % of injected cells after 8w was observed. Human PRs appeared as clusters or sporadically closely to the apical side of host RPE. Proliferation could not be detected. Ultra-structural analysis by electron microscopy revealed typical characteristics of photoreceptors including synapses and structures corresponding to inner segments. No increased GFAP reactivity of Müller glia or microglia activation could be detected in the transplant area. In the retina the cellular immune response showed 0.13 ∓ 0.07 $CD8^+$ cells/mm (2w) and 0.80 ∓ 0.4 $CD8^+$ cells/mm (8w) in the transplanted eyes vs. 0.12 ∓ 0.02 $CD8^+$ cells/mm(2w) and 0.20 ∓ 0.02 $CD8^+$ cells/mm (8w) in control eyes.

Conclusion: In this work, the safe application of human iPSC-derived PRs into the subretinal space of a large animal model was demonstrated. No major immune responses to the transplants were observed. This work represents a further step towards the translation of cell replacement therapies in the retina into clinical application.

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 CXCL10, CCL2, CCL3 and CCL4 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.

Identification of PARP-associated pathway as novel therapeutic targets in 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. Over-activation of poly-ADP-ribose-polymerase (PARP) has been associated with the pathogenesis of RP. PARP produces poly-ADP-ribose-polymers (PAR), which are further catabolized by poly-ADP-ribose-glycohydrolase (PARG). To date it is unknown whether the catabolism of PAR is also part of the cell death mechanism.

Methods: Here, we investigated the possible catabolism by inhibiting PARP and PARG, using the well-established experimental system of organotypic retinal explant cultures. The activity of calpain, PARP, and histone deacetylases (HDAC) was detected *in situ* on sections from post-natal day (P) 11 retinal degeneration 1 *(rd1)* mouse. Retina explants were derived at P5 from *rd1* mice and then treated from P7 to P11 with the PARP inhibitor olaparib, the PARG inhibitor JA2131, or both drugs in combination. After histological workup, the effect of different treatments on cell viability was studied via the TUNEL assay.

Results: Calpain activity *in situ* showed a significant reduction after treatment with olaparib and JA2131. JA2131 did not increase PAR, while Olaparib reduced that. Yet, the PARG inhibitor JA2131 significantly reduced the numbers of TUNEL positive cells in the outer nuclear layer of the retina and sirtuin-type HDAC activity. Remarkably, the inhibition of PARG did not appear to reduce PARP activity.

Conclusion: We found that both the PARP inhibitor, olaparib, and the PARG inhibitor, JA2131, significantly decreased calpain activity, increased cell viability, and HDAC activity. However, the PARG inhibition did not increase PAR. This indicates that 1) PAR catabolism is part of the cell death pathway, that 2) both enzymes are likely to act upstream of sirtuins-type HDACs, that 3) PARP and PARG activity together contribute to activation of calpain, and that 4) PARG is not responsible for PARP avtivity.

Fuel for vision: Evidence for a Müller glia-to-cone photoreceptor glutamine shuttle

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Background: Müller Glia (MG) are the principal glia cells of retina responsible for maintaining the health of photoreceptor (PR) along with other neurons. One of the vital functions is removing the bulk of extracellular glutamate released from photoreceptor synapses. The glutamate taken up can be synthesized to glutamine via glutamine synthase (GS), while glutaminase C (GAC) is responsible for the reverse reaction of converting glutamine back into glutamate. In addition to acting as an excitatory neurotransmitter, glutamate is also one of glucogenic amino acids which can be metabolized to support intermediary metabolism and energy production in TCA cycle. In this study, we found evidence for a glutamine-glutamate cycle between the MG and the PR in the retina of wild type mouse.

Methods: Standard immunofluorescent (IF) staining was performed on cryosections from wild type (WT) mouse retina to study the expression pattern of related key enzymes, including GS, GAC, cone arrestin (Arr3). All images were acquired and analysed by using Zeiss Zen microscope software.

Results: IF result showed expression of GS throughout the MG, with higher expression in the end feet, outer limiting membrane and the cell bodies. Co-staining of Arr3 and GAC revealed that cone PR inner segments expresses GAC.

Conclusion: The details of retinal energy metabolism are still largely unknown, yet, this study indicates that a glutamate-glutamine shuttle may exist between MG and PRs. Glutamate uptake from the surrounding by MG can be converted to glutamine by GS, then exported out of the MG. Free glutamine is imported into PR and converted to glutamate by GAC to be used as energy source. Future studies may investigate the metabolite itself using isotopic tracking, while interventional studies blocking enzymes in the glutamate-glutamine pathway may provide for a better understanding of the importance of glutamine metabolism for cone survival.

Distribution of cGMP-dependent protein kinase in the mouse retina

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Background: The cGMP-dependent protein kinase or protein kinase G (PKG) signaling is essential for numerous processes, including neuronal cell death. However, the localization of specific PKG isoforms in the mammalian retina has yet to be explored comprehensively. Our study aimed to find out the protein and mRNA distribution patterns of PKG 1α, 1β, and II in the mouse retina, focusing on PKG isoform expression in retinal ganglion cells (RGCs) and the optic nerve.

Methods: We used C3H wild-type mice at postnatal 30 days to obtain sagittal, central cryosections. In these sections, the protein expression was assessed using specific anti-PKG antibodies whose isoform-specificities had been validated in the previous studies. The mRNA expression was analyzed using RNAscope technology. The results were evaluated using fluorescence microscopy, taking negative and positive controls into account.

Results: For protein expression, PKG Iα showed high signals in the ganglion cell layer (GCL) and the inner nuclear layer (INL), while PKG Iβ and II detected no signals in the GCL. Moreover, all PKG isoforms showed strong signals on the nerve fiber layer (NFL) and inner segments (IS) of photoreceptors. Here, the signal intensity of PKG Iβ was higher than for the other isoforms. For mRNA expression, all PKG isoforms showed signals on the GCL and inner plexiform layer. However, PKG II showed much stronger signals on GCL compared to the other isoforms. Furthermore, PKG Iβ and II showed signals on INL and IS while PKG Iα detected no signals among them.

Conclusion: Overall, our data indicate that the protein expression of PKG Iα, Iβ, II in mouse retina partially corresponds to the respective mRNA expression. Furthermore, PKG II may be more strongly expressed in RGCs, implying that PKG II could potentially play a role in neurodegenerative diseases affecting RGCs or the optic nerve. Further validation for the protein and mRNA distribution needs to be performed using co-localization with specific RGCs marker on the whole-mount retina, perhaps also including models for RGC degeneration or optic nerve atrophy.

Keywords: PKG, retina, retina ganglion cell, inner segment, immunofluorescence, RNAscope

cGMP analogues as the potential neuroprotective compounds in autosomal dominant retinitis pigmentosa

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Purpose: About 30% of non-syndromic retinitis pigmentosa (RP) cases are considered to have an autosomal dominant RP (adRP) inheritance pattern. The *Rho*^{Ile256del} mouse is a new, human homologous mouse model for adRP. Previously, different mouse and rat models for RP, carrying mutations in the *Rho* gene had shown an excessive accumulation of cyclic guanosine-3',5'-monophosphate (cGMP) in photoreceptors (Arango-Gonzalez *et al.*, PLoS One, 9:e112142, 2014). Moreover, inhibitory cGMP analogues targeting protein kinase G (PKG) have shown strong, functional neuroprotection in mouse models for autosomal recessive RP (arRP), including in *Pde6b*- and *Prph2*-mutant *rd1, rd10,* and *rd2* mice (Vighi et al., PNAS, 115:E2997-E3006, 2018). Here, we wanted to explore the efficacy of PKG-targeting cGMP analogues in the *Rho*^{Ile256del} mouse model for adRP.

Methods: An immunostaining for cGMP was performed in mutant and wild-type (WT) retina. The cGMP analogues DF003 and DF238 were used for the treatment of organotypic retinal explants derived from *Rho*^{Ile256del} adRP mice, as well as from WT mice. Retinas were cultured from postnatal 12 day (P12) until either P18, P20, or P24. cGMP analogues were added to the culture medium every second day, with non-treated (NT) retinal explants serving as controls. At the end of the culture period, the retinas were fixed in 4% PFA and cryosectioned. Photoreceptor cell survival was evaluated by quantifying cell rows in the outer nuclear layer (ONL) and cell death detection using the TUNEL assay.

Results: The in *Rho*^{I256del} retina, the cGMP immunostaining showed labeling of photoreceptor segments not seen in WT counterparts. The rows of photoreceptors in the ONL continually declined from P18 to P24 for NT and DF003-treated groups. For DF003 treatment, the percentage of TUNEL positive cells decreased significantly at P18 and P20, even though the photoreceptor row count at P18 and P20 remained similar to NT. At P24, the photoreceptor row count in DF003 treated samples showed a significant increase compared to NT. Compared to DF003, the compound DF238 reduced TUNEL positive cells in the ONL more substantially and demonstrated a marked neuroprotective effect already at P20.

Conclusion: cGMP analogues showed a neuroprotective effect and reduced photoreceptor cell death. The lead compound DF003 confirmed its protective effects seen earlier in other RP models also in the *Rho*^{I256del} adRP mouse model. At the same time, DF238 appears to be more efficient at protecting photoreceptors. Overall, our data strongly suggest that cGMP-dependent cell death pathways are also at play in models for adRP.

Keywords: autosomal dominant retinitis pigmentosa, neuroprotection, pharmacological agents, in vitro studies

Forward programming of photoreceptors from human induced pluripotent stem cells

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One of the major causes of adult visual impairment and blindness is the progressive dysfunction and death of photoreceptors caused by retinal degenerative diseases. The biggest bottleneck for testing the transplantation of human photoreceptors, which is currently extensively tested as a treatment option for late stages of retinal degeneration, is the lack of cell material in high quantity and quality.

To establish a quick and efficient protocol for photoreceptor differentiation, we aim to use transcription factor (TF) overexpression in human induced pluripotent stem cells (hiPSCs). We have developed a TF screening platform based on a photoreceptor-reporter hiPSC line. It allows combinatorial screening of more than 1500 TFs and downstream single cell sequencing analysis. Such screening experiments identified three factors: OTX2, NEUROD1 and GON4L, that differentiate hiPSCs into up to 50% cone photoreceptor precursor cells in only 10 days. While OTX2 and NEUROD1 are known players in photoreceptor development, the GON4L was never before associated with retinal development indicating that *in vitro* differentiation may be uncoupled from *in vivo* programs. The engineered cone precursor cells were morphologically characterized and showed positive for photoreceptor-specific markers, which made them suitable for coming transplantation studies. Furthermore, co-cultures with human retinal pigment epithelium cells significantly increased photoreceptor differentiation.

This protocol will facilitate fast, efficient and unlimited production of human photoreceptors *in vitro*. The inducible photoreceptors may serve as essential human testbeds for drug screenings, basic and biomedical research as well as for photoreceptor replacement therapies.

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