Roles of Disulfide Bonds for Outer Segment Disc Rim Curvature

Submitted by

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Contents

Abstract

The protein peripherin-2/rds (P/rds) is a protein located within the photoreceptor cells (rods and cones) of the eye and is essential to vision. P/rds is an integral membrane protein that is required to form outer segments organelles, and is assembled as polymers that are joined by disulfide bonds. Outer segments contain stacks of discs with highly curved, hairpin like rims and it is still unknown how these rims attain their highly specific shape. To determine if the disulfide bonds that hold together P/rds higher order polymers are responsible for the shaping of these membranes, *Mus musculus* (mouse) and *Xenopus leavis* (frog) eye cups were treated *ex vivo* with a disulfide reducing agent and analyzed by western blotting and transmission electron microscopy. It was determined that outer segment discs treated in this way possessed rim diameters 25% greater in size (in comparison to control rims) in both species. This treatment was effective at reducing P/rds, so we conclude that there is a correlation between disulfide bond reduction between P/rds polymer constituents and the increase in outer segment disc rim size and shape change.

Introduction and Background

For vision to occur, light must first pass through the cornea, the clear outer layer of the eye. The lens, a tissue that is also normally perfectly clear, then focuses the light onto the retina. The retina is an extension of the brain that projects into the back of the eye and is responsible for directly detecting light. The brain receives a signal from the retina and an image is generated. The retina has many layers, with various cell types, with one key type referred to as photoreceptor cells of two subtypes. The constituents of photoreceptor cells are cones and rods. (Goldberg et al., 2016) (Fig. 1). Cones work to provide color vision and function best under well illuminated conditions and rod cells function in dimly lit conditions to provide basic black and white vision (Dahl, 2015).

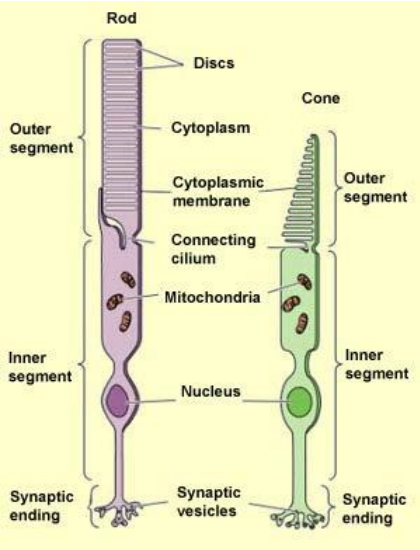


Figure 1: A Rod and Cone Cell
Rod and cone cells are a different shape, but consist of the same major parts. (Image source – The Brain From Top to Bottom)

Photoreceptor cells are made up of a three part cellular architecture (Goldberg et al., 2016). This includes the synaptic terminus, which works in communication with other neurons and the inner segment, which works to maintain cell viability (Dahl, 2015). The third region of photoreceptors is the outer segment, which contains long stacks (1000 or more) membranous discs (Wrigley,

Ahmed, Nevett, & Findlay, 2000). The main structural difference between rod and cone cells is their rim boundary shapes. Rod cells possess only fully internalized discs with "hairpin" (Fig. 2) shaped rims (the exception being nascent discs) and cone cells possess the aforementioned rims and "U" shaped edges along shared boundaries by adjacent partially internalized discs (Goldberg et al., 2016). The lamellar (flattened) region of these discs contain the molecule rhodopsin which absorbs photons of light (Dahl, 2015)

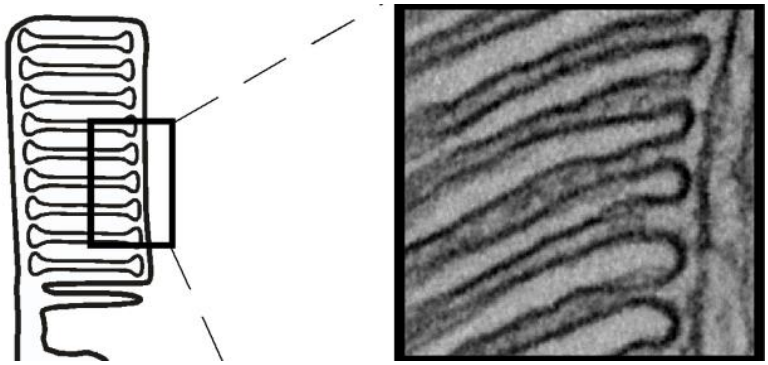


Figure 2: An Outer Segment and Disc Rims (TEM)
An outer segment of a rod photoreceptor cell (left)**.** The outer segment contains tightly packed discs with highly curved rims (right).

P/rds is a protein located on the tight, hairpin-like rims and not on the U-like edges of the discs (Arikawa et al., 1992; Ding et al., 2015). This protein is approximately 35 kDa (35,000 daltons) in its monomer form – when its disulfide bonds have been broken and approximately 70 kDa in its dimeric form – when its disulfides have not been broken (Molday et al., 1978). It is an integral-membrane glycoprotein localized exclusively to the rim regions of photoreceptor outer segment discs (Wrigley, Ahmed, Nevett, & Findlay, 2000). P/rds molecules polymerize as dimers, tetramers and higher-order polymers through the use of disulfide bonds, which are bonds produced through the oxidation of two sulfhydryl groups (Fig. 3). The disulfide bonds form between two cysteine-150 residues (Chakraborty et al., 2010). Disulfide bonds help to maintain the tertiary and quaternary structures of proteins ("Protein Structure", 2009). Proteins are capable

of producing many highly varied membrane shapes through different bending mechanisms (Zimmerberg & Kozlov, 2005) and it is believed that the P/rds protein is essential for the high curvature of outer segments and discs (Goldberg et al., 2016).

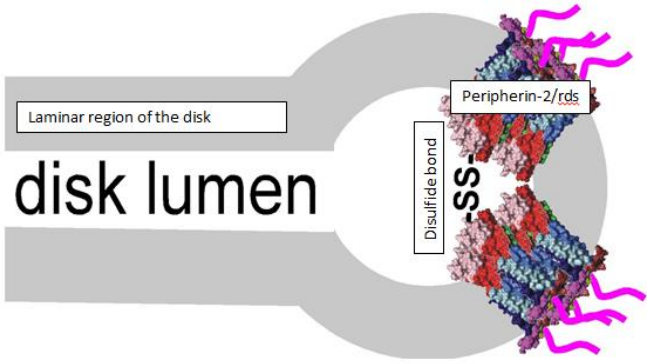


Figure 3: Peripherin-2/rds Localization and Polymerization
P/rds tetramers are polymerized through disulfide bonding. This image represents the current hypothesis for how the polymers give the outer segment disc rims their shape.

There is more direct evidence that suggests that this protein contributes to the formation and maintenance of disc rim shape. It has been shown that the flattening of microsomal vesicles is dependent on the formation of disulfide bonds between P/rds molecules, as vesicle flattening was only seen in condition conductive to disulfide formation (Wrigley, Ahmed, Nevett, & Findlay, 2000). Thus, it is possible that the formation of the energetically unfavorable disc rim curvature depends upon of the disulfide bonding of P/rds polymers.

This study will provide knowledge on how disc rims maintain their highly specialized shape, and determine if disulfide bonds joining P/rds tetramers are required for this curvature. New information on the effects of disulfide reduction in P/rds will be obtained, and this work may lead to further studies on the effect that disulfide reduction has on membrane morphology for various proteins. This will be tested through the use of a membrane permeable reducing agent that will break the polymers' disulfide bonds in various species. It is important to note that in the species used, *Mus musculus* (mouse) have approximately 97% rod cells and 3% cone cells (Jeon,

Masland, and Strettoi, 1998). *Xenopus laevis* (frog) have approximately equal amounts of rod and cone cells (Green, LaRue, Hayasaka, 2010). The primary experimental approaches used were done via western blotting and transmission electron microscopy (TEM). A blinded observer was used to validate results obtained by TEM and image analysis.

Objectives

1. Determine the concentration of reducing agent DTT required to break the disulfide bonds in P/rds since the experimental system includes supplemental oxygen, which can inactivate DTT, and its concentration as a function of time must also be determined.

2. A proper incubation chamber that *can maintain sample viability* must be developed because portions of the eye being used in this project must be incubated in either a reducing or non-reducing environment while maintaining viability.

3. Determine the role of the disulfide bonds between P/rds polymers in disc rim shape maintenance.

Specific Aims

1. Develop an assay to determine the concentration of the reducing agent DTT after sparging with 95% O_2 / 5% CO_2 for varied times.
2. Develop an incubation system for eyecup tissue explants that maintains retinal integrity.
3. Determine conditions required for the reduction of $\geq$ 90% P/rds present.
4. Overall: Determine if P/rds disulfide bond loss is correlated with change(s) in outer-segment disc rim curvature.

Reagents Used

The reducing agents used include *tris*(2-carboxyethyl)phosphine (TCEP) which is *not* membrane permeable, so it is not expected to reduce P/rds in-situ (further testing was done to confirm this, results not shown), and dithiothreitol (DTT) which is membrane permeable and is expected to reduce P/rds in-situ. N-Ethylmaleimide (NEM) was also used to cap any remaining free sulfhydryls. Extra cellular solution (ECS) is an incubation solution used that is designed to keep retinal tissue healthy in mice. Wolf-Quimby amphibian culture media is a cell-culture media used to maintain amphibian cell health.

Methods

Incubation-Perfusion Design

An incubation-perfusion chamber was designed using: glass slides, hybridization chambers (Grace Bio-Labs), silicon isolators (Grace Bio-Labs), press fit tubing connectors (Grace Bio-Labs), and gaseous 95% O_2 / 5% CO_2. This incubation set up was designed to allow a flow rate of approximately 0.2 ml – 0.4 ml per minute of ECS or Wolf-Quimby media in the presence or absence of reducing agent (Fig. 4). Eyecups (eyes with the cornea, iris, and lens dissected away) are secured into the base of the chambers with superglue, and perfused with the oxygenated solution. After several experiments (results not shown), it was determined that performing perfusions for 2 hours each with 5 millimolar (mM) DTT and 2.5 mM TCEP was sufficient to reduce $\geq$90% (mouse) and $\geq$40% (frog) of the total P/rds to its monomeric form. After ECS or Wolf-Quimby treatments were completed, samples were perfused with 3 ml of 100 mM NEM over the course of three minutes. Reduced samples (experimental) were treated with 5 mM DTT and 2.5 mM TCEP, and non-reduced (control) samples were treated with only 2.5 mM TCEP.

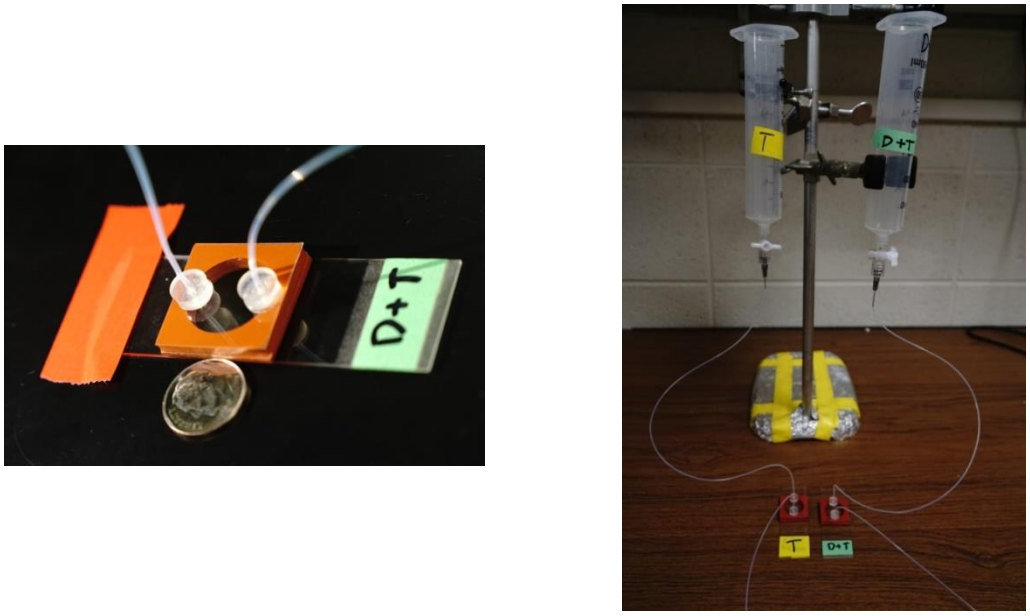


Figure 4: Incubation Chamber Design
The incubation chamber design (left) used allowed for continuous perfusion of eyecups with oxygenated solution designed to maintain retinal integrity. Parallel chambers (right) were utilized to compare an experimental and control sample.

DTT Assay

The reducing agent DTT is susceptible to oxidation by oxygen which compromises its ability to reduce disulfide bonds. The solution containing the DTT is sparged with 95% O_2 to maintain retinal integrity. In order to determine if a sufficient amount of DTT would remain viable in the experimental DTT containing solution, a colorimetric analysis with Ellman's Reagent (DTNB) was performed to assay DTT concentration. It was determined that less than 10% oxidation of DTT occurred over a 2 hour period (results not shown). Therefore, it was concluded that this method of O_2 sparging left a sufficient amount of reducing DTT in solution to perform the desired experiment.

Western Blotting

After sample perfusion-incubations were complete, whole or partial retinas (depending on the trial) were removed from the eyes and processed. Retinas were homogenized with an Eppendorf pestle in 8 molar (M) urea and Laemmli Sample Buffer (LSB) and boiled. The samples were then loaded into 10 or 15 well, 10% SDS-polyacrylimide gels and electroprocessed.

Some samples were loaded with 2-Mercaptoethanol (β-ME) to test whether control samples could be reduced. The gels were transferred to Immobilon® FL membrane and western blots were developed using the primary antibodies anti-P/rds PabMPCT (for mouse) and Mab1G9 (for frog). Tubulin (molecular weight 65 kDa) was commonly used as the internal control with the anti-tubulin antibody Mab12G10 (mouse) and Pab 15246 (frog) and also beta-actin with the anti-beta actin (molecular weight 55 kDa) antibody Pab8227 (frog). Blots were then processed with fluorescent secondary antibodies. The blot scans were performed using a Li-COR Odyssey® scanner.

Transmission Electron Microscopy (TEM)

To prepare samples for TEM, perfused eyecups were dissected into quarters or eights. These quarters were then fixed with a mixture of glutaraldehyde and formaldehyde and then treated with 50% ethanol (EtOH), 70% EtOH, 90% EtOH and 100% EtOH, as well as propylene oxide for dehydration purposes. They were also treated with osmium tetroxide to bind lipids, uranyl acetate, and lead citrate to provide contrast. Samples were then placed in Poly/Bed® 812 resin and allowed to harden into blocks. Sectioning of the blocks was done via a diamond knife and the samples were again treated with uranyl acetate and lead citrate. Images of the samples were taken on a FEI Morgagni™ 268 transmission electron microscope. Digital imaging was performed by Nicole Roussey and/or Drs. Victoria Kimler and Andrew Goldberg.

NIH Image J Analysis

Disc rim diameters were measured using the program Image J, provided by the Nation Institute of Health. Rims with a blurry or doubled appearance were not included in the analysis. The best 8 rims present in each image were analyzed, with three images used for each sample.

The outer diameter of the rims was measured (Fig. 5) at the widest point. Statistical analysis of the results was then done to calculate the average rim diameter for each sample.

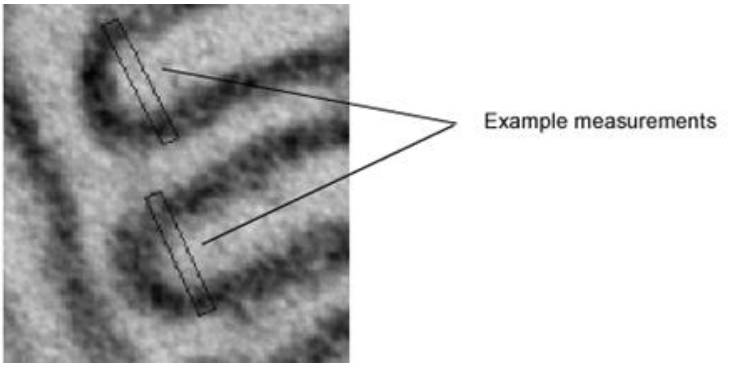


Figure 5: Sample Rim Measurements
Rims with the most well defined appearance were measured. The outer diameter measurements include the thickness of the membrane.

Proof of Concept - Rudimentary Experimentation

It is known that P/rds can be reduced in-vitro (not shown), but it was then necessary to experimentally determine if it is possible to perform an *in-situ* reduction of the protein. This was done using four mouse eyecups in Dulbecco's Modified Eagle Medium (DMEM) either with or without the reducing agent (5 mM DTT) for varying time periods and exposure to 95% O_2 / 5% CO_2. Retinas from the four samples were then analyzed via a Western blot (Fig. 6). Reduced (monomeric) P/rds is known to have a molecular weight of approximately 35 kDa, and unreduced samples are known to have molecular weights of approximately 70 kDa, its dimeric weight, *as well as* 35 kDa. The results obtained were consistent with these predictions.

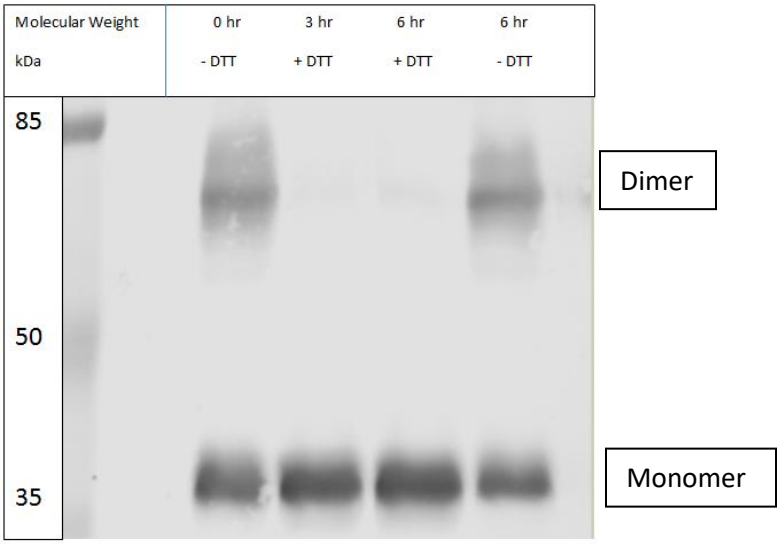


Figure 6: *In-Situ* Reduction of P/rds Western Blot
Samples not exposed to DTT (0 hours (the control sample) & 6 hours) regardless of time outside of the donor animal had the expected results of 35 kDa and 70 kDa P/rds present. Reduced samples (3 hours & 6 hours +DTT) had a nearly complete reduction of the protein, with only 35 kDa protein present.

This blot demonstrated that P/rds can be reduced *in-situ* by 5 mM DTT within 3 hours and that the reducing agent is the required factor for the reduction, since the –DTT, control 6 hour sample displayed no reduction. This experiment was then repeated using a 3 hour time point only and had one reduced eyecup and one control eyecup. A Western blot (results not shown) and TEM imaging were performed (Fig. 7).

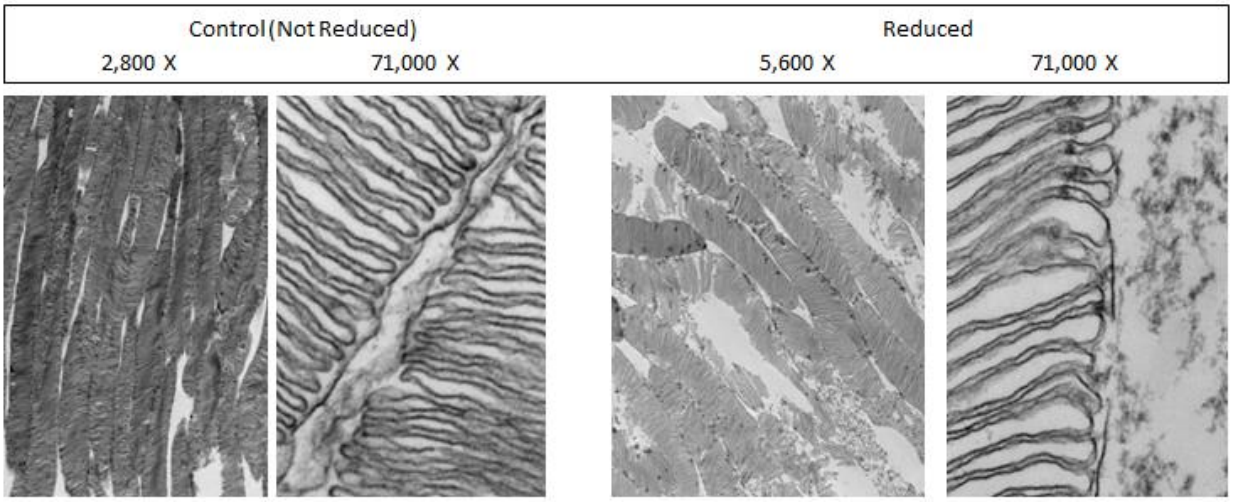


Figure 7: TEM Analysis of *In-Situ* Control and Reduced Photoreceptor OSs and Rims
TEM results of the control sample (left) presents normal looking, well organized vertical outer segments as well as disc rims with a normal appearance. Results of the reduced sample (right) presents disorganized and broken outer segments as well as swollen and abnormally shaped rims.

TEM results for the proof of concept experiment determined that sections of the outer segments remained healthy and intact and there was no change in the disc rims for the non-reduced samples (Fig. 7). The reduced samples had broken and disorganized outer segments as well as enlarged and abnormally shaped rims (Fig. 7). These results suggest that the reduction of P/rds

disulfide bonds is possible *in-situ* and that this reduction is accompanied by a change in the shape and size of outer segment disc rims.

Results - Mouse

Primary Perfusion Western Blot

An initial trial of the incubation-perfusion experiment (see Fig. 4) was performed using mouse eyecups to test the perfusion method and to determine if the parameters designed were sufficient for maintenance of retinal health and P/rds reduction. In the initial trial of the current method of incubation and reduction one sample was perfused with 5 mM DTT + 2.5 mM TCEP in ECS (2 hours – sample DT) and another sample was perfused with 2.5 mM TCEP in ECS (2 hours – sample T) and both following with 100 mM NEM. The western blot showed proof of reduction in the sample exposed to the membrane permeable reducing agent DTT. This is known because the DT lane only has one prominent band present at approximately 35 kDa, the monomeric weight of P/rds. However, the sample only exposed to the non-membrane permeable TCEP did not show signs of reduction, as anticipated. The non-reduced lane, T, has two bands present at approximately 35 kDa and 70 kDa, the dimeric weight of P/rds. The anticipated 2:1 ratio of dimeric (~66%) to monomeric (~33%) P/rds was also present (Fig. 8). The DT sample had approximately 90% monomeric P/rds, confirming that the new method of perfusion was successful.

(a) (b)

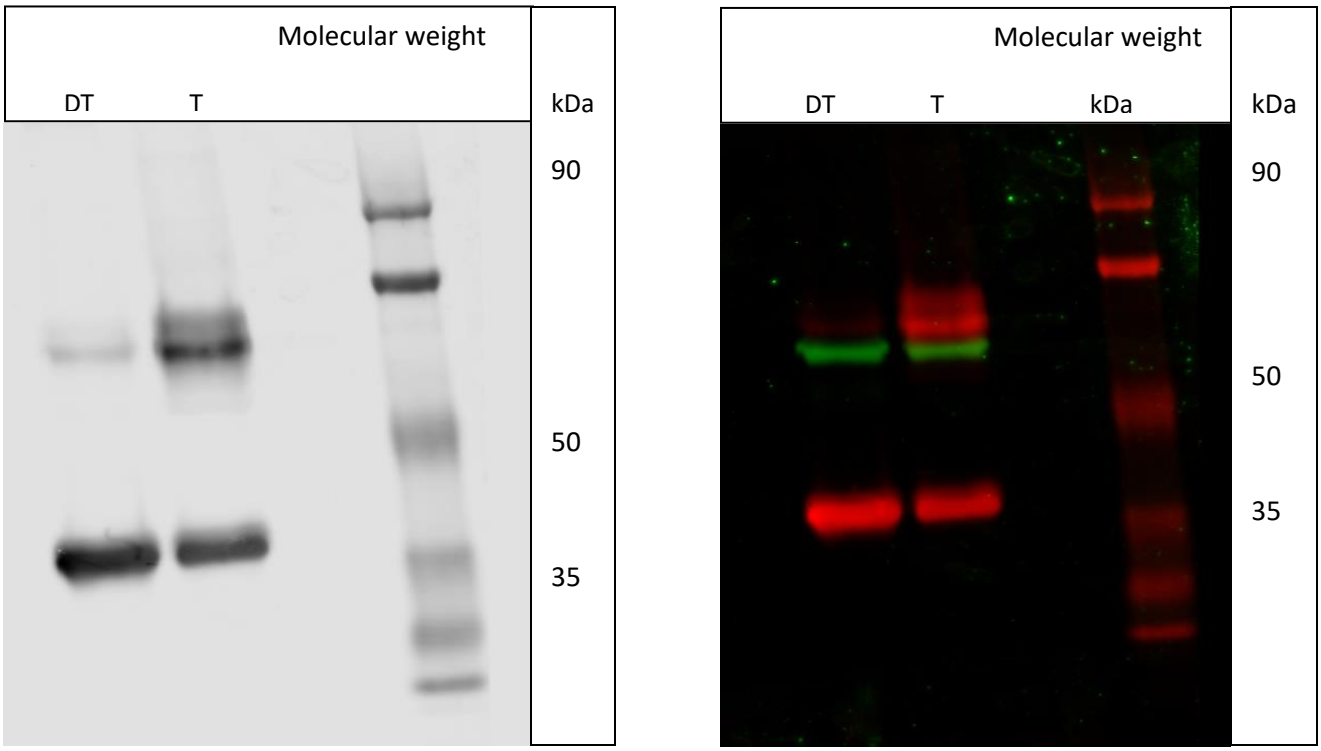
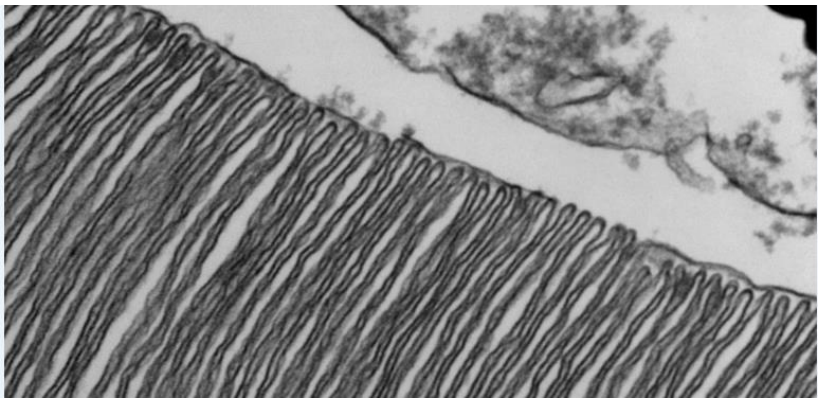


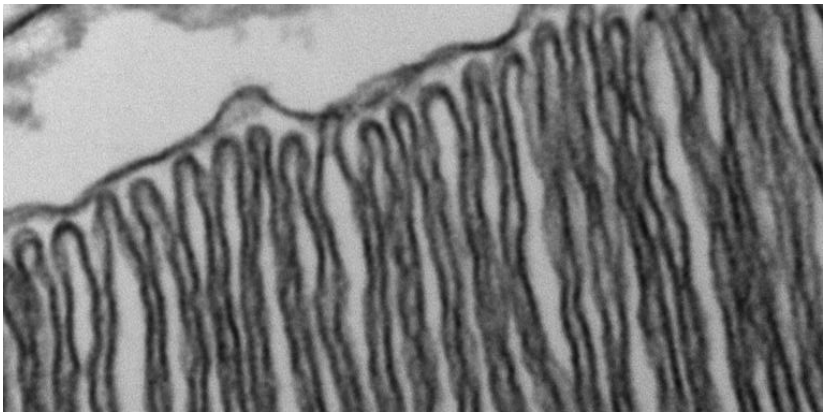
Figure 8: Primary Perfusion Western Blot Displays Reduction of P/rds
This western blot was treated with anti-P/rds PabMPCT and anti-tubulin Mab12G10 antibodies. A and B are different versions of the same scan. Red = P/rds, Green = Tubulin.

Primary Perfusion TEM

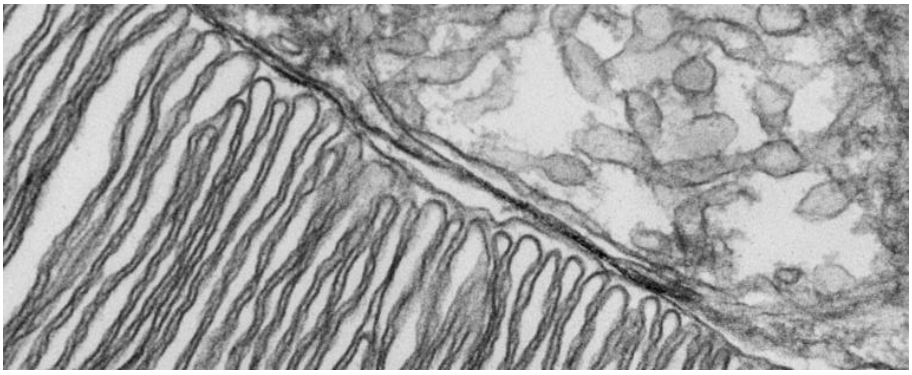
Transmission electron microscopy was performed on the experimental eyecup samples. The following results were received using 56,000 times magnification and 140,000 times magnification (images may not be true to scale) of outer segments and their disc rims. The T sample had organized outer segments and normal looking rims, and the DT sample had semi-organized outer segments with enlarged, abnormally shaped rims (Fig. 9).



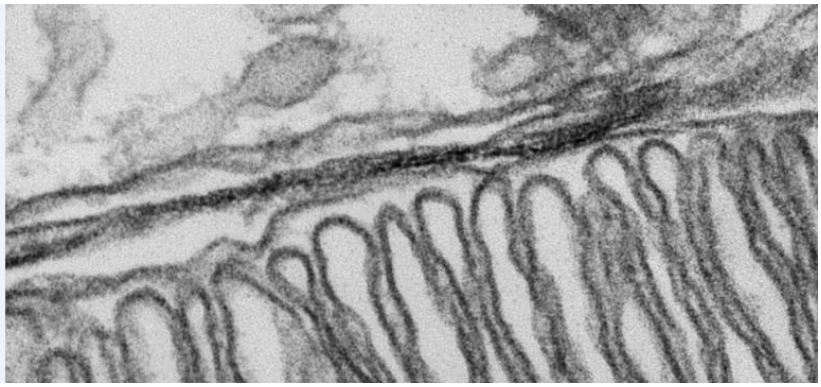
A. “T” Sample



B. “T” Sample



C. “DT” Sample



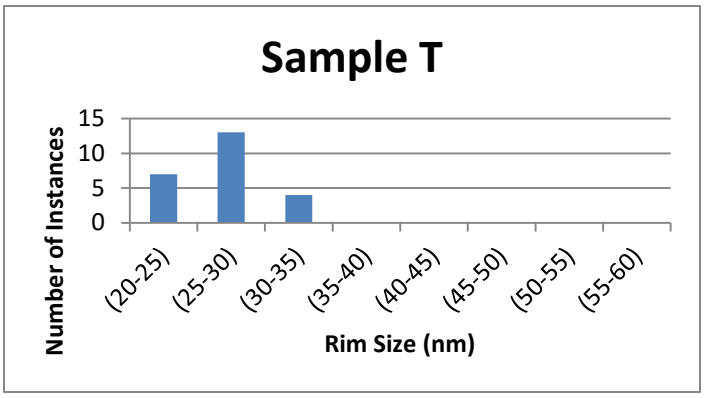
D. “DT” Sample

Figure 9: Primary Perfusion TEM Results Display Disc Rim Enlargement
A. T -56,000 magnification B. T - 140,000 magnification. C. DT - 56,000 magnification D. DT - 140,000 magnification.

Primary Disc Rim Measurements (Image J Analysis)

Measurements of the outer segment rims were done through the program Image J. It was determined that the best method of data collection was by measuring the eight most viable rims in three images for each sample (24 rims total). A standard length was used to determine the actual diameter of the rims in nanometers (nm, 10^{-9} m). Histograms were made of the results (Fig. 10). Sample T had an average of approximately 27nm and DT had an average of approximately 43.3nm, an increase of approximately 52% (n = 8 rims, 3 images).

A



B

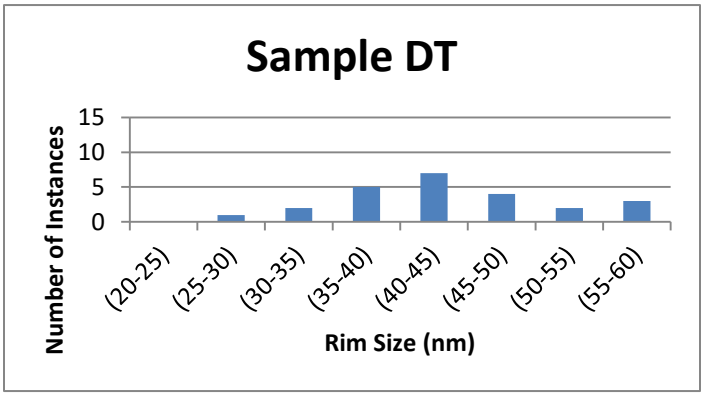


Figure 10: Image J Analysis Shows Disc Rim Enlargement
A. A histogram of sample T's measurements. B. A histogram of sample DT's measurements.

Second and Third Perfusion Western Blot

After the initial trial displayed positive results, further trials were performed. In the

following trials of the current method of incubation and reduction two samples were perfused with 5 mM DTT + 2.5 mM TCEP in ECS (2 hours – samples DTA and DTB) and two other samples were perfused with 2.5 mM TCEP in ECS (2 hours – sample TA and TB) and all following with 100 mM NEM. The western blot showed proof of reduction in the samples exposed to the membrane permeable reducing agent DTT. This is known because the DTA and DTB lanes only have one prominent band present at approximately 35 kDa. However, the samples only exposed to the non-membrane permeable TCEP, TA and TB, did not show signs of reduction, as anticipated. The non-reduced lanes, TA an TB, have two bands present at approximately 35 kDa and 70 kDa (Fig. 11) Samples not exposed to DTT (TA and TB) had the anticipated ratio of 2:1 dimeric (~66%) to monomeric (~33%) P/rds. Sample DTA had approximately 90% monomeric P/rds and DTB had approximately 78% monomeric P/rds.

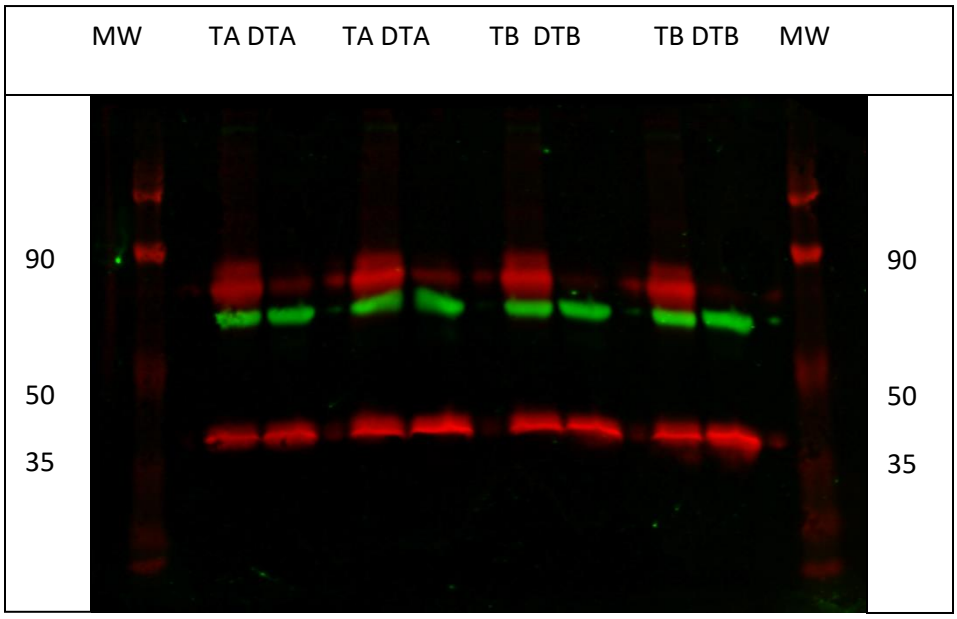


Figure 11: Western Blot Displays P/rds Reduction in Further Trials

This western blot was treated with anti-P/rds PabMPCT and anti-tubulin Mab12G10 antibodies. Red = P/rds, Green = Tubulin.

Second and Third Perfusion TEM

Transmission electron microscopy was performed on samples TA, DTA, TB, and DTB. For this trial the following results were received using 140,000 times magnification of outer segment rims (Fig. 12). Samples TA and TB had well organized outer segments with normal disc rims, and samples DTA and DTB had semi-organized outer segments with enlarged and abnormally shaped disc rims.

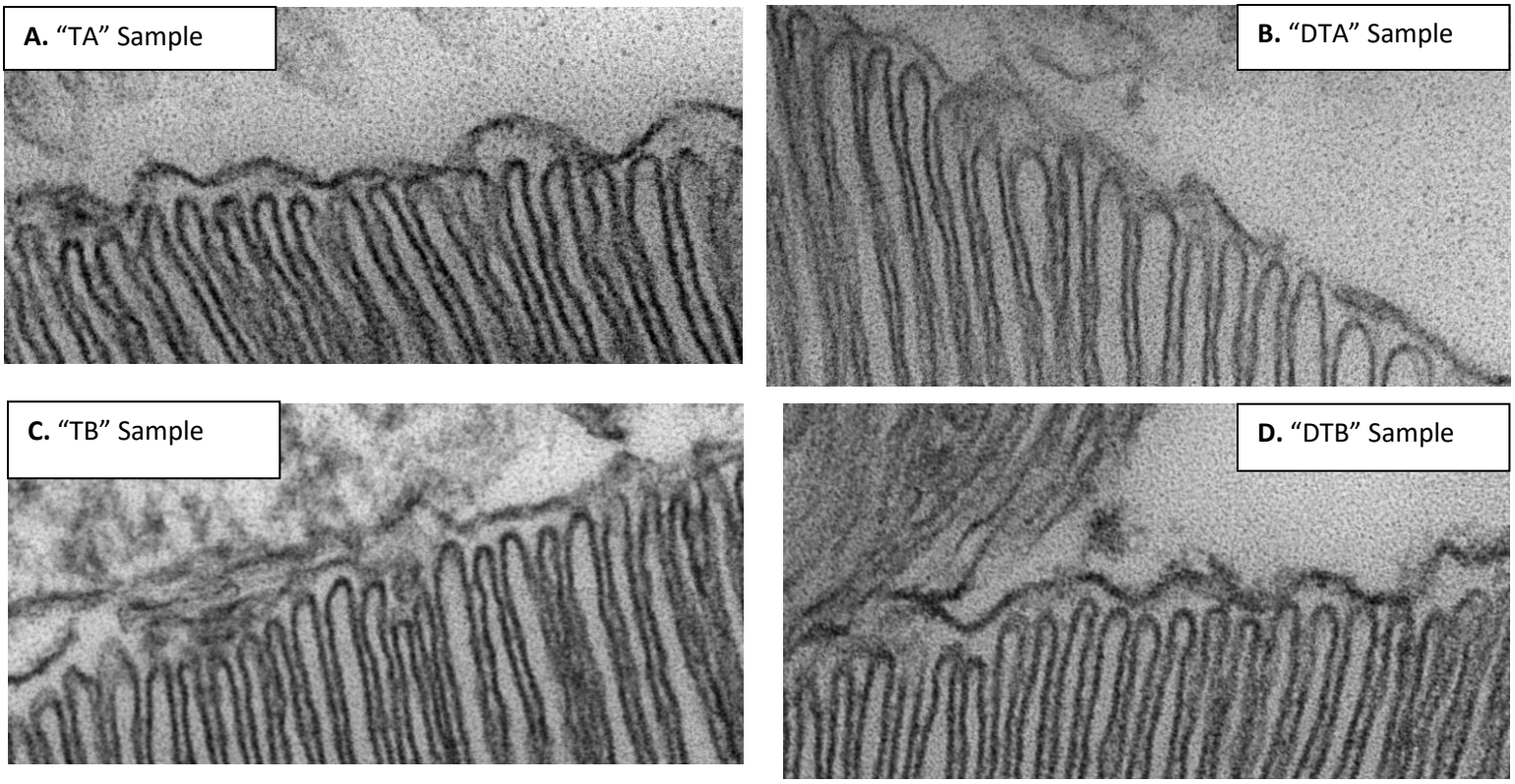


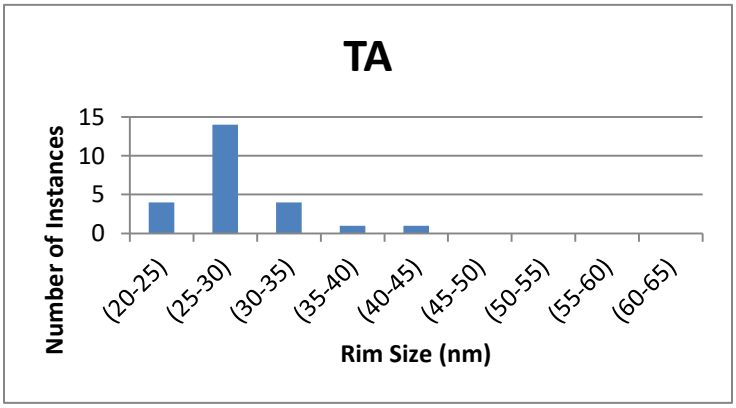
Figure 12: Second and Third Perfusion TEM Results Display Disc Rim Enlargement
A. TA - 140,000 B. DTA - 140,000 magnification C. DTA - 140,000 magnification D. DTB - 140,000 magnification.

Second and Third Perfusion Disc Rim Measurements (Image J Analysis)

For these trials, eight rims were measured per image with three images for each sample, totaling 24 rims each. Histograms were made of the results (Fig. 13). In these trials, the DT samples had a rim diameter increase of approximately 35% or ~10 nm (n = 8 rims, 3 images).

Sample TA had an average rim diameter of 28.44nm, DTA – 42.93nm, TB – 27.93nm, and DTB – 34.00nm.

A



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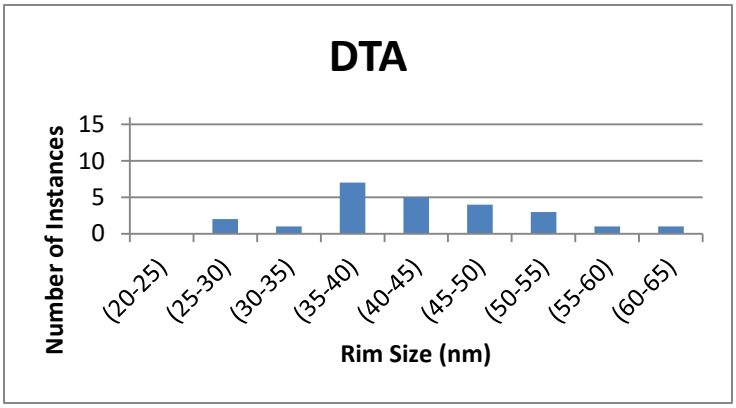

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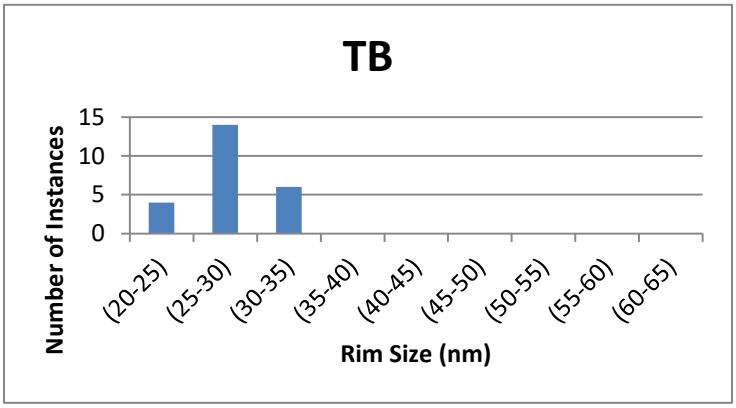

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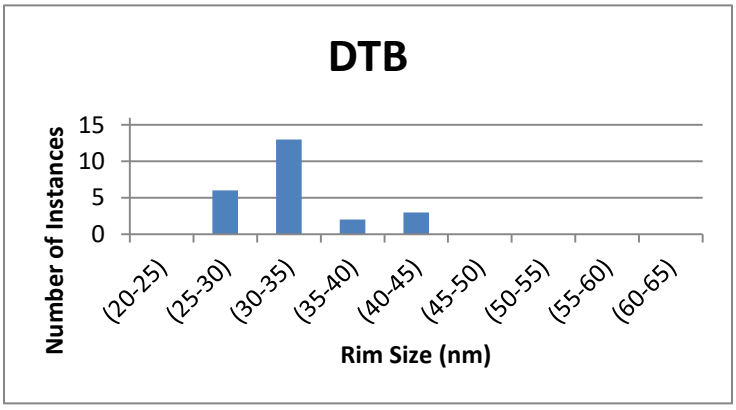


Figure 13: Further Trials Image J Results Displays Disc Rim Enlargement
A. A histogram of sample TA's measurements. B. A histogram of sample DTA's measurements. C. A histogram of sample TB's measurements. D. A histogram of sample DTB's measurements.

Summary of the Mouse Model System

Analysis of all three *trials* was then done and it was determined that on average in non-reduced (TCEP only) samples, approximately 63% of *total* P/rds is dimeric. In reduced samples, on average approximately 14% of total P/rds is dimeric. Approximately 20% of dimeric P/rds remains unreduced after 2 hours (Fig. 14).

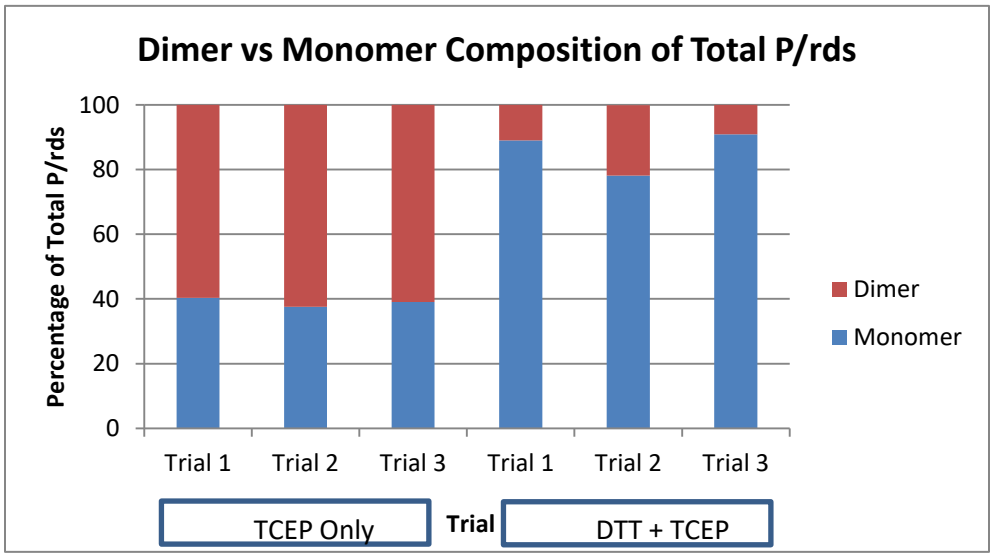
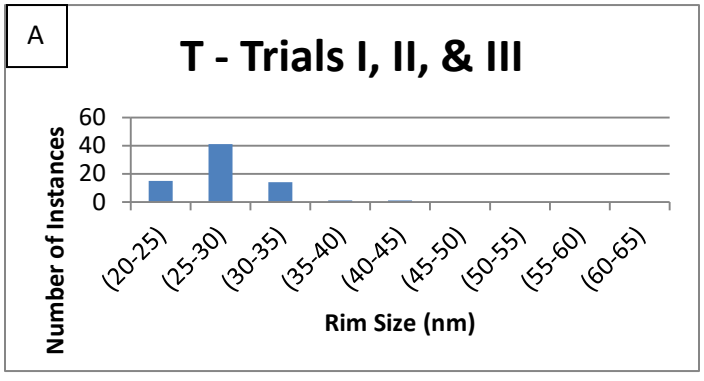


Figure 14: P/rds Dimer and Monomer Compositions in All Mouse Western Blots
This graph shows the composition of total P/rds in all three mouse trials performed.

Image J analysis data was used to create graphs of the average values retrieved for reduced and unreduced samples in all three trials (Fig. 15). The T samples had an average rim diameter of 27.8nm, and the DT samples had an average rim diameter of 39.7nm. There was an average increase of 40% in rim size from the non-reduced to the reduced samples.



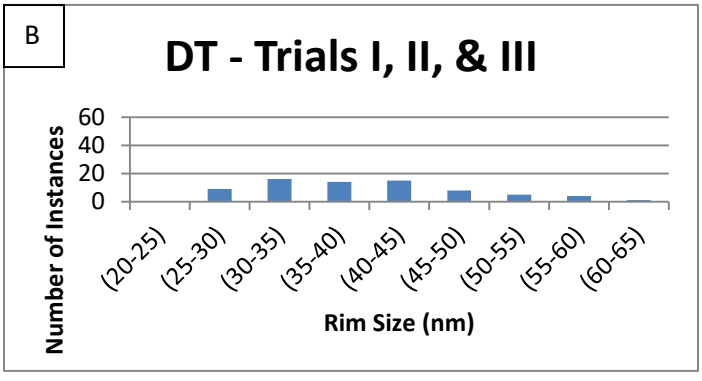


Figure 15: Image J Analysis Shows Disc Rim Enlargement
A. A histogram of the T samples' measurement results. B. A histogram of the DT samples' measurement results.

These results tell us that there is a correlation between reducing the majority of the disulfide bonded P/rds in a mouse animal model and the production of abnormally large rims. This however, is not a direct causation. A blinded observer separately performed the TEM imaging process and performed independent Image J analysis to confirm that results were not the result of "cherry-picking." A similar average rim size and histogram distribution was received, confirming the results that the reduction of P/rds affects disc rim size. After a sufficient number of trials were performed in the mouse model, a frog model was then used using the same experimental design.

Results - Frog

Primary Perfusion Western Blot in Frog Model System

An initial trial of the current method of incubation and reduction was done using frog eyecups to determine if this method was sufficient for frog samples. One sample was perfused with 5 mM DTT + 2.5 mM TCEP in Wolf-Quimby (Amphibian Culture Media) (2 hours – sample DT) and another sample was perfused with 2.5 mM TCEP in the same media (2 hours – sample T) and both following with 100 mM NEM. The western blot showed proof of reduction in the sample exposed to DTT. This is known because the DT lane only has one prominent band present at approximately 35 kDa. However, the sample only exposed to TCEP did not show signs of reduction, as anticipated. The non-reduced lane, T, has two bands present at approximately 35 kDa and 70 kDa, the dimeric weight of P/rds (Fig. 16). It is important to note that the anticipated ratio of dimer to monomer was unknown for this model.

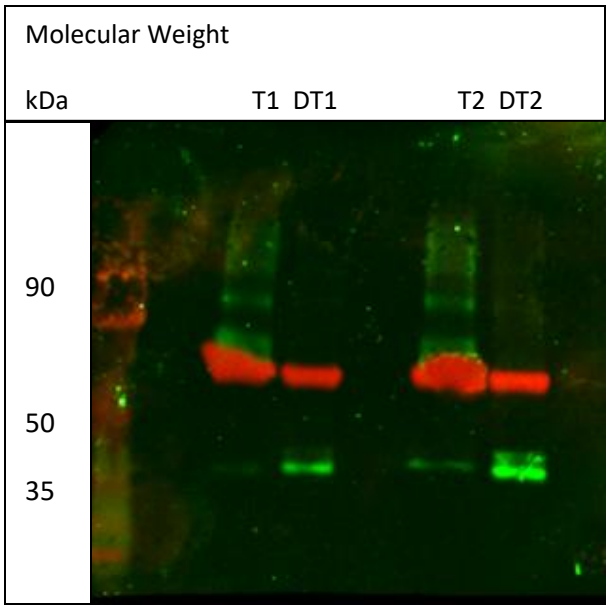


Figure 16: Frog Model Western Blot Results Displays P/rds Reduction
This western blot was treated with anti-P/rds Mab1G9 and anti-tubulin Pab15246 antibodies. Red = Tubulin, Green = P/rds.

Technical duplicates of the T and DT samples were run. The DT sample had, on average, approximately 95% monomeric P/rds. This was determined using tubulin as an internal control. It is important to note that the tubulin bands partially occluded the P/rds dimer bands, likely skewing the dimer to monomer ratio results. A graphical representation of the western blot results was created for this trial (Fig. 17).

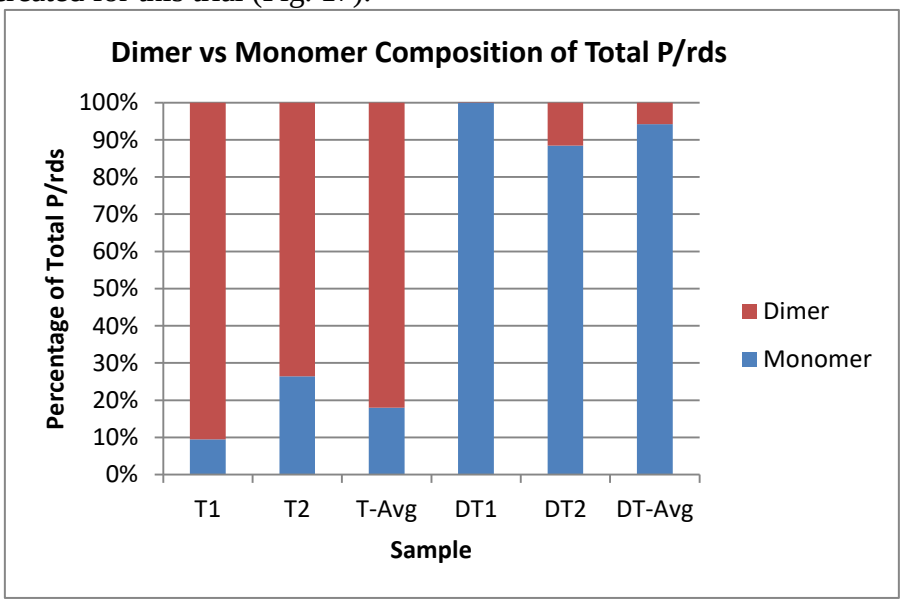


Figure 17: P/rds Dimer and Monomer Compositions in Primary Western Blot
This graph shows the composition of total P/rds in the initial frog trial that was performed.

This western blot determined that DTT is a sufficient reducing agent for use in the frog model, and that the vast majority of the P/rds present can be and was reduced to its monomer form. Further quantitation showed that in frogs there is approximately 15% monomer in unreduced samples (T), a significant variation from the mouse model's 2:1 dimer to monomer ratio.

Second Perfusion Western Blot (with Different Internal Control)

In the second trial of incubation and reduction was performed using the same setup as the initial trial, however a larger retinal percentage was used for the western blot. In this trial beta-actin, (molecular weight 55 kDa) was used as an internal control to avoid occluding the P/rds dimer bands, which occurred in trial 1. This western blot also showed proof of reduction in the sample exposed to DTT. This is known because the DT lanes only have one prominent band present at approximately 35 kDa. However, the sample only exposed to TCEP did not show signs of reduction, as anticipated. The non-reduced lane, T, has two bands present at approximately 35 kDa and 70 kDa (Fig. 18).

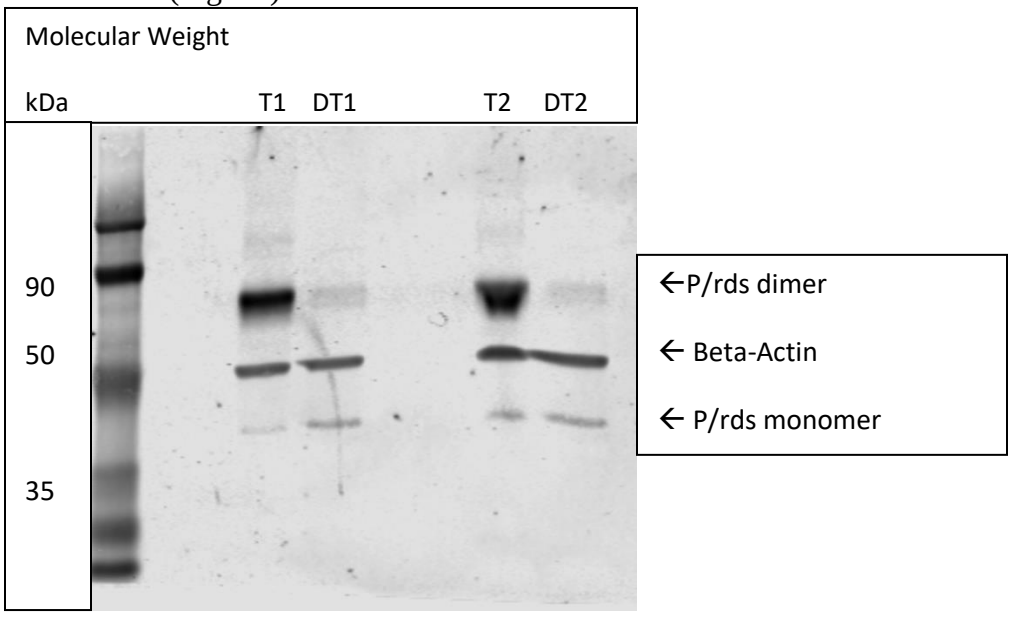


Figure 18: Frog Model Western Blot Results Displays P/rds Reduction
This western blot was treated with anti-P/rds Mab1G9 and anti-beta actin Pab8227 antibodies. Technical duplicates of the two samples were run.

A graphical representation of the western blot results was created for this trial (Fig. 19).

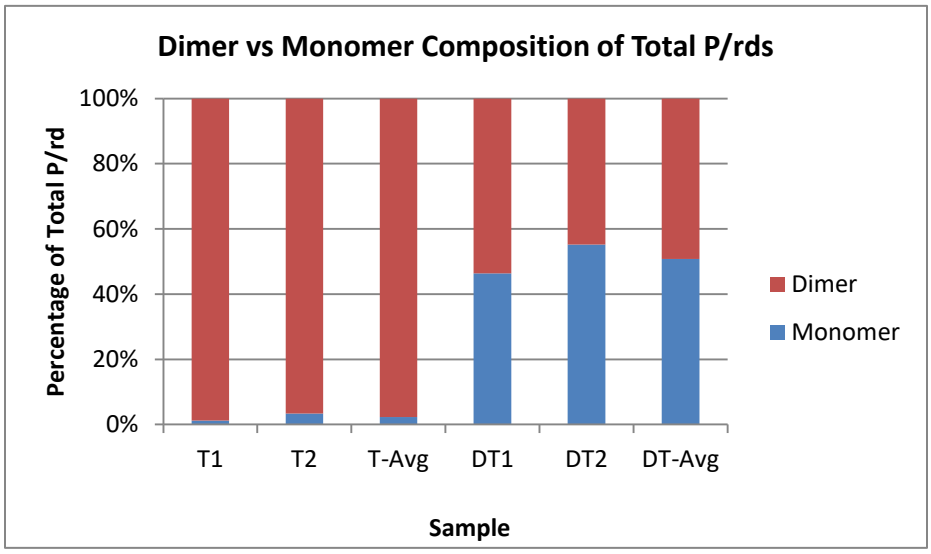
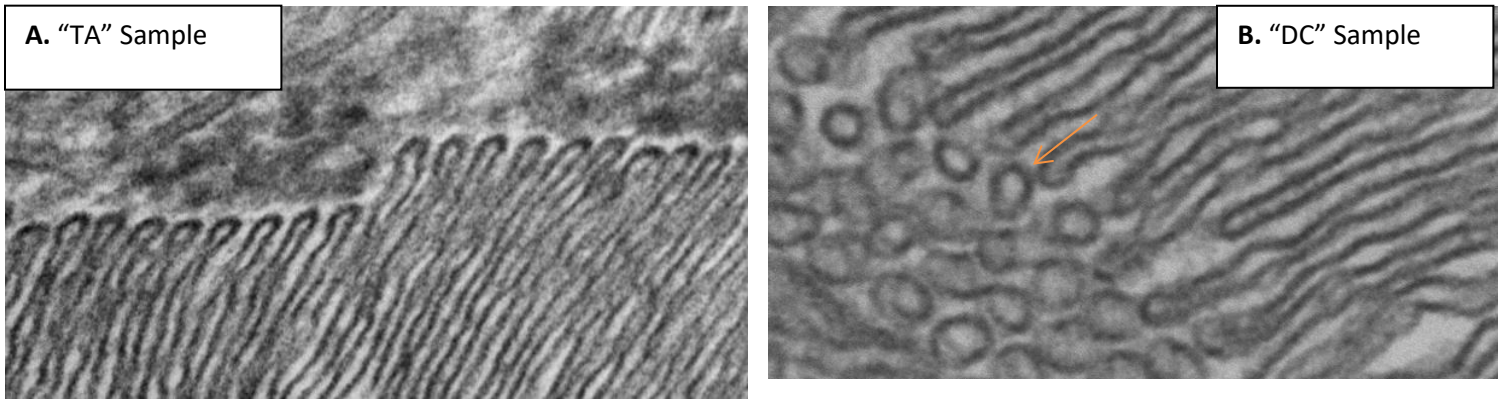


Figure 19: P/rds Dimer and Monomer Compositions in Second Western Blot
This graph shows the composition of total P/rds in the second frog trial that was performed.

Further quantitation showed that in this trial, there is approximately 2.32% monomer and 97.68% dimer in unreduced (T) samples, a drastic variation from the mouse model system and a slight variation from the initial frog model trial. The "DT" sample had, on average, approximately 50% monomeric P/rds.

Frog Model TEM

Transmission electron microscopy was performed on samples TA & DC (trial 1's T and DT) and T2A & D2B (trial 2's T and DT). For this experiment the following results were received using 56,000 times magnification (not shown) and 140,000 times magnification of outer segment rims (Fig. 20).



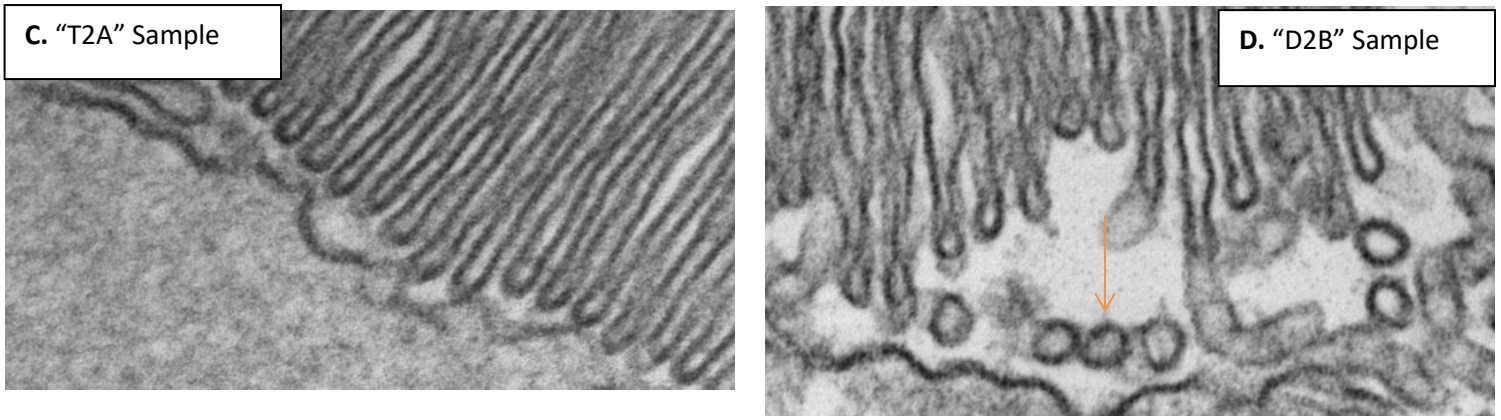
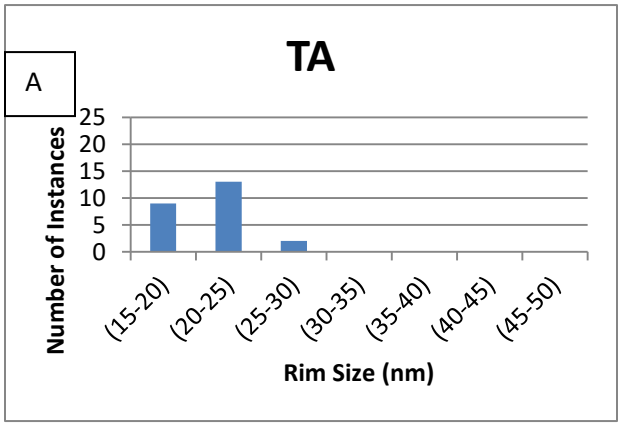


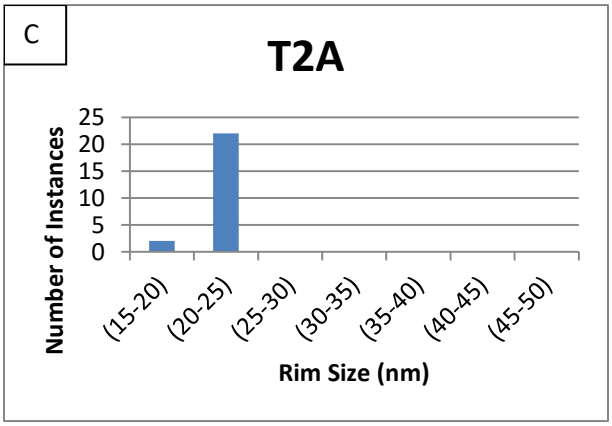
Figure 20: Frog TEM Results Display Rim Enlargement and Vesicle Formation
All images 140,000 magnification. A - TA, B - DC, C - T2A, D - D2B. Arrow – vesicular structure.

The T samples from each trial had discs and disc rims with a normal appearance, and the DT samples had enlarged disc rims, but they maintained their shape more so than the reduced mouse samples. In these trials in the reduced samples DC and D2B unknown vesicular and tubular structures were observed. Their origin is unknown, but it possible that they are related to the abnormal reduced samples' results in the western blot trials (the DT samples had significantly less total P/rds than the T samples). However, this remains an untested hypothesis.

Frog Model Disc Rim Measurements (Image J Analysis)

For these trials, eight rims were measured per image with three images for each sample, totaling 24 rims each. Histograms were made of the results (Fig. 21). The T samples had an average diameter of 21.5nm, and the DT samples had an average of 27.5nm. In these trials, the DT samples had a rim diameter increase of approximately 28% or ~6 nm (n = 8 rims, 3 images). In this trial, the unknown vesicular structures were also measured across their minor axis. These were observed to be 167% of the size of the unreduced rims.





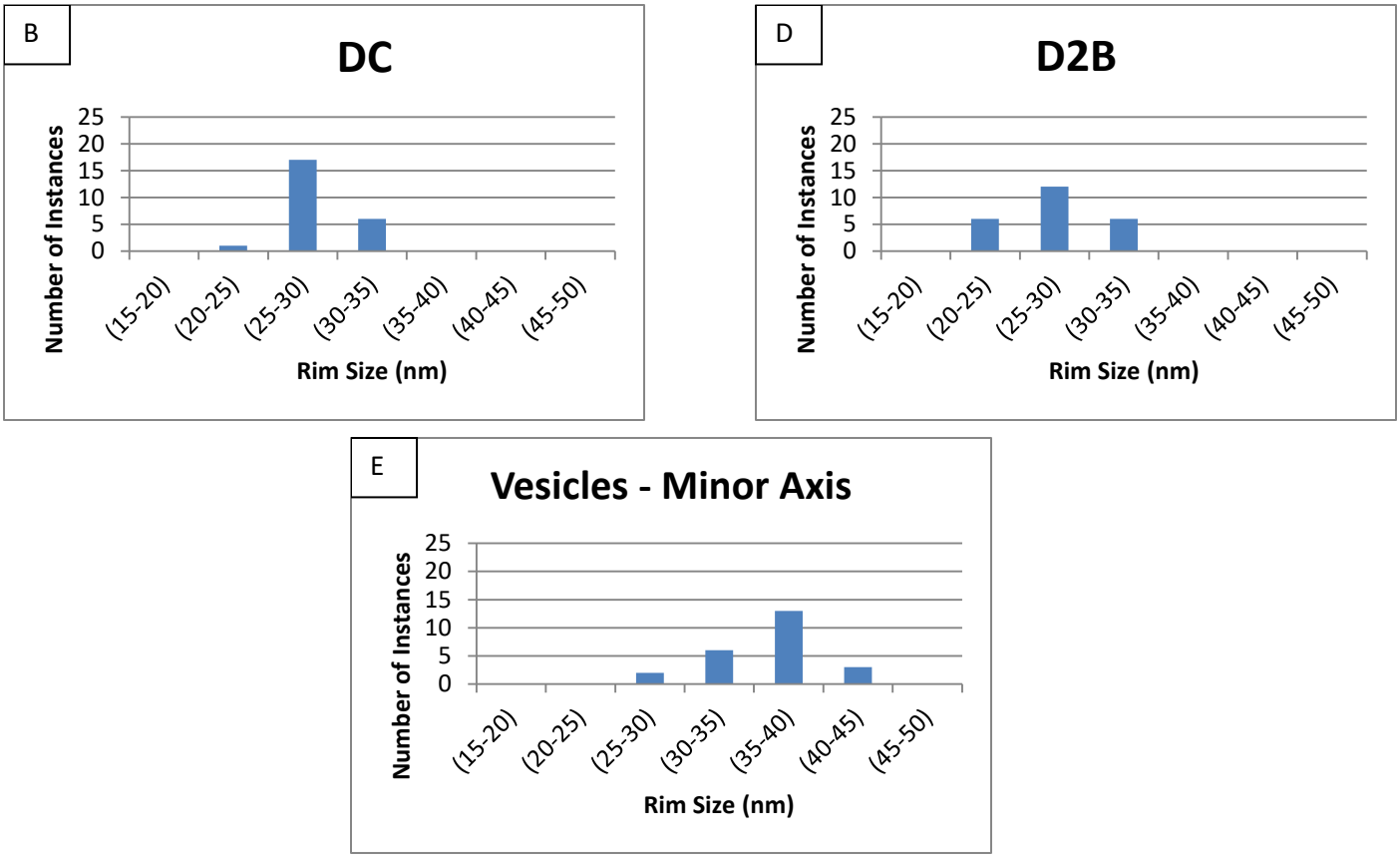


Figure 13: Image J Analysis Results Show Disc Rim Enlargement and Large Vesicular Diameters

A. TA - average rim diameter = 21 nm. B. DC - average rim diameter = 28 nm. C. T2A - average rim diameter = 22 nm. D. D2B - average rim diameter = 27 nm. E. Minor axis of the vesicular structures - average diameter = 36 nm.

Summary of the Frog Model System

In the frog trials, there were several issues that arose. The P/rds in the western blots of the reduced samples was only present in miniscule amounts (approximately 10% of the total P/rds in the non-reduced samples). It is possible that this is the results of some biological anomaly, or an issue with the DTT and the anti-P/rds antibody used in western development. In the TEM images, unknown, large vesicular and tubular structures of unknown origin were observed. The TEM preservation and sectioning of the frog trials was also not as well done as in the mouse trials. It is likely that this occurred because the TEM preparation method was optimized on mouse samples,

and changes will need to be made to continue frog trials. These issues have led to the conclusion that before any more trials are done on frog samples, it is essential to spend more time learning about the biochemistry of the several forms of frog P/rds. However, it does appear that there is a correlation between P/rds reduction and an increase in disc rim size in the frog model system.

Discussion

From the data obtained, it is apparent that there is a correlation between the reduction of the polymerized P/rds protein to monomeric P/rds and an increase in outer-segment disc rim size and a change in shape. The mouse trials resulted in a clear and concise result that showed an increase in rim size of approximately 40% with little other changes occurring in the tissue samples. However, the frog trials displayed an unclear result, with aberrant results in the western blot as well as the TEM. The TEM performed had unanticipated results with the formation of large vesicular structures and large tubular structures, but there was also an increase in rim size of approximately 28%. The breaking of the disulfides between the constituent monomers of P/rds polymers does appear to correlate to an increase in rim size and change in disc rim shape in the various species tested.

Future Plans

The current frog data will be analyzed with different criteria in Image J. No more trials with frog samples will be performed at this time due to our limited knowledge of frog P/rds. Current plans include working to develop experiments that will allow for further understanding of the (frog) protein's biochemistry. There are also tentative plans to continue perfusions using different mammalian samples. It is also likely that immuohistochemsitry (IHC) and/or immunogold TEM may be done on future perfusion samples to see how reduction by DTT affects P/rds localization in outer segments compared to their non-reduced counterparts.

Undefined experiments utilizing purified rod outer segments (ROS) from mice will also be performed to help to prove causation and not just correlation between the pieces of data collected.

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