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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Blast-related injuries can result in retinal detachment (RD). RD causes a separation of the neural retina from the retinal pigmented epithelium, uncouples photoreceptors from their synaptic partners, and ultimately leads to blindness. We discovered that synaptic disjunction by photoreceptors is due in part to an increase in the activity of Rho kinase (ROCK). We proposed therefore to prevent the damage to retinal synaptic circuitry after injury by using a highly efficacious ROCK inhibitor, AR13503. The work is done on adult pigs, whose retina is similar to humans, to increase the translational potential of the results. This past year we extended the time frame of our examination of the ROCK inhibitor by looking at retinas one week after detachment. Drug treated retina appeared to have both better rod synaptic morphology as well as function,as tested by ERGs, than untreated retina after a week. Our work this past year however was primarily focused on cone synapses. Cone synaptic ribbons shorten and disappear with detachment within 2 hours. This damage is prevented by ROCK inhibition. Two days after detachment and spontaneous reattachment, cone synapses look as though they have recovered, however ERG recordings still indicate cone dysfunction. The same result is apparent after one week: normal cone synaptic morphology but abnormal function. This year we also examined delayed treatment. Delaying injection of AR13503 for 2 hrs after a detachment still promotes reduction of damage to both rod and cone synapses. This is important for a transition of our findings to clinical practice, as it allows for treatment of soldiers and civilians who cannot be treated immediately after injury. We will finalize analysis of ROCK inhibition of cone synaptic damage this year and look forward to summarizing our work on both rod and cone synaptic trauma.

15. SUBJECT TERMS

Retinal detachment, Rho kinase, synaptic disjunction

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1. INTRODUCTION

Our research is directed toward preventing trauma-induced visual loss. Eye trauma is the 4th most common injury in combat. In blast-related injuries, the most common cause of ocular damage, the retina frequently is detached from its underlying supportive pigment epithelium. This injury results in the disjunction, or breaking, of the synapses between the rod and cone cells and their postsynaptic bipolar cells. The loss of the first synapse in the visual pathway necessarily results in visual loss. We reported that detachment causes rod synaptic disruption very quickly (within 2 hours). Moreover, we discovered an approach that can significantly reduce the loss of synaptic connectivity at the first synapse. By reducing the activity of Rho kinase (ROCK), we can reduce trauma-induced cytoskeletal changes in photoreceptors and decrease the extent of rod synaptic terminals retraction and separation from their postsynaptic partners. Moreover, this inhibition of rod synaptic disjunction is correlated with improved scotopic electroretinographic (ERG) responses. This past year we focused on long-term (1 week) results after a single drug dose, and on the effects of ROCK inhibition on cone synapses. We are using adult pigs so that our results have the potential for translation to human patients.

2. KEYWORDS

Retinal detachment, retinal reattachment, rod photoreceptor, cone photoreceptor, rod spherule, cone pedicle, synaptic ribbon, synaptic retraction, synaptic disjunction, Rho A, Rho kinase, Lim kinase, ERG, scotopic or photopic responses, confocal microscopy, trauma

3. ACCOMPLISHMENTS

-What were the major goals of the project?

The major goals as stated in the SOW were the following:

Specific Aim 1- Test the ability of the new ROCK inhibitor netarsudil-M1, AR-13503, to stabilize photoreceptor synapses via single injection and/or a sustained–release delivery system over several days

Milestone #1 Determination of the dose response curve for soluble AR-13503- 100% complete

Milestone #2 Direct determination of whether a sustained-release drug application is an improvement over a single injection- 100% complete

Milestone #3 Assessment of an expansion of the length of time over which the injured, detached retina can be protected with the ROCK inhibitor AR-13503- 70% complete

Specific Aim 2- Determine how long after a retinal detachment injury a drug can be applied and still reduce synaptic disruption

Milestone #4 Determination of effectiveness of ROCK inhibition in a delayed treatment- 75% complete

Specific Aim 3- Determine if drug (either a ROCK or LIMK inhibitor) at the time of surgical reattachment helps promote recovery

Milestone #5(previously #7 in SOW) Determination of the efficacy of a drug application as an adjunctive treatment with retinal reattachment surgery-10% complete

-What was accomplished under these goals?

Milestone #1: Prior to the beginning of grant funding, we had determined that a subretinal injection of 0.5µM AR13503 significantly saved rod photoreceptor synapses 2 hours after detachment, by 63.8%. When our DoD grant started, we began by looking at 3 doses of an intravitreal injection applied immediately after creation of a detachment. Both eyes receive a detachment, drug dissolved in balanced salt solution (BSS) is injected into one eye BSS into the other, and then detachments remain for an additional 2 hours before euthanasia and enucleation. We compare synaptic damage in the treated eye with damage in the untreated eye by examining the retraction of rod terminals into the outer nuclear layer, signifying the breakage of the rod-bipolar synapse, in immunohistochemically-labeled confocal sections of retina. All 3 doses administered by intravitreal injection (0.5µM, 0.75µM, 1.5µM) reduced the disjunction of photoreceptor-bipolar synapses in the detached retina, however, the 0.5µM dose of soluble AR13503 proved to be the optimal dose for saving rod synapses for both subretinal and intravitreal injections.

Our original analyses of 2-hour detachments included the surprising finding that not only did the detached retina show synaptic disruption but so too did areas of attached retina adjacent to the detachment and as far as 1-2 cm from the border of detached-attached retina. In other words, attached retina also had synaptic disruption, albeit at lower levels than the detached retina. Indeed, subsequent examination demonstrated that after creating a localized detachment in the inferior nasal quadrant, rod synaptic injury is apparent in *every other quadrant* of the eye as well. Thus, detachment can injure much of the retina even if it is quite localized. Present results indicate that ROCK inhibition does not significantly reduce synaptic disjunction in these attached areas, even when ROCK inhibition induces significant reduction of disjunction in the detached retina.

The details of the procedures, results, and analysis leading to these conclusions are found in the paper published this year titled “ROCK inhibition reduces morphological and functional damage to rod synapses after retinal injury” in the Appendix.

This past year we examined the effects of detachment and application of AR13503 on cone synapses. Sections were triple labeled for PSD-95, which labels all presynaptic terminals, CtBP2, which labels all synaptic ribbons, and peanut agglutin (PNA) which is specific for cone cells. Fluorescently tagged PNA labels the membrane of the entire cone cell but is particularly prominent at the outer and inner segments and the cone pedicle. In this way, we could distinguish cone from rod synapses.

We learned that not all our specimens survived the long periods in the freezer made necessary by the pandemic when physical presence at the University for employees was prohibited unless they had clinical responsibilities. In some cases, dehydration and perhaps oxidation had occurred (‘freezer burn’ in layman’s terms). Although at low magnification the specimens appeared to be in good condition, at the higher magnifications needed for the analysis of synaptic morphology, the variability in the penetration of the immunohistochemical labeling was poor, and, thus, for selected specimens we did not feel confident in the results. We have completed the process of analyzing all the old material to determine what was usable and to decide what additional data we will need. We have now repeated experiments on animals 2 hrs, 2 days, and 1 week after detachment. In some cases, we now have adequate data for statistical analyses, and in other cases, we will need 1-2 more animals. The results obtained this past year follow.

Two-hour survivals. Using older material from 2 animals and one animal from a new experiment, we found that 0.75 uM AR13503 injected intravitreally at the time of detachment (a localized 4-5 disc diameter-wide detachment in the inferior nasal quadrant as done previously) reduced damage to the cone synapses. This dose was selected because the old material from experiments using this dose had survived without freezer burn. Without drug, cone pedicles in detached retina tend to change shape, either flattening or rounding as described by Fisher et al. (2005). Synaptic invaginations can also be less deep, and ribbons shorten. We quantitated the length of ribbons in cone pedicles and found a reduction in length, by about 50%, in pedicles after detachment. The number of ribbons also decreased. The results suggest there is protection of ribbons, both in length and number, in eyes treated with AR13503. Statistical analysis is pending.

Thus, damage to cone synapses over two hours seemed to be reduced by ROCK inhibition. Additionally, these results demonstrated that rapid damage to rod synapses is accompanied by rapid damage to cone synapses, within hours of detachment. (Please note, the optimal dose for rod synapses was 0.5 uM, although 0.75 uM was also effective in some eyes. In additional experiments, described below, we used retina treated with 0.5 uM AR13503 to examine cone synapses; 0.5 uM AR13503 was effective for both rod *and* cone synapses.)

Milestone #2: We examined drug administration with sustained-release fibers. In its fiber-based form, AR13503 is available in a bioerodible, polyester amide (PEA) polymer, 0.24x2mm. For a single implant, which contains a total of 14 µg of drug and maintains about 600 ng of drug in the vitreous cavity, the dose is equivalent to our successful dosage of 0.5µM with soluble drug. The results from this form of drug administration were variable in our hands, and the treatment benefit did not reach statistical significance. We are no longer pursuing this avenue of drug administration.

Milestone #3:

*Two-day survivals***.** To examine longer term effects of the ROCK inhibitor, we had previously examined eyes with detached retinas 2 days after the detachment injury. One eye was treated with a subretinal injection of 0.5µM AR13503 dissolved in BSS, and the other eye with BSS. By 2 days after surgery, most retinas had spontaneously reattached, so we could also look at retinal function with ERG.

The morphology and function of rod synapses, determined by confocal microscopy and scotopic ERG responses, respectively, were improved with subretinal injection of the ROCK inhibitor AR-13503. In addition, the anatomy and function correlated with one another, i.e., more synaptic disjunction resulted in lower scotopic responses. These results also are included in the attached publication "ROCK inhibition reduces morphological and functional damage to rod synapses after retinal injury."

This past year we began to look at the effects of detachment/reattachment on cone synapses. Material from one animal from our old experiments was not freezer damaged and when analyzed showed no difference in ribbon length in cone pedicles from detached retinas. In other words, this particular parameter of the cone pedicle recovers after spontaneous reattachment. This result is consistent with extant literature indicating that cone

photoreceptors tolerate detachment better than rods. We will be doing additional experiments to confirm this result.

We have also now looked are cone photoreceptor function after spontaneous reattachment of detached retina. Since the ERG recordings remain useful from the older experiments, we have been able to examine cone-specific responses from the same animals (n=5) that we used to examine rod-specific, scotopic responses. First, we did an additional analysis of ERG recordings to examine intra-animal variability of ERG responses. We found that there can be considerable variability in responses between eyes in the same animal. This observation confirms the necessity of our statistical approach- to assess changes over time compared to the original baseline in each eye individually. For photopic flicker responses, which test cone function primarily, we did not observe any statistical difference in eyes treated with ROCK inhibition compared with those treated with BSS. However, the data were very variable. In other words, some animals (3 of 5) did show improved function by 14-32% in the treated eye compared to the untreated eye. However, for the photopic b-wave (0 dB) responses, which also test cone function primarily, we did find a small but significant increase in the amplitude of the response in the eyes treated with the ROCK inhibitor compared to the untreated eye (GEE statistics).

Seven-day survivals. To look at longer time periods after treatment of a single subretinal injection of AR13503, we have maintained pigs for 1 week after detachments are made. Again, we treat one eye with the drug dissolved in BSS and the other with BSS. The animals are examined with ERG, making baseline recordings before any surgery and then again at 7 days. After the day-7 ERG recording and examination by fundus photography and OCT, the animal is euthanized and enucleated. The retinas are then examined morphologically for retracted rod spherules and for ribbon length in the cone pedicles. In the 3 animals examined so far, preliminary results suggest some improvement in rod synapses morphologically and improved scotopic responses in the treated eye 7 days after a single dose of AR13503. All animals, however, showed the existence of retracted rod terminals in the ONL. This finding indicates, as has been discussed by Fisher et al. (2005), that reattachment of the retina may reduce the amount of synapse retraction but does not appear to substantively reverse the damage initially caused by detachment. This result reinforces the importance of rapid treatment of damaged retina to prevent early injury to the retina.

For cone synapses, no difference in ribbon lengths was detected in two animals. Thus, of the morphological parameters that we examine, the rod synaptic changes seem longer lasting (possibly irreversible) than the cone changes. Functional analyses await. We will continue these experiments until we have examined enough animals to enable robust statistical testing (estimated to be 3-4 animals).

We also tested the drug, AR13503, over a 1-week time point in control animals (no detachments). The controls (n=3) consisted of injection of one eye with AR-13503 and the other eye with BSS. There was no difference in the amount of rod synaptic retraction, analyzed from sections with confocal microscopy, or in the rod specific responses by analysis of amplitudes in response to scotopic, -24 db., stimuli. Thus, AR-13503 is not toxic to the normal healthy eye.

Milestone #4: We have examined delayed treatment with ROCK inhibitor after detachment. We used a delay of 2 hours. Both eyes undergo BSS-induced retinal detachment; 2

hours later drug is applied by intravitreal injection of 0.5µM AR13503 into one eye and BSS into the other eye. After 2 more hours the animal is euthanized and enucleated, and the retinas are examined for synaptic damage.

For rod synapses, we have completed analysis of data from 5 animals. The result was significant (GEE and with mixed random effects statistics). Synaptic disjunction of rod synapses was reduced by 35.3% by intravitreal injection of 0.5uM AR13503.

We have examined 3 animals for the response of cone synapses after a delayed treatment. The preliminary results suggest that ROCK inhibition provides some protection for cones as well as rods. Eyes treated with AR13503 had longer ribbons, but there was considerable variability. In some untreated eyes, for instance, there was a total absence of ribbons. It appears that 4 hours of detachment can be very damaging for cone ribbons. The statistical analysis of treated versus untreated cone pedicles has not been done yet.

Despite the limitations mentioned, these results are exciting, as they suggest that treatment of patients with a ROCK inhibitor that is delayed by a few hours after injury will provide some protection to the retina. Delayed treatment will be important for translation to clinical practice, as it will allow for treatment of soldiers and civilians who cannot be treated immediately after injury.

Milestone #5: We have tested an inhibitor of LIM kinase called BMS-5. Two animals received 30 uM in DMSO and one animal received 300 uM in DMSO subretinally. All three experiments were 2-hr detachments. LIM kinase is a downstream effector of the RhoA pathway and another potential therapeutic target. There was no significant protection for rod synapses with this drug. Further, it appeared that the drug caused some apoptosis, i.e., was toxic. We are not sure whether DMSO contributed to this toxicity. But unfortunately, DMSO was needed to dissolve the BMS-5. At this time there are no alternative LIM kinase inhibitors that we can try. We are no longer pursuing this line of inquiry.

***Traumatic brain injury (TBI) in mice*.** In our grant, we proposed that ocular problems, associated with 70% of TBI patients, are due to retinal damage similar to what we observe with retinal detachment. We took advantage of the opportunity to examine mouse eyes from animals subjected to a single Controlled Cortical Impact (Garg et al. 2018) by a laboratory in our department. This work is being done with all required approved animal protocols at the New Jersey School of Medicine. In this preliminary examination of 2 eyes, we found that both eyes showed pronounced retraction of rod axons and terminals. This finding confirms previous data that we presented in our grant from a rat TBI protocol showing retinal damage after TBI. Further, the data support the hypothesis that ocular problems after TBI are due in part to damage to the retina at the level of the photoreceptor synapses. We are currently collaborating with two additional labs at Rutgers University to acquire more eyes from rodents subjected to TBI.

-What opportunities for training and professional development has the project provided?
Nothing to report

-How were the results disseminated to communities of interest?

The PIs and the postdoctoral fellow (who is currently a consultant) supported by this grant are all members of the Association for Research in Vision and Ophthalmology. We will submit an abstract for the annual ARVO meeting and hope to present our work in the spring. An abstract has also been submitted and accepted for the Vail Vitrectomy meeting, which is an invitation-only conference, to take place in the spring. Some of our results on cone synapses will be presented there by Marco Zarbin. We also have been invited to review our work at an ARVO symposium focused on translational science. Drs. Townes-Anderson and Zarbin will present the work.

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

Having examined our existing preserved retinal material, we determined what additional experiments are needed to examine the effects of detachment on cone synapses. We have now completed some of these experiments but still need a few animals at the one-week time point as well as a 2-hour delayed treatment after detachment. Addiitonally, we will add another normal animal to our controls for the experiments. Next, we will increase the delay of treatment after injury to 4 hours to test if such a delay still provides protection to the retina in terms of synaptic disruption and retinal function. Finally, we plan to begin our studies on retinal reattachment (*Milestone #5*), which will test if using ROCK inhibition as an adjunct to reattachment surgery is beneficial to retinal repair after detachment. The experiments on retinal reattachment will only occur if there are adequate funds remaining in the grant.

We will also complete our work on the analysis of cone synaptic structure and function. Specifically, we will complete our statistical analysis of cone synapses 2 hours, 2 days, and 1 week after detachment, comparing retinas with drug treatment to those without. We will also do a qualitative analysis of the cone synapse using the relatively new confocal imaging called Stimulated Emission Depletion (STED). Recently, we have had success in reconstructing cone pedicles after STED imaging and will present on these results in our next report. We will also examine cone synapses using Go alpha, an antibody against cone and rod bipolar cells. These experiments will determine if there are changes in the postsynaptic processes of the cone pedicle, i.e., in the bipolar cell dendrites.

Finally, as we have done with rod synapses, we will examine retina that is at some distance from the detachment to see if disruption of cone synapses spreads to other regions of the retina. We also will determine whether the area centralis in the porcine retina, which has some similarities to the macula in humans because it is an all-cone region of the retina, shows synaptic disruption. This analysis may be especially interesting as to-date there is an assumption that macula-sparing detachments are not associated with damage to central vision in contrast to macula-involving detachments. We can test in pig if detachments in the inferior nasal retina do or do not affect the area centralis.

4. IMPACT

-What was the impact on the development of the principal discipline(s) of the project?

Our work, both over the past two years and previously, leading up to the award of our grant, has demonstrated 3 things that are truly novel for the field of retinal trauma and care. First,

injury to the synaptic circuitry of the retina occurs very rapidly after retinal detachment, within 2 hours. This observation applies to the rod synapses as well as to the cone synapses. The rapidity may indicate a new urgency in how fast retinal detachment should be treated. Additionally, it may place retinal detachment on par with other central nervous system traumas such as spinal cord injury and stroke, which are now treated within hours. Second, our results show that retinal injury by detachment is not confined to the detachment but occurs all over the retina, i.e., in attached retina as well. Thus, the injury is larger than its gross manifestation and emphasizes the potential importance of rapid treatment. And third, we have demonstrated that a ROCK inhibitor, AR13503, is effective in reducing rod and cone synaptic injury if applied immediately after or during detachment. Our data on cone synapses remain to be tested statistically but seem very promising. Additionally, we have data suggesting that delayed application of the drug, by up to 2 hours, also will protect rod and cone synapses, although statistical analysis remains to be done. The practical application of this demonstration would be the use of ROCK inhibition for iatrogenic detachment used for gene therapy and transplantation of cells or implants. But additionally, we now are suggesting that delayed treatment would apply to retina trauma suffered on the battlefield or in accidental trauma.

-What was the impact on other disciplines?

It seemed possible to us that the RhoA pathway is involved in other traumas of the central nervous system and thus that ROCK inhibition might be useful for treating traumatic brain injury, for instance. Indeed, others have now published on this idea and confirmed our suggestion. But we also reasoned that the visual abnormalities seen with TBI may be due to synaptic disruption in the retina after brain injury. We had and now have more evidence that TBI does produce synaptic retraction in the retina and, thus, disjunction of the rod synapses. Thus, our studies on retinal detachment impact brain and visual injury more broadly.

-What was the impact on technology transfer?

Nothing to report

-What was the impact on society beyond science and technology?

The possibility of new therapies for ocular trauma should improve the quality of life for those who experience these traumas by reducing visual loss.

5. CHANGES/PROBLEMS

-Changes in approach and reasons for change

In Milestone #2 we had proposed the use of a drug-infused implant to provide a continuous release of the ROCK inhibitor AR13503. As described above, we tried several experiments, none of which yielded statistically significant results. In contrast, injection of soluble drug was consistently positive in preventing synaptic disjunction. We have discontinued the use of implants.

In Milestone #5, we proposed to test LIM kinase as another target for drug therapy. The drug we used was very insoluble and therefore required the use of DMSO. The drug in DMSO proved to be toxic to the retina, and we have discontinued this line of inquiry as other LIM kinase inhibitors are not available presently.

-Actual or anticipated problems or delays and actions or plans to resolve them

The Animal Facilities at our school was impacted by COVID-19. Initially, the facility was closed. It is now open, but personnel support is limited. Indeed, we had to proceed with experiments with animal technicians who were not well acquainted with our protocols, and this obstacle has delayed our progress.

More significantly, preserved retinas, fixed and kept frozen, showed deterioration akin to freezer burn over the months they were stored in the freezer. The period of storage was prolonged by the COVID-induced directives at the University. As a result, several experiments had to be repeated to get good quality images of cone synapses.

Finally, there have been several changes and adjustments to the personnel working on the grant. Celia Nunes left for another job, and Eva Halasz moved to Switzerland. We have been able to recruit Ilene Sugino to work with us. She formerly worked full time in Dr. Zarbin's lab and is well acquainted with most procedures. She and Dr. Townes-Anderson now do the ERG recording. Qian Sun’s duties have been expanded to include collection and fixation of the eyes after the experiments are completed and immunohistochemical labeling. Sectioning of the retina and imaging of the retina is now done at the medical school core facilities by Luke Fritzky, Director of the Core, with oversight by Ellen Townes-Anderson. Eva Halasz continues to work on the projects and is critical to image and ERG analyses, which are done digitally and therefore can be accomplished long distance. All members of the team stay in touch via the internet and zoom meetings are held periodically. Thus, we feel our personnel situation has restabilized, and we are enthusiastically completing various aspects of the grant.

-Changes that had a significant impact on expenditures

Although there has been rearrangement of budget items, i.e., we now pay for histology core services, there have been no significant unexpected expenses.

-Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

6. PRODUCTS

Publications, conference papers, and presentations

Halász É, Zarbin MA, Davidow AL, Frishman LJ, Gombkoto P, Townes-Anderson E. ROCK inhibition reduces morphological and functional damage to rod synapses after retinal injury. Sci

Rep. 2021 Jan 12;11(1):692. doi: 10.1038/s41598-020-80267-4. PMID: 33436892; PMCID: PMC7804129. Federal support acknowledged.

Townes-Anderson E, Halasz, E, Weiwei W, Zarbin, M. Coming of age for the photoreceptor synapse. Investigative Ophthalmology & Visual Science September 2021, Vol.62, 24. doi:https://doi.org/10.1167/iovs.62.12.24. Federal support acknowledged.

Zarbin, M, Townes-Anderson E, Halasz, E, Sugino IK. Rho Kinase Inhibition Reduces Photoreceptor Damage After Retinal Detachment: Possible Implications for Gene and Cell Therapy. 2021 Subspecialty Day. New Orleans, LA. November 12-13, 2021

Zarbin, M, Townes-Anderson E, Halasz, E, Sugino IK. Role of Rho Kinase Inhibitors in Managing Retinal Detachment. Moorfields UAE 2021: A Year in Focus. Dubai, UAE. January 12-15, 2022

Zarbin, M, Townes-Anderson E, Halasz, E, Sugino IK. Impact of ROCK Inhibition on Morphological and Functional Changes in the Cone Synapse after Retinal Injury. Vail Retina Meeting, Vail, CO, March 2022.

Townes-Anderson E. Potential Causes of Superior Visual Outcome Observed with Pneumatic Retinopexy vs. Vitrectomy. Comment on article, *JAMA Ophthalmol.* 2021;139(6):620-627. doi:10.1001/jamaophthalmol.2021.0803, July 1, 2021.

Website- Nothing to report
Technologies- Nothing to report
Inventions- Nothing to report
Other products- Nothing to report

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Ellen Townes-Anderson
Project Role: Principle Investigator
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 4
Contribution to Project: Supervisor of experiments, analyses, and publications, also participates in ERG recordings and oversees histological sectioning and imaging at the medical school's core imaging facility

Name: Marco Zarbin
Project Role: Co-Principle Investigator
Researcher Identifier): 0000-0002-7811-7132
Nearest person month worked: 1
Contribution to Project: Performs animal surgeries, reviews data analyses and publications

Name: Eva Halasz
Project Role: Postdoctoral Fellow, now Consultant
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 6
Contribution to Project: Performs histological and ERG analyses, and participates in development of experiments and publications

Name: Qian Sun
Project Role: Research Teaching Specialist
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 1
Contribution to Project: Surgical assistant in animal surgeries, maintains surgical equipment, harvests eyes after euthanasia, and performs immunocytochemical staining

Name: Ilene Sugino
Project Role: Research Interventionist
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 1
Contribution to Project: Assists in surgeries, in ERG recordings, and in OCT and Fundus photography

Name: Amy Davidow
Project Role: Statistician
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 1
Contribution to Project: Involved in all statistical analyses

Has there been a change in the active other support of the PD/PIs or senior /key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization name: University of Houston College of Optometry
Location: Houston, Texas
Contribution: Collaboration with Dr. Laura Frishman

8. SPECIAL REPORTING REQUIREMENTS

Quad Charts- attached

9. APPENDICES

scientific reports

Check for updates

OPEN

ROCK inhibition reduces morphological and functional damage to rod synapses after retinal injury

Éva Halász[1], Marco A. Zarbin[2], Amy L. Davidow[3], Laura J. Frishman[4], Peter Gombkoto[5] & Ellen Townes-Anderson[1,2]✉

Retinal detachment (RD) causes damage, including disjunction, of the rod photoreceptor-bipolar synapse, which disrupts vision and may contribute to the poor visual recovery observed after retinal reattachment surgery. We created a model of iatrogenic RD in adult female pigs to study damage to the rod-bipolar synapse after injury and the ability of a highly specific Rho-kinase (ROCK) inhibitor to preserve synaptic structure and function. This model mimics procedures used in humans when viral vectors or cells are injected subretinally for treatment of retinal disease. Synaptic disjunction by retraction of rod spherules, quantified by image analysis of confocal sections, was present 2 h after detachment and remained 2 days later even though the retina had spontaneously reattached by then. Moreover, spherule retraction occurred in attached retina 1–2 cms from detached retina. Synaptic damage was significantly reduced by ROCK inhibition in detached retina whether injected subretinally or intravitreally. Dark-adapted full-field electroretinograms were recorded in reattached retinas to assess rod-specific function. Reduction in synaptic injury correlated with increases in rod-driven responses in drug-treated eyes. Thus, ROCK inhibition helps prevent synaptic damage and improves functional outcomes after retinal injury and may be a useful adjunctive treatment in iatrogenic RD and other retinal degenerative diseases.

Retinal detachment (RD), the separation of the neural retina from the underlying retinal pigment epithelium (RPE), is a well-known cause of visual loss and has a major impact on quality of life[1]. Although the retina can be reattached by various surgical procedures, the final visual outcome is often unsatisfactory[2–8].

The reasons for poor visual recovery are not completely understood. Recognized factors include the duration, extent, and height of RD as well as involvement of the macula, which contains the cone-rich fovea[2,3,5,7,9,10]. Another factor may be injury-induced rearrangement of neural circuits in the retina. First described by Erickson et al. in 1983[11], rod presynaptic terminals retract from the outer plexiform layer (OPL) after RD, resulting in disjunction of the first synapse in the visual pathway, the photoreceptor-bipolar synapse. The synaptic disjunction has been conclusively documented in retinas days after detachment by electron microscopic examination of serial sections[12]. We have observed synaptic disjunction of rod-bipolar synapses after only 2 h of detachment using confocal microscopy[13]. Cone photoreceptors also respond to detachment and exhibit shape changes of their presynaptic pedicle and active zone, but they do not retract their terminals[14]. Besides the synaptic remodeling shown by the photoreceptors, other cells in the retina react as well. Bipolar and horizontal cells, for instance, sprout extensively into the outer nuclear layer (ONL)[15]. Reattachment of the retina does not fully repair these synapses. On the contrary, reattachment results in additional abnormalities, including sprouting of new neurites from rod photoreceptor terminals into the inner nuclear layer[16,17]. Histopathology of human retinas that have undergone retinal reattachment surgery shows very similar structural changes[18]. Thus, incomplete structural recovery has led us (and others[14]) to propose that synaptic changes contribute to the incomplete visual recovery observed in patients who undergo surgically successful retinal reattachment.

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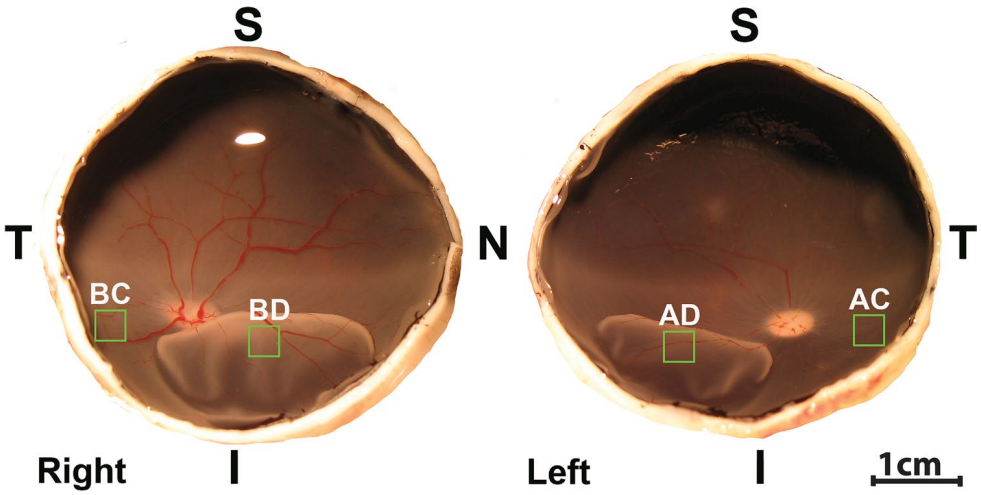

Figure 1. Eyecups from a right and left eye illustrating retinal detachments in the nasal-inferior quadrants. Location of retinal samples (green box) taken from BSS-treated eye attached (BC) and detached area (BD) and from the AR13503-treated eye corresponding attached (AC) and detached areas (AD). *S* superior, *T* temporal, *I* inferior, *N* nasal.

Our previous work with in vitro and in vivo RD models demonstrated a significant link between the activation of the RhoA pathway and rod axon retraction[13,19,20]. We showed that inhibition of activated Rho kinase (ROCK) by the ROCK inhibitors Y27632 and fasudil can reduce synaptic disjunction[13,21]. However, the effective concentration of these ROCK inhibitors was high, 1 and 10 mM, respectively, suggesting the possibility of off-target effects such as inhibition of protein kinase C or protein kinase A[22,23]. These concerns led us to evaluate AR13503, which is the active metabolite of Netarsudil[24], a clinically-approved ROCK inhibitor. AR13503 inhibits both ROCK isoforms, ROCK 1 and 2, 100-fold more potently than Y23632 or fasudil and therefore could have high efficacy at lower doses[25].

Here, we test whether AR13503 is effective in reducing rod-bipolar cell disjunction after RD and whether this inhibition improves retinal function 2 days after retinal reattachment. We tested ROCK inhibition in pigs because porcine eyes are similar to human eyes in size, retinal anatomy, and vasculature[26–28]. Pigs are diurnal, have both rod and cone photoreceptors, and the retina has an area centralis rich in cone cells that is similar in function to the macula in humans. Moreover, the porcine and human electroretinograms (ERGs) are similar[29,30].

To investigate the potential for possible clinical translation, we chose: (1) to use small detachments, similar in size and height to iatrogenic detachments used for subretinal injection of stem cells and viral vectors[31–33], and (2) to allow for spontaneous retinal reattachment, as often is done after such subretinal procedures. We show that the destructive structural and functional changes of the retina that occur after retinal injury are partially mitigated by the inhibition of ROCK activity. Thus, we propose a potential adjunctive therapy for iatrogenic detachments that uses ROCK inhibition to stabilize the synaptic circuitry of the neural retina. Stabilization of synaptic circuitry by ROCK inhibition may have wide application in other traumas and diseases of the central nervous system[34,35].

Results

Subretinal administration of AR13503 decreased the amount of rod terminal retraction in 2-h detachments. AR13503, a ROCK 1 and 2 inhibitor, is the active metabolite of the FDA-approved netarsudil (AR13324) developed by Aerie Pharmaceuticals Inc. (Durham, NC). AR13503 has a Ki of 0.2 nM for both ROCK 1 and 2, and Ki's of 1 nM and 27 nM for PKA and PKC, respectively[25]. It is likely to have higher efficacy in the eye than other ROCK inhibitors we have tested, as Y27632 has Ki's of 22 nM and 41 nM, and fasudil has Ki's of 76 nM and 47 nM for ROCK 1 and ROCK 2, respectively[25].

In order to compare the effects of AR13503 with our previous experiments using Y27632 and fasudil, we followed exactly the same protocols for the experimental RD and the morphological analysis of the retina as before[13,21]. We created detachments in both eyes of a single animal and treated 1 eye with subretinal injection of the ROCK inhibitor diluted in BSS and the fellow eye with BSS alone. We chose a concentration of 0.5 µM AR13503, in consultation with Aerie Pharmaceuticals. Once created, detachments remained for 2 h before euthanasia and enucleation. The presence of RD was confirmed after fixation and bisection of the eyes (Fig. 1). We examined retraction of rod spherules in confocal images of SV2-labeled retinal sections.

We have shown previously that SV2-immunolabeling in normal retina is observed only in the inner segments of photoreceptors and in the synaptic layers, i.e., the OPL and inner plexiform layers (IPL)[36]. However, after RD, SV2-labeling occurs in the ONL. Label in the ONL is due to retraction of the rod axon terminal and rearrangement of synaptic vesicles within the cells resulting in label in rod cell somata as well as in individual rod spherules[13,21]. Disconnection of the rod bipolar dendrites and the rod synaptic terminals in retina detached for 2 h has been described previously in our porcine retinal detachment model using rod bipolar and synaptic

markers[13].Thus, the synapses are broken. This interpretation is consistent with the phenomenon of synaptic disjunction observed in cat retinal detachment model using both light and electron microsocopy[11,12].

We have described previously the spread of retraction to attached regions in the inferior part of the retina after 2-h detachments[13]. In line with this observation, in the present experiments, rod synaptic terminal retraction into the ONL, occurs in detached retina (BD, AD) and also in attached retina approximately 1 cm away, inferotemporally, from the detachment (BC, AC) albeit at lower levels (see Fig. 1 for location of sampling; Fig. 2A–D). Here, we also addressed the question of how far the injury spread superiorly after RD. Using eyes in which the detachments were made with BSS, samples were taken from the nasal- and temporal-superior (NS, TS) quadrants of the retina approximately 1 and 2 cms away from the edge of the RD. Rod synaptic terminal retraction was present in all sections; the level of retraction was less than in the detached area (BD) (BD = 55.4 ± 13.5; NS = 13.3 ± 3.4; TS = 14.4 ± 3.6; n = 4 animals, all in pixels/μm of ONL length, + /− SD). Normalizing the amount of retraction in the attached retina by looking at the amount of retraction in attached retina/amount in detached retina, it appeared that retraction in attached retina was about 25% of the amount in detached retina both in the inferior and superior attached retina. Thus, synaptic injury appears to occur extensively in the retina 2 h after detachment and at least as far away as 2 cms from the edge of the detachment.

AR13503 treatment significantly decreased the number of SV2-labeled pixels in the detached retina (AD) by 63.8% (n = 3 animals, p = 0.001) compared to the untreated detached retina (BD) (Fig. 2E). However, we found no difference between the drug-treated attached retina (AC) compared to the corresponding attached retina in the control eyes (BC). The reduction of retraction in the detached retina by 0.5 μM AR13503 (63.8%) was greater than either 1–10 mM Y27632 (34.5–43.7%[13]) or 10 mM fasudil (51.3%[21]).

Thus, we conclude that (1) the synaptic disjunction spreads in the retina well beyond the detachment, and it is not confined to the detached area; (2) the drug primarily reduced the synaptic damage in the bleb area where the injury was induced, and the drug was applied; (3) AR13503 is more efficacious than previously used ROCK inhibitors.

Intravitreal administration of AR13503 decreased the amount of rod terminal retraction in 2-h detachments. Intravitreal injection of drugs is more straightforward clinically than subretinal injection because intravitreal injections can be done in an outpatient office setting whereas subretinal injections require surgery in an operating room. Therefore, we also administered AR13503 intravitreally at the time of retinal detachment to test for reduction of photoreceptor axonal retraction after RD. In order to compare with previous intravitreal injections of fasudil, we again followed our previous procedures [21]. Final AR13503 concentrations in the vitreous cavity were calculated to be 0.5, 0.75, and 1.5 μM for these experiments.

For 0.5 μM AR13503, there was a significant, 40.2% ($p < 0.0001$, n = 3 animals) difference between the extent of synaptic disjunction in the saline- and drug-treated detached areas (Fig. 3A). For the other doses, 0.75 and 1.5 μM AR13503, there was a reduction of 21.6% (n = 3 animals, p = 0.48) and 13.5% (n = 4 animals, p = 0.43) in SV2-labeled pixels in detached retinae; however, these reductions were not significant (Fig. 3B,C). The reduction in the detached retina by intravitreal 0.5 μM AR13503 was smaller than for subretinal injection of 0.5 μM AR13503.

These results indicated that immediate treatment with AR13503 via intravitreal injection reduces axon retraction after RD, primarily in the detached retina, and of the doses used, the 0.5 μM dose gave the best outcome.

Histopathology in the outer retina after retinal detachment and spontaneous retinal reattachment. With 2-h detachments, the application of subretinal 0.5 μM AR13503 during or immediately after the creation of a RD was more efficacious than fasudil or Y27632 in reducing the disjunction of the rod-bipolar synapses in the detached retina by preventing rod terminal retraction. Thus, we pursued experiments at a longer time point using the same dose to test the efficacy of AR13503 over time. Two days after detachment, most detachments have reattached (Fig. 4A). Only animals with fully reattached retinae were used for analysis. This time point thus served not only to test for the longer-term effects of the drug, but it also allowed for iatrogenic detachments to reattach spontaneously.

In all eyes, treated and untreated, SV2 labeling in the ONL, indicating rod axon terminal retraction, is present at 2 days after reattachment (Fig. 4B). Drug-treated detached areas showed 36.6% less synaptic retraction in the ONL than BSS detached areas (BC = 13.8 ± 4.3; BD = 22.3 ± 12.6; AD = 14.1 ± 5.4; AC = 11.3 + /− 3.9; all in pixels/μm of ONL length, + /− SD; paired t-test, n = 6 animals, p = 0.047). In order to compare the morphology to the full-field ERG responses, which provides information from the entire retina, we also examined the average numbers of retraction in each eye. Samples from the detached and attached areas of drug-treated eyes showed significantly less SV2 labeling in the ONL by 29.7% vs. the combined corresponding areas from the BSS eyes (Fig. 4C. combined BSS = 18.1 ± 7.2; combined AR13503 = 12.7 ± 3.4; all in pixels/μm of ONL length, + /-SD; n = 6 animals, p = 0.04).

Although SV2 labeling in the ONL is still present at day-2, we noted that the amount of retraction was less than in the sections from 2-h RDs. Statistical analysis showed that the BSS detached-reattached area had a significant 54.9% decrease in pixels in the ONL at day-2 compared to the 2-h detachment (BD = – 26.65 (-54.9%); average reduction in pixels/μm, n = 9 animals, p = 0.003, using 144 sections, 1080 images). Other areas showed slight decreases in the number of pixels, indicating rod photoreceptor synaptic terminal retraction in ONL, but these decreases were not significant (BC = – 0.76 (– 6.0%), p = 0.71; AD = – 4.19 (– 21.0%), p = 0.11; AC = – 3.67 (– 23.3%), p = 0.33; average reduction in pixels/μm of ONL length, reduction in percentage).

One plausible explanation for a decrease in SV2-labeled pixels in the ONL would be the death of rod photoreceptors. One of our earlier observations was that when the pig retina is detached for 4 h, rod photoreceptor cell death is present[21]. However, in the current 2 h and 2-day retinal samples we did not encounter pyknotic rod photoreceptor nuclei suggesting that reduced labeling is not due to cell death. Another plausible explanation for

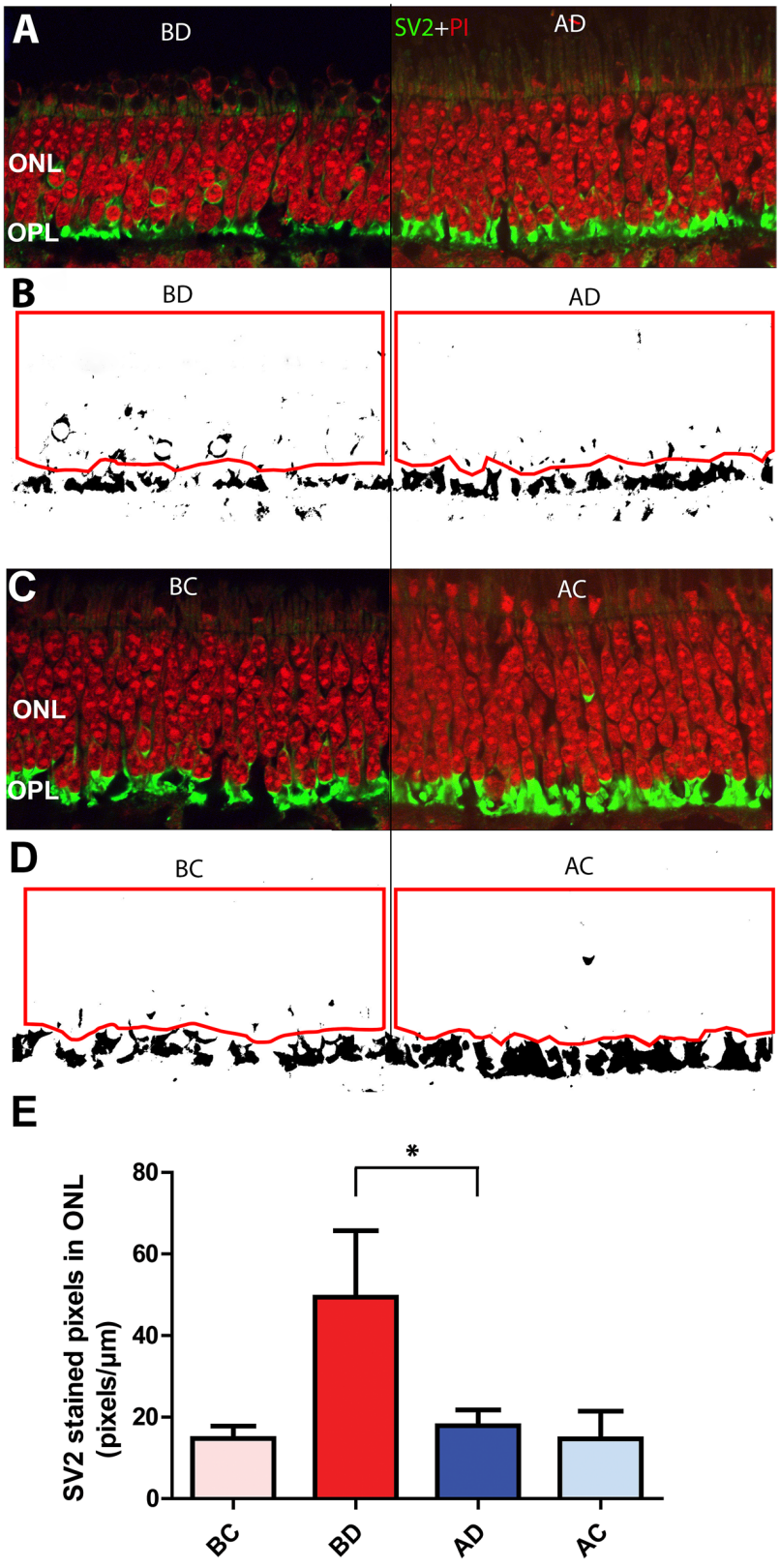

Figure 2. Effect of a subretinal dose of 0.5 µM AR13503 2 h after detachment. **(A,C)** Retina labeled for synaptic vesicle protein (SV2, green) and nuclei (propidium iodide, PI, red). Retracted rod spherules in the ONL are abundant in the sample from BSS eye detached area (BD)**. (B,D)** Binary mask created for SV2 channel for data analysis. Red line indicates the outline of the ONL, where the amount of SV2 labeling was measured. (**E)** There was significantly less rod spherule retraction in the AR13503-treated detached area (AD) compared to the detached area from the untreated eye (n = 3 animals, *p = 0.001, using 120 images/animal, +/− SD). BC, AC, attached areas of the untreated and treated retina.

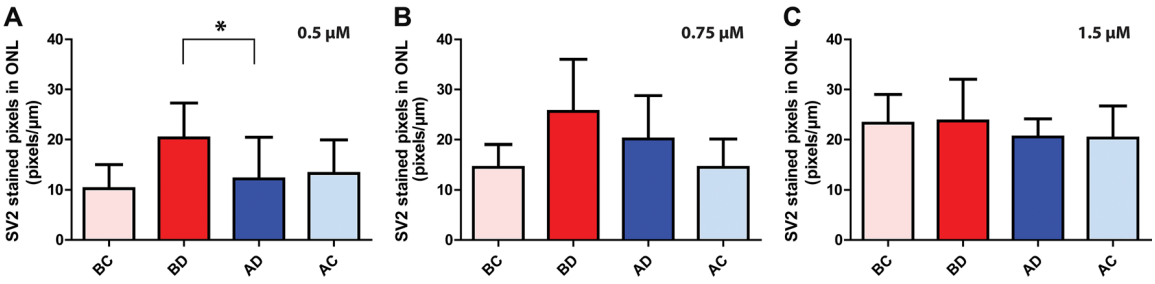

Figure 3. Effect of AR13503 intravitreal injection on axonal retraction of photoreceptors 2 h after retinal detachment. **(A)** 0.5 µM AR13503 treatment. There was a 40.2% decrease between the treated (AD) and the untreated (BD) detached areas (n = 3 animals, *p < 0.0001, using 120–180 images/animal, + /− SD). (**B)** 0.75 µM AR13503 treatment. Retraction was reduced by 21.6% in the treated detached area (AD) compared to the untreated detached area (BD) (n = 3 animals, p = 0.48, using 120–180 images/animal, + /− SD). (**C)** 1.5 µM AR13503 treatment. There was a 13.5% decrease in axonal retraction in the treated detached area (n = 4 animals, p = 0.43 using 120–180 images/animal, + /− SD). Only the 0.5 µM AR13503 dose showed a significant reduction in retraction. BC, AC, attached areas of the untreated and treated retina.

a decrease in SV2-labeled pixels can be a reduction in synaptic proteins or vesicles over time. The amount of total labeling in the outer retina (OPL plus ONL) was measured and compared in the 2-day and 2-h samples (n = 9 animals). There was no statistically significant difference in the number of labeled pixels in the outer retina over time, thus a decrease in synaptic proteins as a possible reason for a reduction of pixels in the ONL after 2 days is unlikely (data not shown). Rather, this result suggests there is movement of some SV2 protein/synaptic vesicles from the ONL back to the OPL.

In addition to rod photoreceptors, other cell types in the retina also react to the detachment injury. In particular, Lewis et al. [15] demonstrated that rod bipolar dendrites sprout into the ONL after detachment. Whether this occurred simultaneously with rod axon retraction was not known. Previously, we found that the rod and bipolar cell connection is disrupted when the retina is detached for 2 h[13]. At that timeframe, there was no evidence of a bipolar reaction. In our current 2-day experiments, we did observe occasional thin, hair-like sprouts from rod bipolar cells, identified by their PKC-alpha labeling (Fig. 4D). In some cases, the sprouts contacted SV2-labeled terminals. This sprouting was present in all eyes, both the BSS- and AR13503-treated eyes, and in both the detached and attached areas. The occurrence of sprouts was too infrequent (11 sprouts in 10.2 cm of examined retina, n = 1 animal) to be able to quantify any differences across the areas.

Thus, retraction is reduced but remains an important finding 2 days after detachment/reattachment, and treated eyes still had less synaptic disruption. In addition, rod bipolar dendritic sprouting is present by 2 days after detachment even in the presence of retinal reattachment.

AR13503 improved the functional outcome after retinal detachment and spontaneous reattachment. Because AR13503 continued to show a significant reduction of synaptic retraction 2 days after injection, we tested for possible functional differences in treated versus untreated retinae. Full-field dark-adapted ERG responses were recorded preoperatively (as baseline) and 2 days later. We focused on scotopic responses to test for rod cell function specifically. To account for variability between animals and between eyes within an animal, amplitudes were normalized as percent of baseline for each eye. Functional outcomes were in line with the morphological results as the b wave amplitude, an indication of the level of transmission between rod photoreceptors and ON-bipolar cells, was improved by 49% at 2 days in the AR13503 treated eyes compared to the BSS eyes (Fig. 5B paired t-test, n = 5 animals, p = 0.017). In 3 of 5 animals, the amplitude recovery not only reached the baseline level but exceeded the preoperative baseline after reattachment. Representative waveforms are shown in Fig. 5C,D. ERGs with larger than normal amplitudes have been termed, "supernormal ERGs"[37]. This phenomenon occurred mainly in the AR13503 treated eyes (3 of 4 eyes showing supernormal ERGs were treated with ROCK inhibitor). The implicit times were similarly delayed in the BSS- and the AR13503-treated eyes by 2 days compared to the preoperative (baseline) status, with an average of 8.7 ms (BSS, n = 5 animals, p = 0.005) and 9.0 ms (AR13503, n = 5 animals, p < 0.0001) respectively. Such delays would suggest that synaptic transmission between photoreceptors and rod-driven bipolar cells was not fully restored. However, based on response amplitude, the data suggest that ROCK inhibition treatment improves the functional outcome measured by ERG at 2 days.

Relationship between structure and function at 2 days. In general, reduced retraction in the detached retina appeared to correlate with increased scotopic ERG responses. To examine this relationship more broadly, we used all eyes that had reattached retinas and in which we recorded ERG scotopic responses after 2 days and analyzed retinal sections. Thus, we included animals that were not included in the analyses of drug efficacy because of the high dose used (animals #52, #53, #54) or the large size of the detachment (animal #52) or because the data were considered to be outliers (animal #50) (see Supplementary Table 1). We calculated the inter-eye differences within each animal for both morphology and ERG using averaged data (#-animal ID in Suppl. Table 1; percent difference in pixels/um and percent difference in ERG recovery; #45A = – 9/– 3; #45B = –

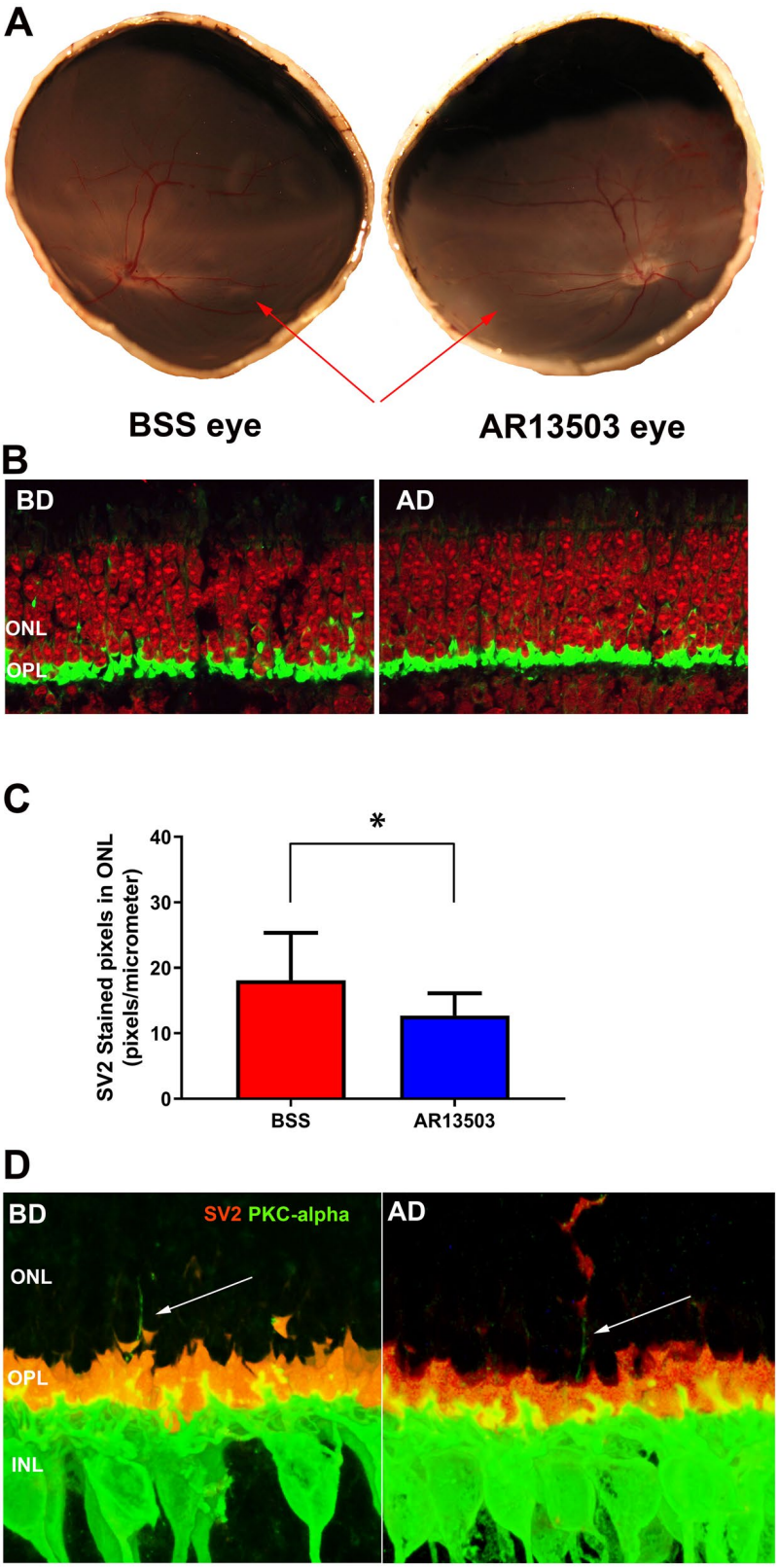

Figure 4. Morphological results 2 days after retinal detachment. **(A)** Detachments have spontaneously reattached (red arrows). (**B)** Retraction in the saline-treated eye detached area (BD) and the drug-treated eye, detached area (AD). Retina labeled for synaptic vesicle protein (SV2, green) and nuclei (propidium iodide, PI, red). (**C)** Axonal retraction was significantly reduced by 29.7% in AR13503-treated eyes compared to the eyes that received BSS alone (n = 6 animals, *p = 0.04, using 60 images/eye, + /- SD). Attached and detached areas of each eye were combined for this analysis (AC + AD vs. BC + BD). Data on the individual areas are reported in the Results. (**D)** Sprouting of bipolar cells. Retinae labeled for synaptic vesicle protein (SV2, red) and rod bipolar cells (anti-protein kinase C-alpha, PKC-alpha, green). White arrows indicate the fine dendritic processes of the bipolar cells extending into the outer nuclear layer (ONL). Pictures were taken of detached-spontaneously reattached areas both from BSS- (BD) and drug-treated (AD) eyes.

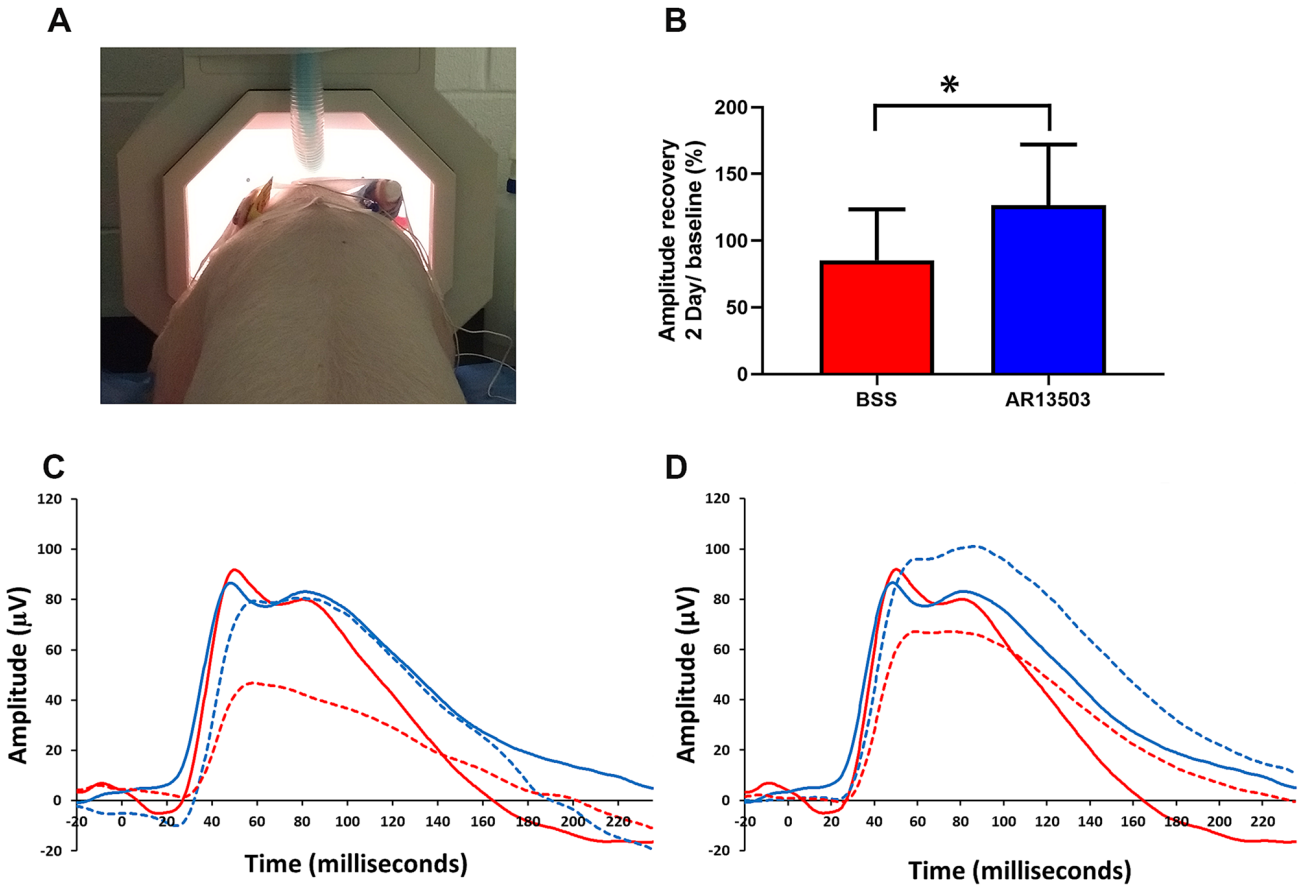

Figure 5. Electrophysiological results 2 days after retinal detachment of eyes with spontaneous retinal reattachment. **(A)** Position of pig in the Ganzfeld stimulator. **(B)** There was a 48.6% difference in amplitude recovery between the AR13503-treated eyes, subretinal injection of 0.5 µM, compared to the eyes that received BSS alone (paired t-test, n = 5 animals, *p = 0.017, + /- SD). **(C,D)** Representative waveforms of rod-specific scotopic responses (0.01 cd s m^{-2}). Red lines, untreated eye; blue lines, AR1303-treated eye. Solid line, preoperative response; dashed line, postoperative response. **(C)** At 2 days in the treated eye (blue solid and dashed lines) the evoked responses recovered to the baseline levels. However, in the untreated eye (red solid and dashed line) the response was lower than the recorded preoperative responses. **(D)** Representative waveform showing supernormal (higher than baseline) response recorded from treated eye (blue dashed line) at 2 days.

30/ + 47; #48 = − 34/ + 79; #49 = − 37/ + 46; #50 = + 44/− 1; #52 = + 36/− 14; #53 = − 48/ + 67; #54 = + 15/ + 30; #74 = − 2/ + 39; Fig. 6).

For morphological data, negative values indicate a reduction, and positive values indicate an increase in synaptic damage in the drug-treated eye compared to the control eye. For the ERG, negative values indicate deterioration, and positive values indicate an improvement in amplitude of the scotopic rod-specific response in the drug-treated eye compared to the BSS eye. Thus, in animals where a minus for morphological data is coupled with a plus for ERG values, the reduction in synaptic retraction was accompanied by improvement in rod-driven function. There was a negative correlation between the 2 variables ($r^2 = 0.681$, n = 9 animals, p = 0.006), suggesting that a decrease in synaptic damage by ROCK inhibition improves retinal function. When we use the morphological values from the detached area only (#-animal ID in Suppl. Table 1; percent difference in pixels/ µm and percent difference in ERG recovery; #45A = + 20/− 3; #45B = − 53/ + 47; #48 = − 33/ + 79; #49 = − 13/ + 46; #50 = + 10/− 1; #52 = + 18/− 14; #53 = − 18/ + 67; #54 = − 17/ + 30; #74 = − 30/ + 39), the correlation is similarly strong ($r^2 = 0.652$, n = 9 animals, p = 0.009). Although this correlation does not capture within eye and within animal variability, overall the relationship suggests that synaptic damage in the OPL is correlated with scotopic function. Moreover, it suggests that reducing retraction by ROCK inhibition may improve the outcomes of iatrogenic detachment/reattachment.

Discussion

Our previous in vivo work showed that RhoA activation occurs in the pig retina within 2 h after retinal injury and that ROCK inhibition can mitigate injury-related synaptic disjunction in the OPL[13,21]. However, both previously used ROCK inhibitors required high (millimolar) concentrations. Here, AR13503 was effective at a more than 1000 times lower dose than fasudil or Y27632. High millimolar doses could mean there are non-specific effects associated with the synaptic rescue, however, the efficacy of the nanomolar dose of AR13503 indicates

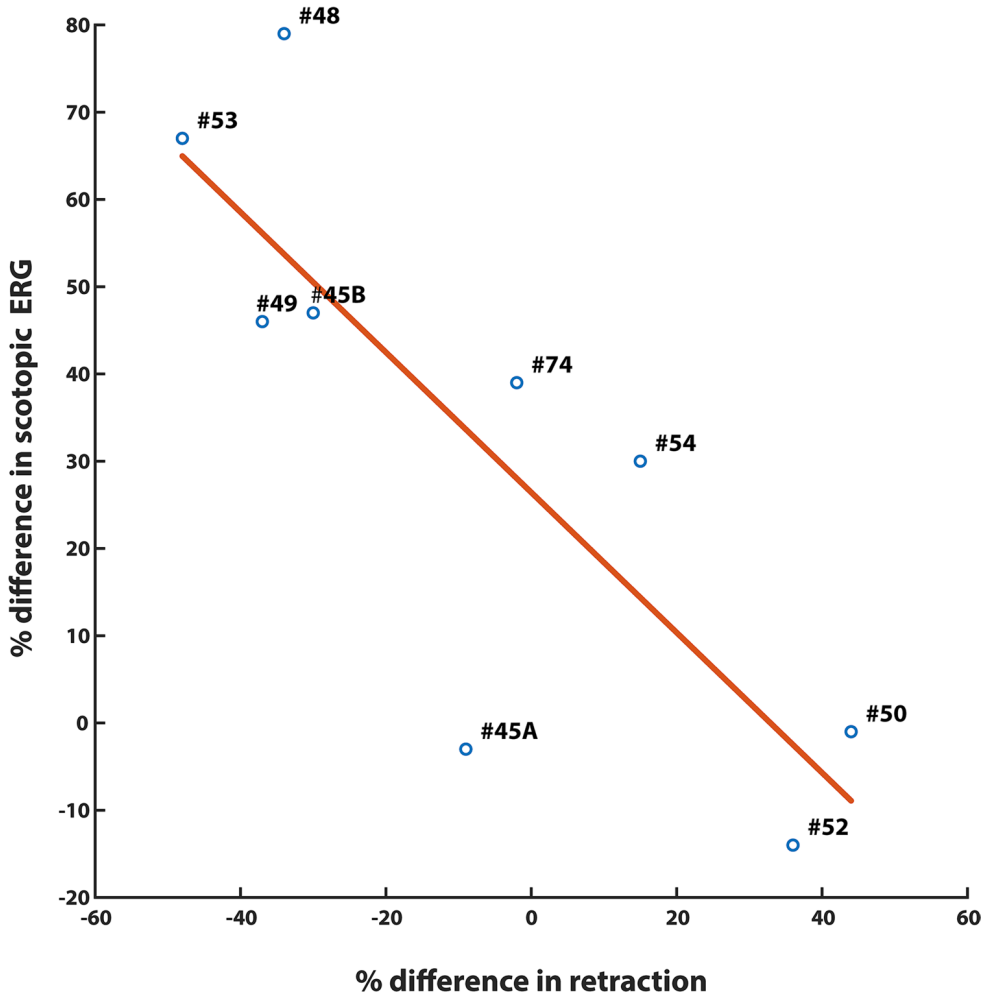

Figure 6. Correlation between the morphological and functional outcomes 2 days after injury. Linear regression model was applied to n = 9 animals. The dose of the drug (0.5 µM, n = 6 animals, and 25 µM, n = 3 animals) and the size of the retinal bleb (quarter-, n = 8 animals, or half of the retina, n = 1 animal) varied among these animals; a correlation between the anatomical and the functional change is present. (#numbers are identification numbers for individual animals, see Suppl. Table 1).

that specific effects against ROCK are the primary cause of the inhibition of synaptic disjunction in the detached retina.

Although both subretinal and intravitreal injection of 0.5 µM AR13503 were effective in the detached retina, subretinal injection showed greater inhibition of axonal retraction. This result may be due to different patterns of diffusion. During subretinal injection, the AR13503 solution is delivered directly to the site of the injury in a relatively limited space between the photoreceptors and the RPE, whereas after intravitreal injection, the drug can disperse widely and has more cellular layers to diffuse through to reach the photoreceptors.

We have reported that RhoA activation increases significantly in the adjacent attached retina[13], and our previous reports[13,21] as well as current results show widespread synaptic disjunction in attached regions of the retina in 2 h, even with these relatively small detachments. The reason for the widespread synaptic damage after injury is not known. Others have also reported changes in the attached retina including upregulation of GFAP[38], upregulation of inflammatory and immune-response related genes[39], and proliferation of retinal cells[40] although the timing of the changes and the methods of retinal injury varied from ours. Mechanical- or ischemia-induced spreading depression is well-known after brain trauma[41–43]. A similar phenomenon occurs in the retina[44]. We suggest that spreading depression may cause the wide-spread activation of RhoA and resulting rod axon retraction in the retina.

For the attached retina, there was no significant effect of ROCK inhibition on rod axon retraction. It is possible that diffusion again plays a role in these results. Transport properties of the RPE in detached and attached retina differ, which could cause more fluid flow across the detached versus the attached retina[45]. Previously we demonstrated that high doses (10 mM) of Y27632 showed significant reduction of retraction in attached retina[13]. High doses may increase the chance of obtaining effective drug levels in the attached retina. Alternatively, the effect could be due to some additional non-specific effect on another kinase, perhaps PKC. In other words, we cannot rule out the possibility that the pathways for retraction might be slightly different for detached and attached retina.

After 2 days, detached retina reattached spontaneously. However, retracted rod terminals remained in both previously detached and attached retina. It is known that some pathologies caused by detachment do repair over time following retinal reattachment. For example, the photosensitive outer segments are restored after reattachment[46]. But, consistent with our observations, disjunction of the rod photoreceptor-bipolar synapse is still apparent after the detached retina has been reattached. Fisher et al.[16] documented synaptic retraction in cat retina 28 days after reattachment of a 3-day detachment. In humans with RD not involving the fovea, approximately 25% have vision 20/40 or worse[7]. We speculate that among other causes, such as cystoid macular edema, macular hole, and epiretinal membrane formation, synaptic disjunction may contribute to suboptimal visual outcome, and its occurrence in fovea-sparing detachments may be due to spreading depolarization.

Fisher and colleagues[15] also described sprouting from rod bipolar cells into the ONL after detachment. Rod bipolar sprouting, as well as rod terminal retraction, have now also been observed in mouse models of retinal degeneration[47,48]. It has been suggested that as rod terminals retract, they "pull" the rod bipolar dendrites with them into the ONL[47]. However, Linberg et al.[12] identified many rod terminals unconnected to rod bipolar dendrites after a 1-week detachment. Previously, we reported that rod axon retraction was observed 2-h after detachment in the absence of bipolar sprouting [13], indicating that the rod-to-bipolar junction had separated. In the present experiments, 2 days after detachment followed by retinal reattachment, a few bipolar sprouts in contact with retracted terminals were observed, but most terminals were unattached to bipolar cells. It seems that after detachment, synaptic disjunction is the more prevalent initial event and that bipolar dendritic sprouting and contact with retracted presynaptic terminals is a subsequent event.

We did observe some significant reduction in synaptic protein labeling in the ONL after spontaneous reattachment of detached retina in untreated eyes, which suggests that some axonal retraction was reversed. This return of synaptic protein/vesicles to the OPL may be part of the growth of rod neuritic processes after reattachment observed by Fisher et al.[14,16,17]. Indeed, a month after reattachment of a 3-day detachment, neuritic processes from rod cells grow into the INL, in a manner consistent with other retinal diseases that affect rod cells[49,50]. To the best of our knowledge, the degree to which rod-bipolar synapses recover after retinal reattachment remains to be investigated. In ROCK-inhibited eyes, no significant change in levels of retraction was observed suggesting that the synaptic circuitry was more or less stable.

Drug treatment reduced retraction, compared to control, over the 2-day period we examined. Anatomical savings in the rod-bipolar synapse were coupled with improved rod-specific scotopic amplitudes in the treated eyes measured by ffERG. One surprising finding was the larger than baseline b-wave amplitudes at 2 days, termed "supernormal ERGs" in the earliest observations of large scotopic b-waves[37,51], primarily in the ROCK-inhibitor-treated eyes. Although increase in ERG amplitudes is uncommon among visual disorders, such observations have been reported in patients with cone dystrophies[37,51,52], early diabetic retinopathy[53], central retinal vein occlusion (CRVO)[54,55] and in animal disease models, e.g., Ant1-deficient mice[56], a rabbit model of retinal degeneration[57], and a rat traumatic brain injury model[58]. In normal porcine retina, intravitreal injection of AR13503 alone did not cause increased scotopic ERG responses after 7 days (n = 3 animals, unpublished data).

Although the mechanism for supernormal ERGs is unknown it has been suggested to be the result of the following. (1) An imbalance of the retinal excitatory and inhibitory signaling, in particular the lack or diminished presence of inhibitory signaling[58–60]. (2) Increased nitric oxide (NO)[61]. ROCK inhibition increases the phosphorylation of endothelial nitric oxide synthetase (eNOS), increasing NO production[62,63]. Thus, increased NO may underlie the development of supernormal ERGs in the ROCK-inhibited eyes. Or (3) VEGF levels in the retina. VEGF is known to increase eNOS and NO levels in the retina[64]. The VEGF effect may occur via VEGF receptor 2 activation and production of the classical NO effector cGMP[65,66]. Kroll et al.[67] suggested that ROCK inhibition enhanced the activation of VEGF receptor 2. Thus, VEGF may contribute to supernormal responses in the ROCK-inhibited eyes via VEGF receptor-induced increases of NO. However, it should be noted that the link between VEGF and ROCK inhibition continues to be investigated.

Whether supernormal ERGs in our ROCK-inhibited eyes mean better vision in the long-term is unknown, as the survival time in our experiments was quite short. However, work in patients is encouraging. Miyata et al.[55] suggested that after anti-VEGF treatment of non-ischemic CRVO, eyes with supernormal ERGs had a better prognosis after 1 year than non-ischemic CRVO eyes without supernormal ERG amplitudes.

This study has limitations. First, for the detachment-spontaneous reattachment experiments we do not know exactly when the retinae reattached. One of our earlier findings was that when the pig retina is detached for 4 h, rod photoreceptor cell death is abundant[21]. Since we did not encounter pyknotic photoreceptor nuclei in the present study, we speculate that the retina reattached within the first 4 h after the blebs were created. Second, at this time we have focused only on the anatomical and electrophysiological changes occurring in the rod-bipolar synapse. In the future, we will examine how other cell types behave in this iatrogenic RD model and whether ROCK inhibition affects them.

Therapeutic approaches for subretinal delivery of stem cells, viral vectors, or visual prostheses involve iatrogenic RDs. In some cases, iatrogenic detachment involves active surgical reattachment[32,68,69]; in other cases, the retina reattaches spontaneously[33,70,71]. Our evidence indicates that even brief, relatively small detachments cause synaptic damage that spreads throughout the retina and reduces retinal function, at least at the 2-day time point. There are undoubtedly multiple reasons for poor visual recovery in therapies involving iatrogenic detachment[31–33]. We suggest that the addition of ROCK inhibition subretinally or intravitreally during or perhaps prior to the detachment may improve post-procedure outcomes.

In addition to RD, there is a list of retinal disorders that exhibit rod synaptic terminal retraction: age-related macular degeneration[72,73]; rat models of glaucoma[74], retinal degeneration[75], and oxygen-induced retinopathy[76]; mouse models of retinoschisis[77] and congenital stationary night blindness[47]. If the synaptic pathology is caused by RhoA activation in these disorders, ROCK inhibition could be beneficial for a broad spectrum of retinal disease.

Our findings may also be relevant more generally to CNS injury. In traumatic brain injury (TBI) for instance, there can be extensive damage to synaptic connections[35,78–80] resulting in synaptic loss as well as structural remodeling of dendritic spines[35,78]. The synaptic damage extends beyond the immediate area of trauma[81]. The downstream effectors of RhoA contribute to synaptic plasticity[82], and RhoA is also upregulated in brain injury[83,84]. Thus, it seems reasonable to suggest that ROCK inhibition can prevent synaptic disjunction in the brain after trauma. Indeed, the use of the inhibitor fasudil has already been successful in preventing synaptic damage and restoring function in a rodent model of TBI[35]. Further, our model of RD is poised to be a useful scenario in which to test RhoA-ROCK inhibition in CNS injury. We have tested, for example, the inhibition of a RhoA downstream effector LIMK that showed effects as robust as ROCK inhibition in RD[85]. Continued investigations to rescue synaptic circuitry after retinal injury may contribute to potential therapies for TBI-related and other neurodegenerations.

Materials and methods

Animals. Three-month-old female Yorkshire pigs, weighing 30 kg, were obtained from Animal Biotech Industries (Danboro, PA, USA) and kept on a 12-h light/12-h dark cycle for at least 1 week prior to use. Animals were housed in an Association for Accreditation and Assessment of Laboratory Animal Care (AAALAC)-accredited pathogen-free facility, 1 animal to a pen. They were subject to overnight fasting with access to water ad libitum before surgery. Experimental procedures and methods of euthanasia were approved by the New Jersey Medical School Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A total of 22 animals and 44 eyes were used. Further description of the animals can be found in Supplementary Table 1.

Retinal detachment and experimental design. RDs were created under general anesthesia. Animals were injected with atropine (0.02 mg/kg; VetUS, Henry Schein, Dublin, OH, USA) and sedated with an injection of ketamine (20 mg/kg; Mylan Institutional LLC, Galway, Ireland) and xylazine (2.2 mg/kg; Lloyd Lab., Shenandoah, IA, USA), all administered intramuscularly. After 5–10 min, a peripheral venous catheter was inserted through the auricular vein, and the animal was intubated with an endotracheal tube. To maintain anesthesia, the animals were supplied with 0.5% to 3.0% isoflurane in oxygen using a ventilator. Lactated Ringer's solution was infused intravenously at a rate of 8 mL/kg/h. Vital signs (oxygen saturation, heart rate, and body temperature) were monitored and maintained within the normal range throughout the experiment.

For surgery, pupils were dilated with topical application of 1% Tropicamide (Bausch&Lomb, Tampa, FL, USA) and 2.5% phenylephrine (Paragon Bioteck, Portland, OR, USA). A standard 3-port vitrectomy was performed using 20-g instrumentation. The posterior hyaloid was detached over the area centralis using active suction and a core vitrectomy was completed. During and after vitrectomy, the vitreous cavity of the eye was perfused with balanced salt solution (BSS; Alcon, Fort Worth, TX, USA) containing 2 µg/mL epinephrine (Henry Schein, Dublin, OH, USA). A 33-g metal cannula was used to slowly inject BSS or Rho kinase inhibitor, AR13503 (Aerie Pharmaceuticals, Durham, NC, USA) dissolved in BSS, subretinally to create a RD (~ 10–15 mm in diameter) in the inferior nasal quadrant (Fig. 1). For intravitreal administration of drug, 150 µL of 10, 15, or 30 µM AR13503 dissolved in BSS was injected with a 30-gauge needle into the vitreous cavity (entering ~ 3 mm posterior to the limbus). Immediately after the procedure, the sclerotomies were closed with 7–0 vicryl suture. The volume of the vitreous cavity was calculated to be ~ 3 ml, and the final intravitreal concentrations were estimated to be 0.5, 0.75, and 1.5 µM, respectively.

After RDs were created the animals survived for an additional 2 h or 2 days. For the 2-h procedures, animals were kept under anesthesia for the 2 h after detachments were made and then euthanized with 7 ml intravenous Euthasol (Vibrac AH, Fort Worth, TX, USA) for enucleation. For the longer survivals, the conjunctiva was sutured after the sclerotomies were closed, 1.6 mg (0.4 ml) Dexamethasone (Fresenius Kabi, Lake Zurich, IL, USA) and 0.1 g (0.5 ml) Cefazolin (WG Critical Care, LLC, Paramus, NJ, USA) were injected subconjunctivally, and Tobradex ointment (Alcon, Fort Worth, TX, USA) was applied topically. Once the animals had recovered, they were maintained in their cage, with constant monitoring, for an additional two days. Animals were administered pre- and postoperative intramuscular injections of buprenorphine (0.01–0.05 mg/kg; Reckitt Benckiser HealthCare, Hull, England) and enrofloxacin (10 mg/ kg; Bayer HealthCare, Shawnee, KS, USA). At the 2-day time point the animals were again anesthetized, using the previous protocol, for ERG recording and structural analysis by fundus photography and optical coherence tomography (OCT) before being euthanized with 7 ml intravenous Euthasol for enucleation.

Full-field flash electroretinogram (ffERG), fundus photography, optical coherence tomography (OCT). The procedures for recording ffERGs, fundus photography, and OCT were done under general anesthesia, as described above. For all 3 procedures pupils were dilated and accommodation relaxed with topical applications of 1% Tropicamide and 2.5% phenylephrine hydrochloride drops. Adjustable lid specula were used to keep the eyelids separated. ERGs were recorded in animals that had 2-day survivals both before retinal surgery and 2 days after surgery. Fundus photography and OCT were performed in the animals 2 days after retinal surgery to confirm the status of the retina.

During electroretinography, flashes were produced and responses recorded using a UTAS ERG system with a BigShot Ganzfeld stimulator (LKC Technologies, Inc., Gaithersburg, MD, USA). The pig's head was placed inside of the ganzfeld bowl (Fig. 5A), and bilateral ERGs were recorded simultaneously using ERG-Jet electrodes (Fabrinal SA, La Chaux-de-Fonds, Switzerland) placed on the cornea. The cornea was kept moist with a hypromellose ophthalmic demulcent solution 2.5% (Akorn Inc, Lake Forest, IL, USA). The reference electrode was placed at the midline of the forehead, about the same distance from both eyes. The ground electrode was placed in midline on

the back between the shoulders of the animal. The stimulus protocol was based on the International Society for Clinical Electrophysiology of Vision (ISCEV) standard for clinical ffERG[86]. Briefly, after 30 min of dark adaptation, the ffERG was recorded to strobe flash intensities of 0.01 cd s m^{-2} with an interstimulus interval (ISI) of 2 s (15 samples) to isolate the rod scotopic response. A notch filter (60 Hz) and 85 Hz low pass filter were applied during data analysis using Matlab (The Mathworks, Natick, MA, USA) to eliminate noise and the oscillatory potentials. The amplitude and implicit time were measured from stimulus onset to b-wave peak, datapoints were automatically identified and values were calculated by custom made script in Matlab (The Mathworks, Natick, MA, USA). Individual responses were analyzed, and aberrant waveforms rejected before averaging.

Sample preparation and immunohistochemistry. After enucleation the eyes were immersed in 4% paraformaldehyde (EMS, Hatfield, PA, USA) for 15 min; a 5 mm slit was made at the ora serrata to aid in rapid fixation. The eyes were then opened; the anterior segment and any remaining vitreous humor were removed carefully, and eyecups fixed overnight at 4 °C. Samples were collected from areas of retina that had been detached and from areas of the retina that had not been detached as diagrammed in Fig. 1. Retinae were immersed in 30% sucrose overnight at 4 °C. On the consecutive day, specimens were embedded in OCT compound (Sakura Finetek, Torrance, CA, USA) at room temperature for 2 h, then frozen and cut into 25-µm-thick sections using a cryostat, as described previously[13].

Procedures for immunolabeling were as previously described[87]. Briefly, sections were washed 2 times with 0.3% Triton X-100 in PBS, blocked with 10% blocking buffer for 1 h at room temperature, and then incubated either in antibody for SV2 (1:100 dilution, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) or antibody for PKC-alpha (1:100 dilution, Cell Signaling Technology, Boston, MA, USA) overnight at 4 °C. The next day, the sections were washed 3 times with 0.3% Triton-100 in PBS and incubated with secondary antibodies conjugated to Alexa Fluor 488, 546, or 647 (1:100 dilution, Life Technologies, Norwalk, CT, USA) for 90 min at room temperature, followed by nuclear staining with 1 µg/mL propidium iodide (1:100 dilution, PI; Sigma-Aldrich, St. Louis, MO, USA) or TO-PRO3 (1:500 dilution; Life Technologies, Norwalk, CT, USA) for 5 min at room temperature. After 2 washes with 0.3% Triton-100 in PBS, sections were covered with Fluoromount-G medium (SouthernBiotech, Birmingham, AL, USA) and preserved under coverslips sealed with nail polish. For all immunohistochemistry, sections from retinal areas to be compared were placed on a single slide so that they were labeled together, under the same conditions; control sections were also processed simultaneously with experimental sections but without primary antibodies.

Quantification of axonal retraction. All data were collected by persons masked to the sample identifications. Sections were examined using confocal microscopy (model LSM510; Carl Zeiss Microscopy, Jena, Germany) by scanning 1 µm optically thick sections with a 63 × oil immersion objective. Brightness and contrast were set to obtain unsaturated images. Laser power and scanning rate were unchanged throughout a single experiment. Enhancements in brightness and contrast were performed (Photoshop 7.0 software; Adobe, CA, USA) only for presentation purposes[13].

Two samples (BC, BD or AC, AD) from each eye, four samples per animal, were obtained (Fig. 1); 30–45 images were taken of each retina sample, and data were collected from two to four sections per sample, examining at least three different areas of each section. SV2 immunolabeling in the ONL was analyzed as described[13]. Briefly, a binary mask of the green channel was created for each image, the ONL was outlined using the PI labeled image as a guide, and the pixels in the ONL of the binary image were counted using ImageJ software (v1.45s; NIH). The measurements are reported as pixels per micrometer of ONL length.

To quantify total SV2 labeling in the outer retina a similar method was used: after the binary mask was created, both the ONL and the OPL were outlined, and pixels in the outlined area were counted.

Statistical analysis. For statistical analysis Student's t-test and generalized estimating equation (GEE[88]) were used. Normality was tested using the Shapiro-Wilks test. Use of the paired t-test was based on the experimental design, one eye was treated, and the other was untreated. Eyes were randomized for BSS or AR13503 treatment. Generalized estimating equation (GEE) was applied to estimate the parameters of a linear model with a possible unknown correlation between outcomes. To capture the strength of the relationship between the anatomical and the functional outcomes, we estimated a Pearson's correlation coefficient from the average change in scotopic ERG and the average change in retraction. While the number of experimental units (animals) was at most 3 to 6 in each experiment, the number of outcomes for each eye-treatment-time combination was large and thus the use of large sample methods that adjust for intraclass correlation, e.g., GEE, is justified.

Statistical analysis was performed with GraphPad Prism 5.1, Matlab (The Mathworks, Natick, MA, USA) and SAS (Version 9.4). The graphics were produced using GraphPad Prism 5.1 and Matlab (The Mathworks, Natick, MA, USA). Data are expressed as mean +/− the standard deviation (SD). We set alpha (type I error rate) at 0.05. Reported p-values were obtained via GEE analysis unless otherwise noted.

Data availability

All relevant data generated or analyzed during this study are included in this manuscript and the supplementary information. Raw data can be obtained from corresponding author.

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Author contributions
E.H., M.Z., E.T.-A.: designed research; E.H., M.Z.: performed research; E.H., M.Z., A.D., L.F., P.G., E.T.-A.: analyzed data; E.H., M.Z., L.F., E.T.-A.: wrote the paper.

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Perspective

Coming of Age for the Photoreceptor Synapse

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Purpose. To discuss the potential contribution of rod and cone synapses to the loss of visual function in retinal injury and disease.

Methods. The published literature and the authors' own work were reviewed.

Results. Retinal detachment is used as a case study of rod spherule and cone pedicle plasticity after injury. Both rod and cone photoreceptors terminals are damaged after detachment although the structural changes observed are only partially overlapping. For second-order neurons, only those associated with rod spherules respond consistently to injury by remodeling. Examination of signaling pathways involved in plasticity of conventional synapses and in neural development has been and may continue to be productive in discovering novel therapeutic targets. Rho kinase (ROCK) inhibition is an example of therapy that may reduce synaptic damage by preserving normal synaptic structure of rod and cone cells.

Conclusions. We hypothesize that synaptic damage contributes to poor visual restoration after otherwise successful anatomical repair of retinal detachment. A similar situation may exist for patients with degenerative retinal disease. Thus, synaptic structure and function should be routinely studied, as this information may disclose therapeutic strategies to mitigate visual loss.

Keywords: photoreceptor morphology, plasticity, RhoA-ROCK, synapse, retinal detachment

Sensory receptors, and photoreceptors in particular, are exquisitely complex cells. At one end, a photosensitive organelle, the outer segment, which transduces energy from visible light into a membrane potential change, connected by a modified cilium, which helps create the membranous outer segment, to an inner segment where metabolic needs are met and proteins synthesized, then the cell body with the nucleus, and a fiber that is both axon- and dendrite-like extends to the final compartment, a presynaptic terminal. But not a conventional terminal; it is a ribbon synapse highly specialized to deliver glutamate in ever changing amounts, in response to light levels, to multiple postsynaptic cells. However, when describing the effects of disease or injury on this complex receptor, reports most often focus on the changes in the outer segment: are the membranous disks disorganized, how many are gone, and has the length of the outer segment returned to normal? We would like instead to turn the spotlight to the synaptic terminal, the first synapse in the visual pathway without which no sensation of light would occur.

Rod Spherules

More than 30 years ago, in a cat model of retinal detachment, changes in the first synapse were noted in response to the detachment injury.[1,2] Because of the ease of immunocytochemical detection, more is known about rod synapses after detachment: in contrast to cone terminals, rod presynaptic terminals retain their characteristic proteins and synaptic markers while undergoing dramatic movements in response to injury, uncoupling from their postsynaptic partners and withdrawing into the outer nuclear layer (ONL).[3] After retraction of the spherule, the rod cell's postsynaptic partners react; rod bipolar dendrites sprout, extending into the outer nuclear layer, and horizontal cell axons grow extensively in the outer and inner retina.[2,5] Surprisingly, and in contrast to the regeneration of outer segments, reattachment of the retina does not restore the outer plexiform layer. In fact, rod terminals continue to exist in the outer nuclear layer weeks after reattachment.[5] In addition, new structural plasticities occur. At rod terminals, neuritic sprouts, visible because of the abnormal diffusion of opsin throughout the rod cell plasma membrane, extend into the inner nuclear layer and develop presynaptic varicosities. Although some normal synaptic structures, like ribbons, have been described in the varicosities along the rod sprouts, normal synaptic contacts with other retinal neurons do not form.[5]

In our more recent studies on retinal detachment using a pig model, we also observed many of these synaptic changes (Fig. 1). Our work has looked at shorter timeframes and therefore has added new information: retraction of the rod presynaptic terminal occurs within hours of

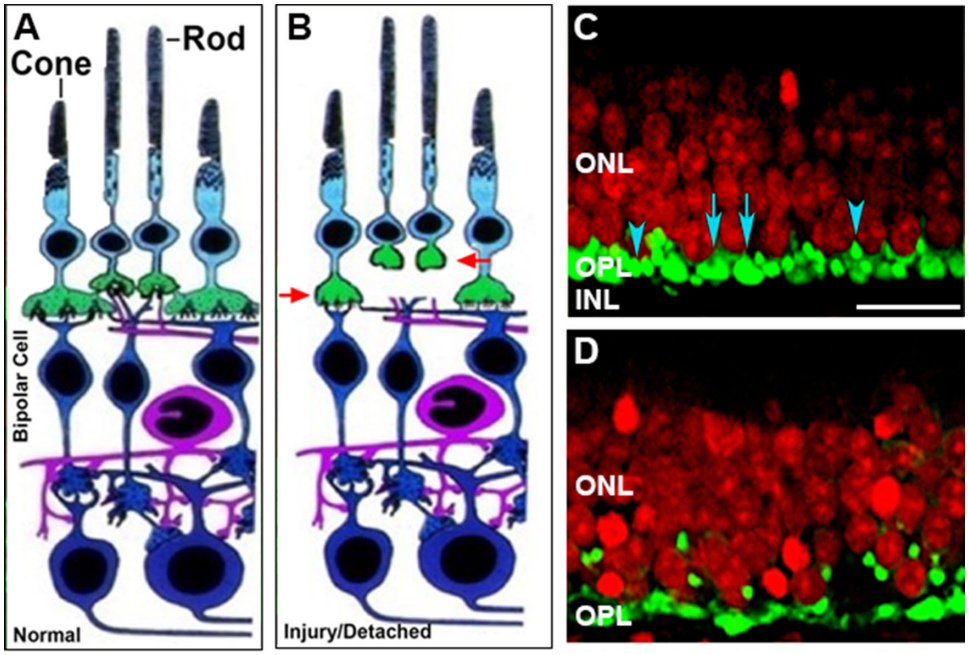

Figure 1. Injury-induced synaptic disjunction. **A.** Diagram of normal retina, modified from Dowling and Boycott 1966.[86] **B**. After detachment, rod axons and terminals retract from the outer plexiform layer and cone terminals round up (*red arrows*). **C**. Detached retina labeled for synaptic proteins (SV2, *green*) and nuclei (*red*). *Top*, within hours after detachment rod (*blue arrowheads*) and cone (*blue arrows*) become rounded in shape. **D**. 24 hours later retracted rod spherules are present in the outer nuclear layer while pedicles appear flattened. *Scale bar*, 10 µm. **C-D**. Porcine retina maintained in vitro, modified from Fontainhas and Townes-Anderson 2011.[40]

detachment, in other words, very quickly,[6] and rod synaptic reactions occur in many places throughout the retina including more than a centimeter away from the detachment in areas that remain attached.[6–8] It appears that there is a wave of change across most of the retina in response to the local injury. Two to seven days later, when the retina has spontaneously reattached, rod terminals remain in the outer nuclear layer, although in reduced numbers compared to two hours after detachment[8] (unpublished data, 2020). Bipolar cell sprouting in our model begins about two days after detachment/reattachment.[8]

Both the previous retinal detachment studies and our own suggest that continued disruption of synapses contribute to the visual disturbances, including lower acuity, consistently observed after anatomically successful reattachment surgery.[9–16] Indeed, we saw a high correlation between the amount of rod synaptic retraction, determined by misplaced synaptic vesicle labeling, and the reduction in scotopic responses two days after detachment/reattachment.[8] In other words, in addition to damaged outer and inner segments, loss of synapses due to synaptic remodeling can contribute to the lack of physiological recovery after retinal detachment.

Genetic Retinal Degeneration

Are rod synaptic changes unique to retinal detachment injury? Published descriptions of synaptic injury in retinal degeneration are now quite common. Retracted rod presynaptic terminals are found in the outer nuclear layer in human retinitis pigmentosa (RP),[17] in models of congenital stationary blindness,[18] glaucoma,[19] retinal degeneration (autosomal recessive RP,[20] X-linked RP[21,22]), oxygen-induced retinopathy (OIR),[23] retinoschisis,[24] and in human and animal models of normal aging and age-related macular degeneration (AMD).[25–28] Bipolar and horizontal cell sprouting has been described in human RP,[17] AMD and aging,[25,26] and models of RP,[21,22] congenital stationary night blindness,[18] and AMD.[27,28] Finally, rod neuritic sprouts in the inner retina have been found in multiple subtypes of human RP,[17,29,30] in animal models of RP,[31–33] in AMD,[34] in rod/cone dysplasia,[35] and after laser damage.[36] Thus, we should add rod synaptic change and loss to the set of problems to be considered and addressed in new therapies for retinal disease.

Sequence of Synaptic Change

If one examines the list of observations for rod terminal retraction, sprouting by bipolar and horizontal postsynaptic partners, and rod neuritic sprouting, it is evident that these phenomena frequently occur in the same injury or disease, suggesting that the neurons involved in the first synapse of the visual system work as a functional unit not only in normal physiology but also in pathology with a stereotypical response. We have reported that rod terminal retraction occurs first[6] in response to detachment. In retinal disease some have suggested that the entire synaptic complex is retracted into the ONL.[18] However, examination of the very early events, which might show that retraction of the spherule occurs first, is often absent. Alternatively, the nature or the magnitude of the perturbation in the circuitry could induce different reactions. Sprouting of postsynaptic cells may be sequential. In a mouse retina, mutant for the presynaptic scaffolding protein bassoon, horizontal cell sprouting occurs before rod bipolar neuritic growth.[37] Finally, it seems that rod neuritic sprouting into the inner retina occurs after sprouting of the secondary neurons as

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it is a phenomenon seen after retinal reattachment, long after detachment-associated changes have occurred. In the mouse, rod cell sprouting does not occur, perhaps because of rapid rod cell death in most mouse retinal degenerations.[29] With this scenario in mind, it is tantalizing to think that if rod terminal retraction is blocked, no further remodeling and synaptic disruption would occur in the rod pathway.

Cone Pedicles

In human cone cell disease, not all functional visual loss correlates with degenerative outer/inner segment changes: in human X-linked RP (XLRP) with mutations in the RP-GTPase regulator gene (*RPGR*), loss of retinal sensitivity to 543 nm light compared with cone inner segment thickness and cell density reductions as seen with high resolution microperimetry, was greater than expected.[38] Recently, in the rd9 mouse, a model for XLRP, rod cell spherule retraction and postsynaptic cell sprouting were described, and, despite the normalcy of cone cell morphology, reduction in photopic b-wave responses was reported.[21] Similarly, in two canine models of RP with *RPGR* mutations, substantial rod circuitry remodeling was reported, which caused reduced retinal function, although no cone synaptic changes were observed.[22] Again, studies of retinal detachment may lead the way to an enhanced understanding of photoreceptor degeneration.

More than a decade ago, changes in cone cell synapses after detachment were described in a feline model of retinal detachment. They included rounding or flattening of the cone pedicles, loss of synaptic invaginations, and reduction in number and size of ribbons.[5] In our pig model, reduction of ribbon length and loss of invaginations occur within hours after detachment along with shape changes to the pedicles (Fig. 1).[39,40] It should be noted that rod terminals also exhibit shallow invaginations and some reduction in ribbon size after detachment, but these changes are less dramatic than the retraction of the spherule resulting in synaptic disjunction. In contrast, cone synapses show no patent synaptic disjunction. However, the cone axons can appear tortuous, perhaps due to movement of the cone cell body inwards into the outer plexiform layer.[5] Changes at the molecular level accompany the pedicles' morphological changes. In contrast to rod cells, most molecular markers specific to cone cells disappear after three to seven days of detachment (i.e., cone opsins, calbindin D, GCAP-1).[4] Although cone opsin mRNA expression returns after reattachment,[41] the structural integrity of cone synapses after reattachment is unknown. If rod synapses are a guide, it is likely that some changes in cone synapses remain after reattachment. Consistent with this hypothesis, patients with retinal detachments present with reduced photopic b-wave responses months after anatomically successful reattachments.[42–45] More work is needed to understand cone synaptic plasticity during detachment and disease and the role of rod and cone synaptic changes among patients with persistent visual loss after outer and inner segment regeneration, whether arising from RP-like disease, retinal detachment, or blunt trauma.

Mechanisms of Synaptic Plasticity

What might be the mechanisms and therefore possible therapeutic targets for control of photoreceptor synaptic plasticity after injury and during disease? We speculated that much could be learned from previous work on the plasticity of conventional synapses during learning and memory, where signaling pathways are well known.[46] Glutamate, calcium, and the cyclic nucleotides, cAMP and cGMP, are among the main actors. Since photoreceptors have no glutamate NMDA receptors, we assessed calcium and cyclic nucleotides. Calcium plays a role in detachment-induced rod synaptic retraction in vitro and blocking L-type channels reduced rod cell plasticity of isolated rod cells[47,48] and intact neural retina in culture.[40] Cyclic AMP via phosphorylation of the transcription factor cAMP response-element binding protein (CREB, another player in activity-dependent synaptic plasticity[46]) also prevents retraction and can stimulate rod sprouting in intact neural retina in vitro.[49,50] We have suggested that activation of rod opsin that diffuses along the inner segment cell membrane in injury and disease, known as mislocalized opsin, is able to stimulate adenylyl cyclase to increase cAMP and CREB activity.[50,51] For cone cells, blocking their cGMP-gated calcium channels prevented the formation of presynaptic varicosities in isolated cone cells whereas addition of the channel agonist 8-bromo-cGMP increased varicosity formation.[48] Although there is currently no evidence of new cone synapse formation after detachment or reattachment, remodeling, including development of a small number of synaptic structures, has been observed in mouse cones after partial loss of cone cells by diphtheria toxin.[52] Furthermore, activation of soluble guanylyl cyclase, to increase cGMP, stimulated neuritic sprouting of isolated cone cells[53] suggesting an explanation for the unusual cone cell sprouting observed in an autosomal recessive form of RP characterized by high cGMP levels in the outer retina.[54,55]

Development of neural connections may additionally serve as a guide to mechanisms of injury. Guidance cues are critical to pathfinding by axonal growth cones as well as synaptogenesis.[56] Some of the signaling pathways activated by these factors are well known. Somewhat surprisingly many of these factors have been shown to increase after retinal injury and disease. For instance, semaphorin 3A (Sema 3A) increases in the retina after retinal detachment,[57,58] optic nerve axotomy,[59] diabetic retinopathy,[60] OIR,[61] and glaucoma;[62] netrin-1 is upregulated in OIR and diabetic retinopathy;[63–66] eph/ephrin signaling is involved in OIR and diabetic retinopathy[67–69] and increases in glaucoma.[70–73] In contrast, ROBO1, a receptor for the repulsive guidance cue slit, and normally present in photoreceptor terminals, decreases in disease.[22] These changes in guidance factors have been observed in both animal models and patients. Additionally, dramatic upregulation of genes for canonical pathways of axon guidance, including for ephrin and semaphorin, is reported in a CNGA3/CNGB1 double mutant mouse that displays extensive horizontal and bipolar cell sprouting. Since guidance cues can promote both axonal and dendritic growth,[74,75] retinal cell sprouting by secondary neurons may be influenced by these factors. In cultures of adult amphibian rod and cone photoreceptors, we found that guidance factors modulate synaptic plasticity. Sema 3A reduced rod neuritic sprouting[58] whereas netrin-1 promoted presynaptic varicosity formation in rod but not cone cells (Fig. 2).

Signaling Pathways

The chemorepulsive factor Sema 3A works through receptors that activate RhoA. We reported that not only did Sema 3A and its receptor neuropilin-1, present on most retinal neurons, increase after injury,[58] so did activated

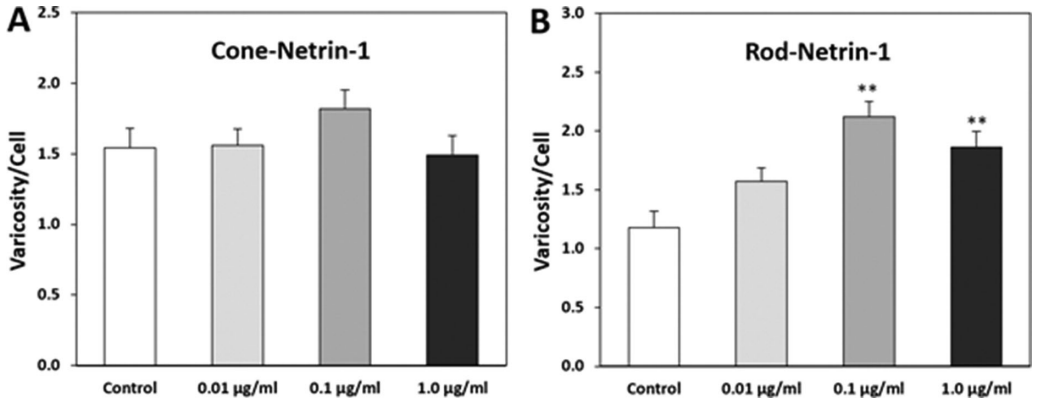

Figure 2. Netrin increases the formation of presynaptic varicosities in isolated rod cells. Data from adult salamander retinal cell cultures. Netrin was added to the culture medium at indicated concentrations. After three days in culture, the higher doses of netrin-1 significantly increase the production of varicosities by rod (**B**) but not cone (**A**) cells. Cultures were stained for rod opsin and synaptophysin to highlight presynaptic formation. $^{**}P < 0.001$, + SEM, $n = 800$ cells, 16 cultures from four animals (one-way ANOVA with Tukey's post hoc test).

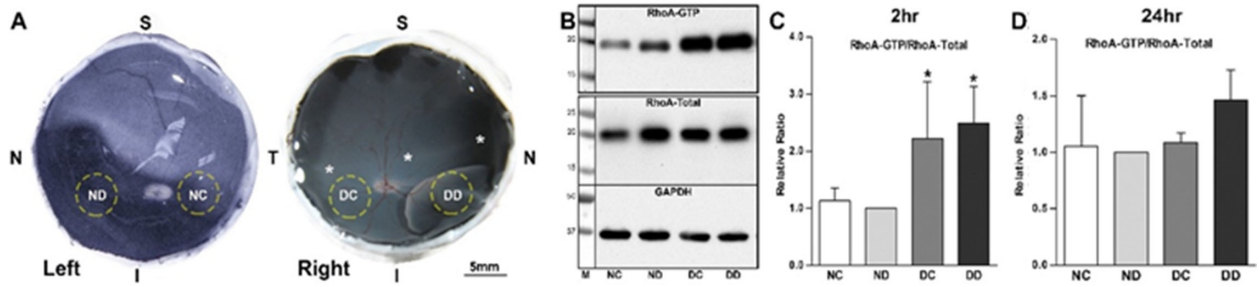

Figure 3. RhoA activation in porcine retina in vivo. **A.** Samples were taken from the detached (DD) and attached retina (DC) in the operated right eye and from the same areas in the normal (unoperated) left eye (ND, NC). **B, C**. Two hours after detachment, active RhoA (RhoA-GTP obtained with a pull-down assay) increases in DD and DC ($^{*}P < 0.05$, $n = 16$ retinal samples, four pigs). **D.** RhoA activation remains above control, but lower than at two hours, in the detached area after 24 hours ($P = 0.07$, $n =$ four pigs). Although activation of RhoA protein increased, total RhoA protein did not change (normalized with GAPDH). S, superior, I, inferior, N, nasal, T, temporal. *Location of cone rich area centralis. Data expressed as mean + SD; normal eye, ND, normalized to 1, one-way ANOVA. Panels A–C modified from Wang et al. 2016.[6]

RhoA, spiking after detachment but frequently remaining at above normal levels for at least 24 hours (Fig. 3). The cause for RhoA activation after retinal injury could relate to the presence of semaphorin, but additional triggers, such as mechanotransduction at the membrane that activates RhoA-associated guanine nucleotide exchange factors (GEFs)[76] and/or injury-induced secretion of ATP, seen after mechanical stimulation and detachment in retina,[77,78] that increases Rho kinase (ROCK) activity by binding to purinergic receptors,[79] may also be involved. In culture, isolated rod cells retract their axonal fiber more quickly with added ATP whereas axon retraction is slowed by suramin, a purinergic antagonist (Fig. 4). Mechanotransduction and ATP secretion, which respond to injury rapidly, may be especially significant at the early times after detachment.

We have reported experiments in which components of the RhoA-Rho kinase (ROCK)-LIM kinase (LIMK) pathway are blocked. In our injury models, both in vitro and in vivo,[6–8,40,49,80–82] anything that reduced the activity of RhoA or its downstream targets reduced rod synaptic disjunction (Fig. 5). The effects of inhibitors are directly on the photoreceptor themselves, as their terminals contain RhoA and LIMK,[80,82] although we do not rule out additional effects on other neurons, epithelial cells, and vascular endothelium. For cone cells we know that ROCK inhibition can also modify synaptic structure. RhoA is present in the pedicles of adult cone cells.[80] In cultures of isolated salamander cones, ROCK inhibition increased neuritic growth and the development of synaptic varicosities. In our in vivo pig model, where cone neuritic growth is not seen, preliminary data indicate that ROCK inhibition prevents the reduction in size of cone synaptic ribbons that occurs in response to a 2-hour retinal detachment (unpublished data, 2021).

Signaling pathways in activity-dependent synaptic plasticity and neural development thus provide a broad canvas for experimentation on ways to preserve synaptic structure at the first synapse. However, an additional consideration could provide more focus in the search for therapeutics. Some elements in these pathways appear almost uniquely after injury. Activated RhoA, for instance, is at very low levels in the retina under normal conditions.[6] Sema 3A is absent in the normal retina.[58] The advantage of targets such as these is that drugs or antibodies blocking their activity are less likely to disrupt normal synaptic function. It can be likened to a conditional gene knockout, a more precise therapeutic tool. Our use of a ROCK inhibitor in retinal detachment seems to be such a focused therapy. However, discovering the timing

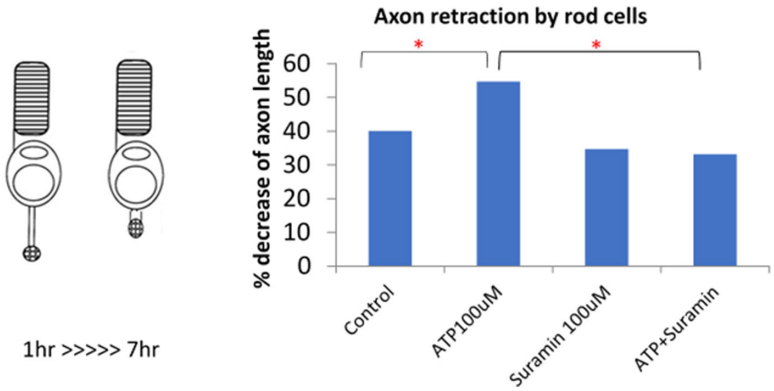

Figure 4. ATP promotes axon retraction in rod cells. *Left*, isolated rod cell in culture showing axon retraction over a six-hour period. *Right*, ATP increases the amount of retraction; suramin, a purinergic receptor antagonist, reduces retraction. *$P < 0.05$, $n = 100$ cells per condition, from five animals (one-way ANOVA with Tukey's post hoc test).

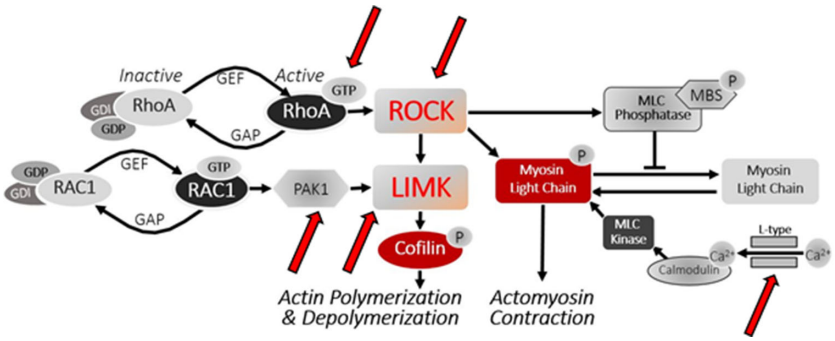

Figure 5. Pathway that contributes to rod synaptic disjunction after detachment. *Red arrows* point to targets of blockers tested: CT-04 against RhoA; Y27632, fasudil and AR13503 against Rho kinase (ROCK); IPA-3 against p21-activated kinase (PAK); BMS-5 against LIM kinase (LIMK); nicardipine against L-type calcium channel. All blockers reduced rod spherule retraction. Data from Nachman-Clewner et al. 1999; Zhang & Townes-Anderson 2002; Fontainhas & Townes-Anderson 2008, 2011; Wang & Townes-Anderson 2015; Wang et al. 2016; Townes-Anderson et al. 2017; Wang et al. 2019; and Halasz et al. 2021.[6–8,40,47,48,80–82]

of the upregulation of these transitory injury-induced targets will be a challenge.

Conclusions

Determining the role of retinal synapses in visual recovery or the lack thereof clearly deserves more attention. Although advances in our understanding may depend in part on the development of new techniques to assess the structure and function of ribbon synapses in disease and injury, much can be learned by application of current high-resolution microscopy and electrophysiology. In terms of treatment, we know that the visual system can tolerate some loss of synaptic connections, perhaps, in part, because of built-in redundancy: 40% or more of cone cells can die, and a patient can retain normal visual acuity and foveal sensitivity.[83,84] This fact may be advantageous by providing time to introduce compounds, such as ROCK inhibitors, to preserve the carefully choreographed synaptic circuitry that remains. Moreover, preservation of the outer retinal synaptic circuitry may also benefit the inner retina, which is known to undergo extensive remodeling after injury and during disease.[5,85] As part of the central nervous system, synaptic preservation in the retina is especially critical as regeneration of appropriate connections is poor.

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