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PRINCIPAL INVESTIGATOR: Luis Pérez de Sevilla Müller

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Luis Perez de Sevilla
Anna Matynia and Nicholas Brecha
E-Mail:luisperez@mednet.ucla.edu

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14. ABSTRACT
The retina has gained significant attention as a potential early biomarker for diagnosing Alzheimer's Disease (AD) and monitoring disease progression. Our study aims to investigate retinal degeneration in two AD mouse models (3xtg and APPSwe) and establish correlations between retinal changes and various functional assessments, including electroretinography (ERG), optical coherence tomography (OCT), pupillary light reflex (PLR), and light aversion. The study utilizes two AD mouse lines, which mimic the pathological features observed in postmortem human AD retinas, to examine the degenerative processes occurring in the retina. By evaluating retinal degeneration in these mouse models, we aim to identify potential early biomarkers that can aid in the diagnosis and prognosis of AD.

15. SUBJECT TERMS
None listed.

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INTRODUCTION:

The retina has gained significant attention as a potential early biomarker for diagnosing Alzheimer's Disease (AD) and monitoring disease progression. Our study aims to investigate retinal degeneration in two AD mouse models (3xtg and APPSwe) and establish correlations between retinal changes and various functional assessments, including electroretinography (ERG), optical coherence tomography (OCT), pupillary light reflex (PLR), and light aversion. The study utilizes two AD mouse lines, which mimic the pathological features observed in postmortem human AD retinas, to examine the degenerative processes occurring in the retina. By evaluating retinal degeneration in these mouse models, we aim to identify potential early biomarkers that can aid in the diagnosis and prognosis of AD.

KEYWORDS: retina, Alzheimer's Disease, behavior, biomarkers, retinal degeneration

ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1: Onset and temporal progression of retinal degeneration in the cone and rod (PR) pathway and ipRGC neuronal circuits in two AD mouse models and AD postmortem human retinas. We will test the earliest visual symptoms and progression of the disease in the retina. We will determine A) the progression of degeneration by loss of neurons in all retinal layers in vertical sections using immunohistochemistry; B) the onset and time course of retina-brain disconnection by RGC degeneration, based on somal size and dendritic structure, and the correlation of Aβ deposition within RGCs with RGC degeneration6 in whole-mount retinas using specific markers; C) altered retinal perfusion by vascular structural abnormalities in retinal layers in whole-mount retinas using dye labeling; D) disease onset as indicated by inflammation with markers in retinal vertical sections and cortex; and E) progression and disruption of energetics by mitochondrial dysfunction with high-resolution respirometry (HRR) assays.

Subtask 1 To determine the progression of degeneration by loss of neurons in all retinal layers in vertical sections using immunohistochemistry. **60% of completion.**

Subtask 2: To determine the onset and time course of retina-brain disconnection by RGC degeneration, based on somal size and dendritic structure, and the correlation of Aβ deposition within RGCs with RGC degeneration in whole-mount retinas using specific markers. **40% of completion.**

Subtask 3: Altered retinal perfusion by vascular structural abnormalities in retinal layers in whole-mount retinas using dye labeling. **0% of completion. These experiments will start during the second year.**

Subtask 4. Disease onset as indicated by inflammation with markers in retinal vertical sections and cortex. **45% of completion.**

Subtask 5. Progression and disruption of energetics by mitochondrial dysfunction with HRR assays. **0% of completion. These experiments will start during the second year.**

Aim 2: Visual functional changes originate from the PR and ipRGC pathways. We will test the correlation of anatomical degeneration with key visual behaviors associated with the progression of AD. Our investigations will determine A) anatomical and functional correlations by in vivo optical coherence tomography (OCT) and electroretinogram (ERG) recordings that estimate retinal bioelectrical function; and B)

AD-related dysfunction in visual behaviors by specifically assessing i) PR and ipRGC specific pathways using the optokinetic response to quantify contrast and visual acuity using metameric paired light stimulation and C) ipRGCs specific pathways using i) circadian rhythms, ii) PLR and iii) light perception/aversion.

Subtask 1: anatomical and functional correlations by in vivo OCT and ERG recordings that estimate retinal bioelectrical function. Expectation: to complete testing of control mice at all ages and AD mouse lines at 6 and 8 month old. **85% of completion.**

Subtask 2: AD-related dysfunction in visual behaviors by specifically assessing i) PR and ipRGC specific pathways using the optokinetic response to quantify contrast and visual acuity using metameric paired light stimulation. Expectation: to complete testing of control mice at all ages and AD mouse lines at 6 and 8 month old. **95% of completion.**

Subtask 3. ipRGCs specific pathways using i) circadian rhythms, ii) PLR and iii) light perception/aversion. Expectation: to complete testing of control mice at all ages and AD mouse lines at 6 and 8 month old for PLR and light perception/aversion. **75% of completion.**

What was accomplished under these goals?

Major Activities:

The major activities in Year 1 for Specific Aim 2 were focused on functional analyses of aging controls and Alzheimer's Disease (AD) model mice, data analysis and preparation of a manuscript.

Specific Objectives:

Specific Objectives in Year 1 for Specific Aim 2 were a) anatomical and functional correlations by in vivo OCT (optical coherence tomography) and ERG (electroretinography) recordings that estimate retinal bioelectrical function, b) AD-related dysfunction in visual behaviors by specifically assessing i) PR and ipRGC specific pathways using the optokinetic response to quantify contrast and visual acuity using metameric paired light stimulation, and c) ipRGCs specific pathways using i) circadian rhythms, ii) PLR and iii) light perception/aversion.

Significant results or key outcomes:

Aim 1:

A. Control mice:

Retinal cell types: We have evaluated the aging effects on retinal thickness, photoreceptors, retinal ganglion cells (RGCs), bipolar cells, and synaptic markers in the outer plexiform layer (OPL) and inner plexiform layer (IPL) of wild-type retinas of males and females. The data obtained from this study will serve as a basis for comparison with retinal neurons/synaptic marker densities and retinal thickness in retinas of AD mice.

1. The analysis of retinal thickness based on DAPI staining has been completed. Preliminary results indicate no variations in retinal thickness between 6 and 12 months of age. Further statistical analysis and data interpretation are underway.
2. The analysis of photoreceptors labeled with PNA has been completed. Morphological changes and distribution patterns of photoreceptors in relation to aging are being assessed.
3. The staining of wholemount retinas with RBPMS to quantify RGC populations is ongoing. Once completed, the collected data will contribute to understanding the effect of RGC death in AD retinas.
4. The identification and assessment of bipolar cells using PKC staining have been successfully performed. Preliminary results indicate no variations in PKC density between 6 and 12 months of age.
5. The staining of synaptic markers in the OPL and IPL using ctbp2 is in progress.

Melanopsin circuitry: We have evaluated the function and anatomy of intrinsically photosensitive retinal ganglion cells (ipRGCs) in wild-type retinas of males and females at 6- and 12-months-old. The primary objective of this study was to understand the effect of aging on M1 melanopsin morphology and ipRGC function before comparing it to the melanopsin circuitry in AD. Our investigation revealed a preservation of function and morphology of ipRGCs in aging mice in males (N=5 mice) and females retinas (N=7 mice). Behavioral tests, including pupillary light reflex, light aversion, visual acuity, and contrast sensitivity, showed stable responses with aging. Immunohistochemistry analysis of retinal wholemounts with melanopsin antibodies confirmed the stability of M1 ipRGC morphology in the ganglion cell layer (GCL) and inner nuclear layer (INL). These findings were presented at a poster at The Association for Research in Vision and Ophthalmology (ARVO) in New Orleans on April 23rd.

Publication:

Based on our findings with ipRGCs, we are preparing a research paper entitled "Preservation of Intrinsically Photosensitive Retinal Ganglion Cells (ipRGCs) in Aging: Implications as a Potential Biomarker for Ocular Degenerative Diseases." to be submitted to Investigative Ophthalmology & Visual Science (IOVS) journal. The paper highlights the age-related changes in ipRGC morphology and associated behavioral functions in mice, emphasizing their potential as a biomarker for ocular degenerative diseases, including AD, PD, glaucoma, and diabetes.

B. AD mouse lines:

Retinas from 3xtg AD mouse model at 6 and 8 months old, both males and females have been stained with RBPMS and melanopsin antibodies to analyze RGCs in wholemount retinas. Our ongoing reconstruction of ipRGCs aims to characterize their morphology and connectivity within the retina and compare it to our controls. Analysis is expected to be complete by the end of September. Additionally, we are in the process of quantifying the RGC population labeled with RBPMS to assess RGC death. Analysis is expected to be complete by the end of September.

Furthermore, we have labeled the vertical sections of AD retinas with DAPI, PNA, ctbp2, and PKC to assess retinal thickness, photoreceptors density, synaptic markers, and bipolar cells density, respectively. These markers will provide insights into potential changes in retinal structure, synaptic connectivity, and cell-specific alterations associated with AD. Ongoing analysis of these markers is underway, and we expect to obtain valuable data on retinal function and connectivity by the end of August.

To investigate inflammation-related markers, we have conducted staining of retinas and brain sections, including the cortex, hippocampus, and cerebellum, with iba-1 and GFAP antibodies. This ongoing analysis aims to evaluate the presence and extent of microglial activation (iba-1) and astrocyte reactivity (GFAP) in the studied brain regions and retinas, indicating potential inflammatory processes associated with AD. Analysis is expected to be complete by the end of September.

Future analysis and data integration will involve conducting statistical analyses to determine significant differences, correlations, and potential sex-specific effects. We will continue to explore the relationship between retinal changes and AD progression (ages 10 and 12), contributing to the development of early diagnostic tools and potential therapeutic targets.

We currently do not have available anatomical studies for the APPSwe mouse line. The high mortality rate observed among the mutants has limited our ability to conduct detailed anatomical analyses of retinal

structures in this specific model. Therefore, our focus with the APPSwe mouse line primarily revolves around behavioral studies to gain insights into the functional aspects of retinal degeneration in relation to AD.

C. Postmortem human retinas:

We have successfully received a postmortem female human retina (66 years old) for our research study from the Georgia Eye Bank. The human retina has been appropriately fixed in 4% paraformaldehyde and prepared by cutting it with a cryostat, ensuring the preservation of tissue integrity and cellular morphology.
Our research objective involves performing immunohistochemistry on the human retina to investigate and compare the observed retinal degeneration in our established AD mouse models to that observed in human retinas. By comparing the findings from our mouse models with the immunohistochemical analysis of the human retina, we aim to gain valuable insights into the similarities and differences in retinal degeneration between these two systems. This comparative approach will provide a comprehensive understanding of the underlying mechanisms involved in retinal degeneration and contribute to the translational relevance of our research. This study is ongoing.

Aim 2

A. Control mice:

Analyses were commenced on control animals while AD mouse models were bred and aged. We determined that between the ages of 6 to 12 months, control mice did not exhibit significant loss of ipRGC function for light aversion or the pupillary light reflex (PLR) of in visual acuity or contrast sensitivity. For light aversion in OPN4$^{+/+}$ mice (Figure 1A), there was an overall increase in light aversion regardless of dilation with atropine (main effect of age ($p=0.03$, $F(1,166)=5.02$) and an increase in light aversion dependent on atropine dilation (main effect of eye drops ($p<0.0001$, $F(1,166)=24.68$), but no interaction ($p=0.50$, $F(1,166)=0.45$). No effect of sex was observed (Figure 1B). In addition, we analyzed data from mice lacking ipRGCs as the standard for ipRGC function. For light aversion in the OPN4$^{dta/dta}$ mice (Figures 1C), there was an increase in light aversion with age in undilated mice (main effect of age ($p=0.01$, $F(1,76)=6.38$), but no effect of dilation ($p=0.32$, $F(1,76)=1.01$) and no interaction ($p=0.13$, $F(1,76)=2.39$). No effect of sex was observed. For pupillometry in OPN4$^{+/+}$ mice (Figure 1D), there was a decreased pupil constriction in mutant mice compared to wild type controls (main effect of genotype ($p<0.00001$, $F(1,90)=84.58$), but not of age ($p=0.91$, $F(1,24)=0.011$) and no interaction ($p=0.76$, $F(1,24)=0.10$). No effect of sex was observed (Figure 1E).

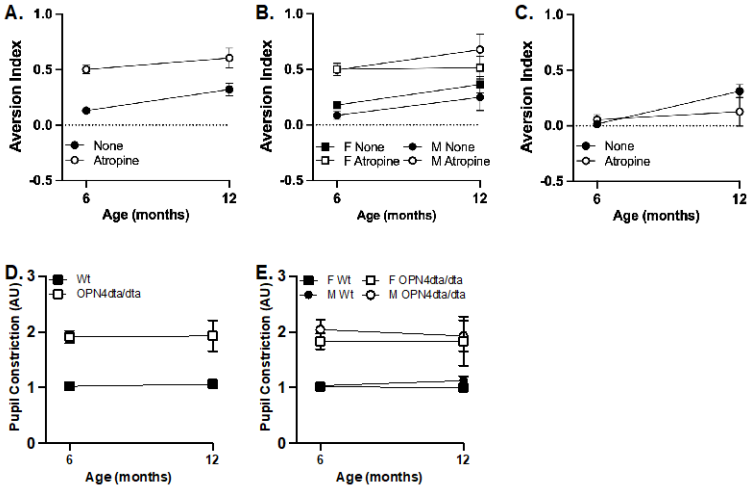

Figure 1. Light aversion and PLR in aging mice. Light aversion was tested at 1000 lux with no or with atropine eye drops in 6- and 12-month-old mice. An aversion index of 0 = no light aversion, whereas 1 = complete light aversion. A) wild type mice, B) wild type mice by sex, C) mice lacking melanopsin-expressing neurons. Pupil constriction was measured using a scale with 1 = pinpoint constriction and 3 = fully dilated. Pupillary light reflex in 6- and 12-month-old mice using a slit lamp. D) wild type and mice lacking melanopsin-expressing neurons, and E) by sex.

For visual acuity and contrast sensitivity, the optomotor response which measures visual tracking of moving stripes with varying width and contrast was used. In OPN4$^{+/+}$ mice, there was no effect of age or sex between 6 and 12 months for visual acuity (spatial resolution, Figure 2A-B) or contrast sensitivity (Figure 2C-D).

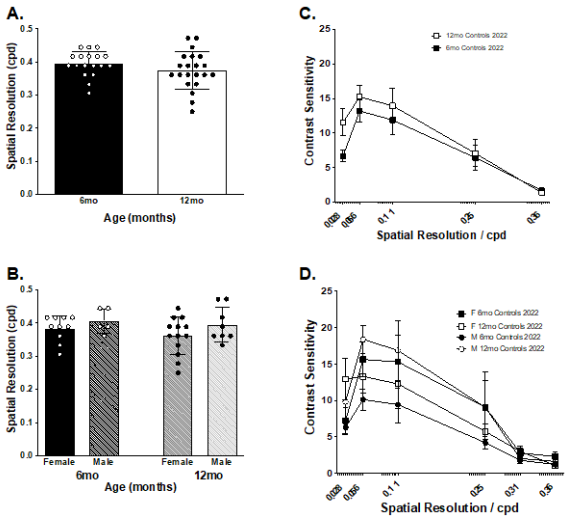

Figure 2. Visual acuity and contrast sensitivity in aging mice. (A) Spatial resolution in 6- and 12-month-old mice. (B) Spatial resolution in 6- and 12-month-old mice by sex. (C) Contrast sensitivity function in 6- and 12-month-old mice. (D) Contrast sensitivity function in 6- and 12-month-old mice by sex. No significant differences were found in visual acuity with age or sex using a student's t-test. There was a significant main effect of contrast ($p>0.0001$, $F(5,136)=23.3$), but no main effect of age ($p=0.10$, $F(1,136)=2.7$), and no interaction ($p=0.72$, $F(5,136)=0.57$).

Retinal structure remains stable whereas retinal function may decrease in control mice aged up to one year. Wild type C57Bl/6J mice were assessed at 6, 8, 10 and 12 months old by ERG and sd-OCT (Figure 3, n=9-11 per test per age). Scotopic *b*-waves maximum amplitudes are not statistically different however, there is a trend towards reduced amplitudes (Figure 3A). By contrast, retinal structure thickness did not change over this age range (Figure 3B).

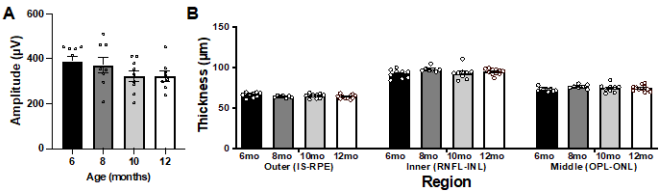

Figure 3. Retinal function and structure remain stable up to year old. A) Scotopic *b*-waves. B) retinal thickness (inner segment-retinal pigment epithelium; retinal nerve fiber layer-inner nuclear layer, outer plexiform layer-outer nuclear layer.

B. AD mouse lines:

Initial experiments in the 3xTG and APPSwe mouse lines include analyses of light aversion, PLR, visual acuity, contrast sensitivity, ERG and OCT of mice but with small sized groups at multiple ages. For light aversion, initial studies indicate a decrease in light aversion in mice without pupil dilation in the 3xTG line (Figure 4A). These mutants exhibit high levels of light aversion at 6mo (n=8), lower light aversion at 8mo (n=7) and significantly reduced light aversion at 10mo (n=3). The remaining 10mo mice will be tested within the next month to increase the n to 7, at which time a statistical analysis will be performed to ensure will ensure robustness and rigor. The approach of testing multiple smaller groups of mice not only guards against temporal conditions that may influence outcomes but is also required due to the experimental design and breeding. No effect was observed for visual acuity or contrast sensitivity in the 3xTG line. Analyses of PLR, ERG and OCT are ongoing. Fewer APPSwe mice survive, therefore, conclusions cannot be made for ongoing experiments.

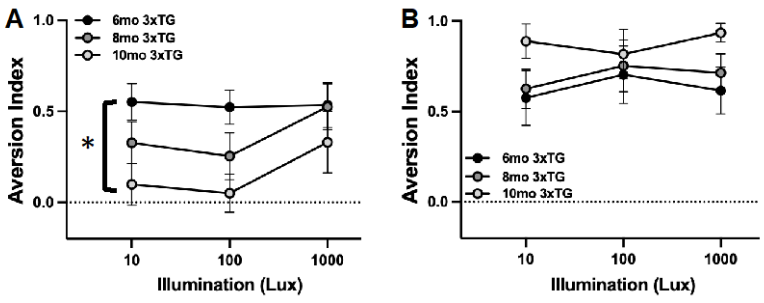

Figure 4. AD mice exhibit changes in light aversion with age. Light aversion was tested at 1000 lux with no or with atropine eye drops A) 3xTG AD mice exhibit age-dependent changes in light aversion ($F(2,49)=5.6$, $p=0.007$). B) 3xTG AD mice exhibit no age-dependent changes in light aversion with atropine dilation.

Other achievements:

Technological advances have been made in pupil measurements. Previously, a semi-quantitative manual assessment using a slit lamp was used, which only assessed the maximal constriction. A high-speed imaging system in semi-restrained mice was developed that allows pupil measurement prior to light presentation (dark adapted baseline), light onset (constriction velocity), maintained light (maximal constriction) and after lights off (PIPR, post-illumination pupillary response). Constriction is dependent on both ipRGCs and rod and cone photoreceptors, whereas the PIPR is dependent on ipRGCs only.

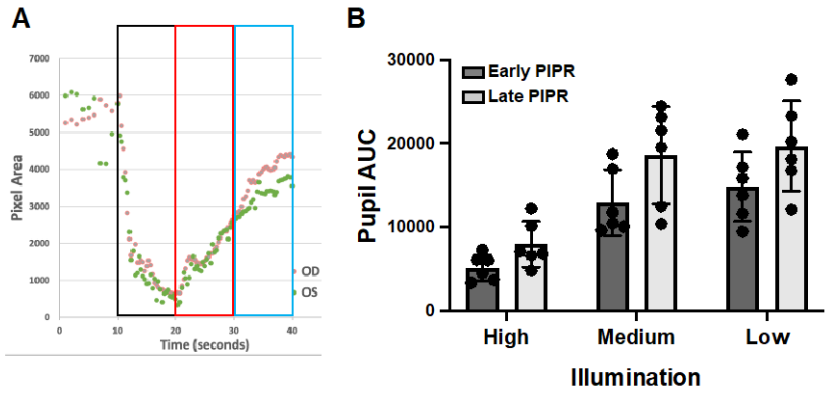

Figure 5. The PLR in mice. A) High speed video imaging captures pupil constriction. Black box indicates lights on, red box indicates early PIPR and blue box indicates late PIPR. B) Quantitation of the early and late PIPR in C57Bl/6J mice.

Stated Goals not met:

The original proposal included visual acuity and contrast sensitivity measurements with metameric pairs to assess the role of ipRGCs. However, loss of melanopsin does not affect visual acuity but does results in decreased contrast sensitivity but loss of rhodopsin or cone opsin affects both visual acuity and contrast sensitivity. Therefore, a more straightforward approach based on this differential effect will be used to assess photoreceptor versus ipRGC involvement. Additional goals not yet met are circadian photoentrainment studies. As this requires a large group of mice that are unable to participate in any other studies, this will be performed in Year 2 of the grant, focused on time points most likely to show deficits.

What opportunities for training and professional development has the project provided?

For this proposal, I am mentoring a volunteer, Brandy Recio, from Sinaloa, Mexico. Brandy has been trained to use advanced immunohistochemistry techniques, confocal microscopy, and analysis software to study the neurodegeneration in the retina during Alzheimer's Disease. She is now a part-time SRA 1 in the lab.

I am also mentoring Amirreza Toloi. He has been a dedicated volunteer for the past two months (+100 hours). Amirreza is actively involved in our research analyzing data of retinas affected by Alzheimer's disease. Amirreza's research includes confocal microscopy, conducting DAPI analysis, and neuron reconstruction. Furthermore, he is extensively working with RBPMS analysis, a vital marker for retinal ganglion cells.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

One ongoing area of our investigation involves the completion of the RBPMS staining analysis in control retinas and AD retinas. This staining technique allows us to specifically label and assess RGCs in wholemounts. Through quantitative analysis, we aim to quantify the RGC population in control animals and compare them to the retinas of the 3xtg at 6 and 8 months of age.
Additionally, we are in the final stages of the ctbp2 staining analysis in control and AD retinas at 6 and 8 months old. This analysis focuses on evaluating synaptic markers in the OPL and IPL of the retina. We aim to conclude this analysis to assess potential alterations in synaptic markers with aging and compare them between control and AD groups.

To gain a comprehensive understanding of our data and draw meaningful conclusions, we will integrate all the obtained data from the analysis of retinal thickness, photoreceptors (labeled with PNA), RGCs (assessed through RBPMS staining), bipolar cells (labeled using PKC), and synaptic markers (evaluated with ctbp2 staining). By performing comprehensive statistical analysis, we aim to identify significant differences and correlations between the measured parameters and aging.

Our future experiments also involve 10- and 12-month-old animals of the 3xtg mouse model late August. However, due to the high mortality rate in the APPSwe mutant mice, data collection is limited to animals up to the age of 8 months old.

To comprehensively evaluate retinal function and responses that could be used as a potential method of diagnosis, we are employing various assessments, including OCT, ERG, PLR, and light aversion at 6, 8, 10 and 12 months old. These assessments allow us to compare retinal function and responses between control retinas and those affected by AD. By elucidating the functional changes in the retina associated with AD, we aim to contribute to the development of diagnostic and therapeutic strategies. We expect to have these studies complete by the end of the year. Analysis will be complete in early 2024.

Through these ongoing and future experiments, we strive to advance our understanding of retinal changes associated with aging and their potential relevance to AD. By uncovering functional alterations, as well as potential biomarkers, we hope to make significant contributions to the field and pave the way for improved diagnostic and therapeutic approaches for AD.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Our study on the function and anatomy of intrinsically photosensitive retinal ganglion cells (ipRGCs) in aging mice reveals a preservation of ipRGC function and morphology in both males and females. These findings have significant implications as ipRGCs could serve as a potential biomarker for ocular degenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), glaucoma, and diabetes. Our research, presented at The Association for Research in Vision and Ophthalmology (ARVO), highlights the stability of ipRGCs and their potential utility in early detection and monitoring of these diseases. We are preparing a research paper for submission to Investigative Ophthalmology & Visual Science (IOVS) journal, which will further emphasize the age-related changes in ipRGCs and their importance in ocular degenerative disease research of additional biomarkers, and the development of innovative diagnostic and therapeutic approaches.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS:

1. Changes in approach and reasons for change

Nothing to report.

2. Actual or anticipated problems or delays and actions or plans to resolve them

We currently do not have available anatomical studies for the APPSwe mouse line. The high mortality rate observed among the mutants has limited our ability to conduct detailed anatomical analyses of retinal structures in this specific model. Therefore, our focus with the APPSwe mouse line primarily revolves around behavioral studies to gain insights into the functional aspects of retinal degeneration in relation to AD.

3. Changes that had a significant impact on expenditures
Nothing to report.

4. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

5. Significant changes in use or care of human subjects
Nothing to report.

6. Significant changes in use or care of vertebrate animals.
Nothing to report.

7. Significant changes in use of biohazards and/or select agents
Nothing to report.

PRODUCTS:

Publications, conference papers, and presentations

Our ipRGC findings in aging were presented as a poster at The Association for Research in Vision and Ophthalmology (ARVO) in New Orleans on April 23rd.

Based on our findings with ipRGCs, we are preparing a research paper entitled "Preservation of Intrinsically Photosensitive Retinal Ganglion Cells (ipRGCs) in Aging: Implications as a Potential Biomarker for Ocular Degenerative Diseases." to be submitted to Investigative Ophthalmology & Visual Science (IOVS) journal. The paper highlights the age-related changes in ipRGC morphology and associated behavioral functions in mice, emphasizing their potential as a biomarker for ocular degenerative diseases, including AD, PD, glaucoma, and diabetes.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

N/A

SPECIAL REPORTING REQUIREMENTS

N/A

APPENDICES

N/A