Mitophagy is a lysosome-mediated autophagic process to cleanse dysfunctional mitochondria thus critical for cell survival. The sigma-1 receptor (S1R) is a unique endoplasmic reticulum protein involved in cellular stress responses. Its regulations in mitophagy remain largely unknown. We hypothesize that S1R plays an essential role in mitophagy and lysosomal organelle fusion.

**Methods:** Retinal explants were isolated from wild type (WT) and S1R knockout (KO) mice. Permanent S1R KO and induced S1R knockdown (KD) were generated using the CRISPR-Cas9 technology in neuronal cell lines. Mitophagy was induced with CCCP, a mitochondrial uncoupler, and detected as decrease of mitochondrial membrane potential (V chase and TIM23) by Western blotting. Autophagic lysosomal organelle fusion was assessed via fluorescent microscopy of cells transfected with a pH-sensitive LC3-II-GFP-RFP construct or organelle markers. S1R protein interactions were studied with co-immunoprecipitation (co-IP) assay.

**Results:** Compared to WT control, S1R KO impaired mitophagy in retinal explants and NSC34 cells (permanent S1R KO) and SH-SY5Y cells (doxycycline-induced KD), but did not affect parkin recruitment to mitochondria, an early event of mitophagy. Re-expressing S1R in the NSC34 cells with a S1R KO background rescued mitophagy. The deficiency of S1R in these tissue or cell samples also caused accumulation of LC3II and SQSTM1 (autophagosome markers), but did not alter the levels of Beclin 1 or ATG7, proteins involved in autophagosome biogenesis. Lysosome pH and protease activities were not negatively affected by S1R KO. However, co-localization of the label of lysosomes with that of autophagosomes or mitochondria was disrupted, in S1R KO NSC34 cells in contrast to WT control, supporting an important role of S1R in lysosomal organelle fusion.

**Conclusions:** S1R plays an important role in CCCP-induced mitophagy. Its molecular function in this context can be narrowed down to autophagosome/lysosome fusion, likely through its interactions with the protein complex essential for this autophagic organelle fusion.
Conclusions: Our findings indicate that dysregulation of mitochondrial fission and fusion affect multiple cellular metabolic pathways. Since NAD⁺ is required for both nuclear DNA damage repair and mitochondrial signaling, depletion of NAD⁺ suggests increased usage of NAD⁺ from the nuclear DNA repair and/or mitochondrial signaling. Alternatively, it may be due to decreased NAD⁺ synthesis.

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Program Number: 1605
Presentation Time: 11:30 AM–11:45 AM
Pink1 deficiency induces Nrf2 dependent EMT and metabolic dysfunction in RPE cells
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Purpose: Defects in anti-oxidative response and mitochondrial functioning have been implicated in the development of AMD, but the interplay of these two pathways in energy demanding RPE cells has not been examined. Here we sought to look at the effect of two important genes –Nrf2 transcription factor, master regulator of the anti-oxidant pathway and Pink1 kinase, initiator of mitophagy, on human RPE structure and function.

Methods: ARPE19 cells were treated with various siRNA (scramble, Nrf2, Pink1, or both Nrf2-Pink1) and stressors and grown for 5 days. Cell morphology was photographed and cells were harvested for RNA and protein that were used for RT-qPCR and Western analysis, respectively. Phenotypic readouts like Mitoxos superoxide, TMRM mitochondrial membrane potential (ΔΨm), ATP and lactate levels were measured and Seahorse mito-stress test was performed using the XF96 analyzer.

Results: Mitotracker-lsotracker assays showed Pink1 knockdown(KD) impaired mitophagy. Cells with Pink1KD or cells treated with EGF started elongating on day 4 whereas the cells with scramble, Nrf2 or Nrf2-Pink1 double KD did not change shape. Both Nrf2KD and treatment with Nrf2 inhibitor Trigonelline prevented cell elongation induced by Pink1KD. This change correlated with a two-fold increase in Twist1 and Zeb1 gene expression and marked Vimentin immuno-staining, suggesting that these morphologic changes are due to epithelial-mesenchymal transition EMT (Fig1). The Pink1KD cells exhibited increased mitochondrial superoxide and lactate levels, decreased (ΔΨm) and ATP production - all of which were ameliorated by Nrf2KD. Seahorse data revealed a 50% decrease in reserve respiratory capacity with Pink1KD which was partially rescued by Nrf2KD (Fig2). Among mitochondrial biogenesis associated genes, PGC1α gene expression showed strong correlation with induction and rescue of the EMT phenotype.

Conclusions: Pink1 deficiency impaired mitochondrial respiration necessitating a switch to glycolysis and induced EMT in RPE cells. Paradoxically, Nrf2 signaling appears necessary for EMT. RPE of AMD patients have been shown to undergo EMT changes and have mitochondrial dysfunction. This study shows direct induction of EMT by Pink1KD in an Nrf2 dependent manner and provides evidence of an interplay of oxidative stress and mitophagy pathways in controlling cell morphology and energy metabolism which can be used as novel AMD therapeutic targets.

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5 days, starting at P10, and were then analyzed for expression of the oxidative stress genes in the RPE.

**Results:** Expression levels of all three types of the TH receptors (Thra1, Thrb1, and Thrb2) in the RPE were 3-5 folds higher than that in retinas. The RPE in mice treated with NaIO, showed severe necrosis/apoptosis and enhanced expression of the oxidative stress genes, compared with vehicle-treated controls. Treatment with antithyroid drug significantly improved RPE morphology, reduced cell loss, and reduced expression of the oxidative stress genes in mice that have been treated with NaIO. In addition, treatment with T3 significantly increased expression levels of the oxidative stress genes in the RPE.

**Conclusions:** This work shows that suppressing TH signaling with anti-thyroid drug improves RPE morphology and survival in mice challenged with oxidative stress. Our findings are in line with the clinical findings, and suggest that suppressing TH signaling may represent a strategy for RPE protection in AMD.

**Commercial Relationships:** Hongwei Ma, Fan Yang, None; Michael Butler, None; Xi-Qin Ding, None

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**Program Number:** 1607

**Presentation Time:** 12:00 PM–12:15 PM

Amyloid precursor protein synthesis, processing, and secretion in retinal pigmented epithelial cells: Effects of hypoxia

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**Purpose:** Amyloid precursor protein (APP) is a ubiquitously expressed protein that is metabolized to amyloid beta (present in drusen in AMD and in plaques in Alzheimer disease brains) and soluble APP alpha and beta (sAPPα and β). We found that hypoxia dramatically decreased APP expression and secretion in retinal pigmented epithelial cells (RPE). Retromer, a multimeric protein complex that transports APP to the cell surface for secretion, was also decreased by hypoxia. In the current study we examined the mechanisms by which hypoxia affects APP levels and secretion by inducing ER stress and silencing retromer.

**Methods:** RPE were isolated from canine eyes and grown to confluence. Cells were placed under hypoxic (0.5% O2) and normoxic (21% O2) conditions for 2 h (for 35S labeling) or 48 h. Cells were treated with either 1µM tunicamycin or 2.5mM 4-PBA (to induce or inhibit ER stress respectively) or siRNA to knock down retromer. Lysates and cell-conditioned media (CCM) were collected and immunoblotted for APP, sAPPα, and sAPPβ.

**Results:** As we previously reported, hypoxia significantly decreased APP cellular levels and secretion by 60% in RPE cells. Importantly, inducing ER stress with tunicamycin caused a 77% decrease in APP in RPE lysates and the ER stress inhibitor 4-PBA decreased hypoxia’s effect on APP levels. Previous data showed that with retromer knockdown, APP secretion was significantly decreased in normoxia and hypoxia. In this study retromer knockdown specifically decreased sAPPβ secretion by 57% in normoxia and hypoxia, but had no effect on sAPPα secretion. Preliminary studies with 35S metabolic labeling indicate that hypoxia does not affect APP synthesis.

**Conclusions:** Hypoxia alters APP expression and secretion in RPE cells. However, preliminary studies indicate that hypoxia may not affect the synthesis of APP. In RPE, ER stress decreased expression of APP by 77%, similar to that seen in hypoxia. This may be the mechanism by which hypoxia caused APP levels to decrease because ER stress inhibition blocked this effect. Interestingly, when retromer was knocked down, sAPPβ but not sAPPα secretion was dramatically lowered. Since formation of sAPPα and β follow different pathways, retromer likely is involved only in sAPPβ processing. Understanding how hypoxia affects APP processing and secretion is vital to our knowledge of RPE pathophysiology.

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**Program Number:** 1608

**Presentation Time:** 12:15 PM–12:30 PM

Chloroquine-induced Retinal Toxicity In Vitro and In Vivo

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**Purpose:** To develop and characterize a murine model of chloroquine-induced retinal toxicity and to determine the mechanisms of chloroquine toxicity

**Methods:** One intravitreal injection of chloroquine (2 mg/kg/2 ul) was administered into adult mice. After one week effects were evaluated by fundus photography, OCT, infrared reflectance, autofluorescence, ERG and by light and electron microscopy. Levels of cell death pathway and anti-oxidant genes in RPE and neurosensory retina were examined by quantitative RT-PCR. ARPE-19 and 661W cells were treated with a dose range of chloroquine (10-500 uM) for 24 hr and cell death was measured by LDH release. The role of lysosomal enzymes, cathepsins and cell death mediators was examined using specific cell permeable cathepsin, caspase, and RIP1 kinase inhibitors.

**Results:** Fundus and infrared reflectance revealed areas of retinal atrophy and subretinal debris. OCT demonstrated disruption of the RPE and ellipsoid layers. Pinpoint autofluorescence was evident in affected areas. ERG exhibited a decreased scotopic response in both the A and B-waves. Histopathology showed loss of the RPE and disrupted outer segments, OPL and photoreceptors. Phalloidin staining of RPE flat mounts confirmed patches of RPE cell loss and cell enlargement. RPE mRNA levels of BAX, NLRP2, NLRP3, ASC, and cathepsins were significantly upregulated (2-4 fold), suggesting the activation of caspase-dependent cell death pathways. Levels of BAX, RIP3, and GPX4 mRNA were increased primarily within the neurosensory retina by 2-4 fold and 10-fold, respectively, pointing to apoptosis, necroptosis and oxidative stress-mediated photoreceptor cell death. Chloroquine induced cell death in ARPE-19 and 661W cells in a dose-dependent manner; 500 uM chloroquine led death of 30-40% ARPE-19 cells and 40-50% 661W cells at 24 hr. In ARPE-19 cells, inhibition of caspase-3, 8 and 9 (mediators of apoptosis) led to a 50, 60 and 30% decrease in cell death, respectively, whereas caspase-1 (a mediator of pyroptosis) inhibition resulted in a 30% decrease in cell death. Inhibition of RIP1 kinase did not significantly decrease cell death.

**Conclusions:** Phenotypic and functional retinal changes induced by chloroquine in the mouse are analogous to those observed in human with chloroquine or hydroxychloroquine retinal toxicity. Chloroquine induces cell death by apoptosis and pyroptosis but does not involve necroptosis, making it distinct from the mechanism of tamoxifen.

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