

# An osmolarity dependent mechanism partially ameliorates retinal cysts and rescues cone function in a mouse model of X-linked Retinoschisis.

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16 **Keywords:** X-linked retinoschisis, Retinoschisin, Disease mechanisms, Gene therapy,  
17 Osmolarity, Edema, Hypertonicity, Electroretinogram

## 18 Abstract

### 19 Introduction

20 X-linked retinoschisis (XLRS) is a vitreoretinal dystrophy caused by *RS1* gene mutations which  
21 disrupt retinoschisin protein function. A vital protein for maintaining retinal architecture, the absence  
22 of functional retinoschisin leads to the development of intraretinal cysts. The preliminary goal of this  
23 study was to investigate a low dose gene therapy in *Rs1* knockout (*Rs1*-KO) mice; however, our  
24 experiments revealed an unexpected therapeutic effect of a hypertonic buffer, which led to further  
25 exploration of this effect.

### 26 Methods

27 10 *Rs1*-KO mice were subretinally injected with an AAV2/4 vector containing the *RS1* gene driven  
28 by an *Ef1α* promoter. 16 *Rs1*-KO mice were subretinally injected with a hypertonic buffer (180 mM  
29 NaCl 0.001% F68/PBS (pH 7.4)) or an isotonic buffer (155.2 mM NaCl 0.001% F68/PBS, pH 7.0) as  
30 a sham control. Endpoints included electroretinogram (ERG), optical coherence tomography (OCT),  
31 and a visually guided swim assay (VGSA). An immunohistochemistry assay was used to quantify  
32 cone density in buffer injected and treatment-naïve eyes.

### 33 Results

34 Unexpectedly, hypertonic buffer-injected eyes had significantly reduced cyst severity at 1 month  
35 post-injection (MPI) ( $p=0.0001$ ), significantly higher amplitudes in cone-dominant ERGs persisting  
36 to 5 months post-injection (5 Hz flicker;  $p=0.0018$ ; 3.0 Flash;  $p=0.0060$ ) and demonstrated

37 improved navigational vision in the light compared to untreated *Rs1*-KO eyes ( $p < 0.0001$ ). To  
38 investigate the role of tonicity on this effect, an isotonic buffer-injected cohort was created (155.2  
39 mM NaCl 0.001% F68/PBS, pH 7.0) (n=6). Surprisingly, hypertonic buffer-injected eyes exhibited a  
40 greater reduction in cyst severity and demonstrated improved cone-dominant ERG metrics over  
41 isotonic buffer-injected eyes. Using an immunohistochemistry assay, we demonstrated greater cone  
42 density in hypertonic buffer-injected eyes than untreated controls ( $p = 0.0147$ ), suggesting a possible  
43 cone preservation mechanism. Moreover, our findings reveal a negative correlation between the peak  
44 severity of cysts and long-term cone-dominant ERG metrics, implying that effectively managing  
45 cysts could yield enduring benefits for cone function.

#### 46 **Discussion/Conclusion**

47 This study presents evidence that cyst resolution can be triggered through an osmosis-dependent  
48 pathway, and cyst resolution can have long term effects on cone signaling and survival, offering  
49 potential insights for the development of novel treatments for patients with XLRS.

## 50 **1 Introduction**

51 Juvenile X-linked Retinoschisis (XLRS), a vitreoretinal disorder, is the leading cause of  
52 macular dystrophy in young males. Its prevalence on a global scale reaches up to 1 in 5,000  
53 individuals (1). The pathology of the disease involves an X-linked autosomal recessive mutation in  
54 the retinoschisin 1 (*RS1*) gene located at Xp22.1-p22.3 (1). Mutations in *RS1*, of which there are  
55 greater than 200 known, impair production of the 224 amino acid protein, retinoschisin (1).  
56 Retinoschisin is primarily secreted by photoreceptors and bipolar cells, and influences cell-cell  
57 interactions through a ubiquitous discoidin domain, facilitating photoreceptor-bipolar cell signaling  
58 and stabilizing the extracellular environment of the retina (2, 3). In the absence of a functional  
59 retinoschisin protein, the laminar architecture of the retina becomes disrupted, causing retinal layer  
60 separation, known as schisis, and the formation of cysts. Cysts, a hallmark of XLRS, spatially divide  
61 the inner nuclear and outer plexiform layers, separating the bipolar cells and photoreceptors.

62 From a clinical perspective, essentially all patients with XLRS experience foveal  
63 involvement, impairing central vision (4). Comparatively, around 50 percent of patients also exhibit  
64 peripheral involvement (2, 4). The progressive impairment of central vision due to macular atrophy  
65 can result in substantial functional vision loss. Additionally, the development of schisis significantly  
66 increases the risk of vitreous hemorrhage and full thickness retinal detachment following even mild  
67 ocular trauma (4). Strategies to manage cyst severity are needed.

68 Currently, Carbonic Anhydrase II Inhibitors (CAIs) serve as the primary medical therapy  
69 available for patients with XLRS. The proposed mechanism of CAIs involves inhibition of  
70 membrane-bound carbonic anhydrase (CA) in the retinal pigment epithelium (5). Fluid transport  
71 from the subretinal space into the choroid is enhanced experimentally following introduction of a  
72 carbonic anhydrase inhibitor (CAI), acetazolamide, suggesting CA is involved in maintaining fluid  
73 homeostasis of the retina (5). However, despite their efficacy in reducing intraretinal cyst formation  
74 in some patients, CAIs are associated with well-known systemic side effects affecting the endocrine,  
75 gastrointestinal, cardiovascular, and central nervous system, potentially impacting medication  
76 compliance (6-8).

77 While CAIs have shown short term benefits including mild decrease in central macular  
78 thickness, reduction of intraretinal cyst formation, and improved vision, it is not known whether they  
79 prevent the long-term progression of XLRS (7). Sudden discontinuation of brinzolamide, a  
80 commonly used CAI in clinical practice, can lead to increased cyst formation and elevated intraocular  
81 pressure during periods of drug holiday (7, 8). There are currently no studies demonstrating long-

82 term efficacy or safety. In addition, it is not clear why reducing cyst severity alone, without rescuing  
83 the *RS1* gene, is associated with vision improvement.

84 Given the lack of effective long-term therapy, ongoing efforts in gene therapy have emerged  
85 as a potential avenue for treatment. A Phase I/IIa trial was conducted on human subjects which  
86 involved a single intravitreal injection of an AAV8 vector carrying the *RS1* gene (9). This safety  
87 study was introduced after good success in a mouse model; however, in human subjects, several  
88 ocular inflammatory outcomes were recorded (9). Additional human studies have also noted  
89 inflammatory outcomes in participants receiving intravitreal high dose AAV gene therapy for XLRS  
90 (10).

91 Compared to intravitreal injections delivering equivalent doses of AAV, subretinal injections  
92 elicited lower levels of humoral immune reactions both in mice and in primates (11). In our study, we  
93 investigate whether a subretinal injection of low-dose adeno-associated virus, AAV2/4-EF1 $\alpha$ -*RS1*,  
94 can rescue the retinal phenotype with reduced risk of vector toxicity and ocular inflammation. In this  
95 study, we subretinally administered a low dose AAV2/4-EF1 $\alpha$ -*RS1* gene therapy to *Rs1*-KO mice, a  
96 model for XLRS. To account for the potential effect of the subretinal injection procedure, a sham  
97 control group receiving a hypertonic buffer, similar in osmolarity to the AAV storage buffer, was  
98 included.

99 We hypothesized that subretinal injections of low dose AAV2/4-EF1 $\alpha$ -*RS1* could correct the  
100 retinal phenotype in the *Rs1*-KO mouse detectable by electroretinography (ERG), optical coherence  
101 tomography (OCT), and a visually guided swim assay (VGSA). However, our experiments revealed  
102 an unexpected therapeutic effect of the buffer, which led to further exploration of this effect.

## 103 2 Methods

### 104 2.1 Study Design

105 Natural history, as well as gene therapy efficacy and effect of subretinal buffer injections,  
106 were evaluated in a mouse model of Juvenile X-Linked Retinoschisis (*Rs1*-KO). The natural history  
107 of this *Rs1*-KO mouse model has been reported, including the variable expressivity of the phenotype  
108 (12). We examined treatment-naïve eyes from postnatal day (P) 15 to 12 months of age. OCT images  
109 were analyzed for both outer nuclear layer (ONL) thickness and cyst severity.

110 To study the effect of gene therapy, eyes treated with a subretinal injection of gene therapy  
111 vector (AAV2/4-EF1 $\alpha$ -*RS1*) (n=10) were compared to hypertonic buffer-injected eyes (n=16) and  
112 treatment-naïve eyes (n=27). Injections alternated between the left and right eyes to avoid systematic  
113 bias.

114 The selection of an AAV2/4 vector was based on previously demonstrated tropism for all  
115 layers of the retina, including the photoreceptor and bipolar cell layers, the primary secretors of  
116 retinoschisin (13).

117 Injections were performed between P24 and P31. Outcome measures include ERG, OCT,  
118 VGSA, and immunohistochemistry. ERG was performed at 1-, 2-, 3-, and 5- months post injection  
119 (MPI) to assess retinal function. OCT was performed at 2- and 3-weeks post-injection (WPI), then 1-,  
120 2-, 3-, and 5 MPI to assess cyst severity and retinal structure. A visually guided swim assay was  
121 performed at 5-6 MPI to assess functional vision. The eyes were collected and fixed at 6 MPI for  
122 immunohistochemistry. For the purpose of ERG and OCT experiments, one eye is considered a  
123 biological data point. For the purpose of the visually guided swim assay, each data point is  
124 representative of the average time-to-platform for a randomly selected platform position. Mice were  
125 treated in one eye with either vector or hypertonic buffer. Mice treated with gene therapy in one eye  
126 and hypertonic buffer in the fellow eye were excluded from the VGSA. Both male (*Rs1* hemizygous  
127 mutants) and female mice (*Rs1* homozygous mutants) were used in this study.

128 To investigate the effect of the buffer, the subretinal injection of the hypertonic buffer was  
129 compared to the subretinal injection of an isotonic buffer. Endpoints included ERG and OCT, and the  
130 same timelines as stated above were used in this group.

131 **2.2 Animal Husbandry and Ethics Statement**

132 This study was performed in accordance with the recommendations set forth by the National  
133 Institute of Health in the Guide for the Care and Use of Laboratory Animals. Animals were handled  
134 in accordance with the approved Institutional Animal Care and Use Committee (IACUC) protocol  
135 #1041421 at the University of Iowa. The *Rs1*-KO C57Bl/6J mouse model was kindly supplied by  
136 Paul Sieving, M.D., Ph.D. at the National Eye Institute. Generation of the *Rs1*-KO mouse model was  
137 previously described (14). This mouse model contains a deletion of exon 1 and a 1630 bp fragment of  
138 intron 1 on the *Rs1* gene (14).

139 Animals were housed according to the IACUC recommendations. Humane endpoints were  
140 observed, and the methods of euthanasia used were carbon dioxide inhalation followed by cervical  
141 dislocation.

142 **2.3 Statistical Analysis**

143 Analysis was performed using GraphPad PRISM 9.0 (GraphPad Software, Inc, San Diego,  
144 California, USA). Two-way ANOVA was performed for ERG and OCT metrics and followed by  
145 multiple comparisons (Tukey's test, non-parametric). One-way ANOVA was performed for VGSA  
146 and followed by multiple comparisons (Tukey's test, non-parametric). The student's t-test was used  
147 in the analysis of cone density. Simple linear regression was used for correlation of peak cyst area vs  
148 5 MPI ERG response.

149 **2.4 Genotyping Information**

150 Genotyping was performed using Taq Polymerase (New England Biolabs, Ipswich, MA)  
151 using the primers listed in Table 1. The PLA2 primer was previously described (14). A 10 microliters  
152 ( $\mu$ L) reaction volume was used containing 2  $\mu$ L of DNA (approximately 25  $\mu$ g/ $\mu$ L), 3.7  $\mu$ L of  
153 ultrapure water, 1  $\mu$ L of 10X Buffer/Mg $^{++}$ , 1  $\mu$ L of 20 mM primer mix (Table 1), 0.2  $\mu$ L of 50 mM  
154 dNTPs, 0.1  $\mu$ L of rTaq, and 2  $\mu$ L of 5X Betaine. Cycling conditions are as follows; initial denaturing  
155 at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 30 seconds, with  
156 a final extension step at 72°C for 4 minutes. This produces a 516-base pair (bp) wild-type band and a  
157 300 bp knockout band.

158 **2.5 AAV Packaging**

159 The human *RS1* gene was cloned into a shuttle plasmid pFBAAV and provided by the Viral  
160 Vector Core at the University of Iowa. The bacterial backbone is from Invitrogen's pFastBac system.  
161 The shuttle plasmid consists of the pFBAAV backbone, an EF1 $\alpha$  promoter, and a bovine growth  
162 hormone polyadenylation signal. The plasmid was packaged into an AAV2/4 capsid by the Budd  
163 Tucker Laboratory at the University of Iowa. The AAV was formulated at  $2 \times 10^9$  viral genomes  
164 (vg)/mL titer, and the composition of the storage buffer is listed in Table 2. Titering was performed  
165 using digital droplet PCR.

166 **2.6 Subretinal Injection**

167 Mice were anesthetized using a ketamine/xylazine mixture (87.5 mg/kg ketamine, 12.5 mg/kg  
168 xylazine). A tropicamide ophthalmic solution of 1% was applied three minutes prior to injection to

169 dilate the eyes. Proparacaine was applied as a topical anesthetic and 10% povidone-iodine was  
170 applied as a topical antiseptic. A partial thickness puncture was made just posterior to the limbus  
171 through the sclera with a 30-G sharp needle, then a 33-G blunt Hamilton needle was inserted and  
172 positioned between the RPE and the retina. Two  $\mu$ L of either gene therapy vector at a concentration  
173 of  $2 \times 10^9$  vg/mL, a hypertonic buffer, or an isotonic buffer was injected into the subretinal space  
174 (formulation available in Table 2). The injection of a solution subretinally creates a separation of the  
175 retina and the RPE known as a subretinal bleb. This bleb was assessed by the injector as seen through  
176 the operating microscope and in the rare cases with no visible bleb or a large vitreous hemorrhage,  
177 the mouse was excluded. Additionally, mice with retinal detachments that persisted until the first  
178 OCT time point were excluded. The mouse was then given topical ophthalmic ointment consisting of  
179 neomycin, polymyxin B sulfates, bacitracin zinc, and hydrocortisone. Mice were injected with  
180 atipamezole to aid in recovery from the anesthetic.

## 181 **2.7 Electoretinogram**

182 Dark adaptation occurred overnight. Mice were anesthetized using a ketamine/xylazine  
183 mixture (87.5 mg/kg ketamine, 12.5 mg/kg xylazine). A tropicamide ophthalmic solution of 1% was  
184 applied to the eyes for pupil dilation. A 2.5% Hypromellose solution (Akron, Lake Forest, Illinois)  
185 was applied to maintain corneal hydration during testing. Testing was performed on the Diagnosys  
186 Celeris (Diagnosys, Lowell, MA). Electrodes were placed on the cornea of bilateral eyes, and  
187 impedance was kept below 10 k $\Omega$ . Eyes were exposed to various light intensities in a modified  
188 ISCEV protocol which includes a dark-adapted 0.01 cd $\cdot$ s/m $^2$ , dark-adapted 3.0 cd $\cdot$ s/m $^2$ , light-adapted  
189 3.0 cd $\cdot$ s/m $^2$ , and a light-adapted 5 Hz flicker of 3.0 cd $\cdot$ s/m $^2$  which is more reproducible in mice than  
190 the standard light-adapted 30 Hz flicker in humans (15). To evaluate rod electrical function in treated  
191 and untreated *Rs1*-KO mice, animals were dark-adapted (DA) overnight to isolate the rod response,  
192 and eyes were subjected to 15 flashes of 0.01 cd $\cdot$ s/m $^2$  light (0.01 dim flash) then 15 flashes of 3.0  
193 cd $\cdot$ s/m $^2$  light (3.0 standard combined response (SCR)). To evaluate cone electrical function in treated  
194 and untreated *Rs1*-KO mice, eyes were light-adapted (LA) for 10 minutes to bleach the responses  
195 from rods and subjected to 15 flashes of 3.0 cd $\cdot$ s/m $^2$  light (3.0 flash) followed by 20 flashes of a 5 Hz  
196 flickering light of 3.0 cd $\cdot$ s/m $^2$  (5 Hz Flicker).

## 197 **2.8 Optical Coherence Tomography**

198 Mice were anesthetized using a ketamine/xylazine mixture (87.5mg/kg ketamine, 12.5mg/kg  
199 xylazine), and Tropicamide ophthalmic solution 1% was applied for pupil dilation. 1%  
200 carboxymethylcellulose was applied to maintain corneal hydration during testing. Volumetric scans  
201 were taken centering on the optic nerve along with nasal and temporal scans. Nasal and temporal  
202 scans involved moving the optic nerve peripherally, as previously described (16). Quantification of  
203 cyst severity was performed using two different methods. The first method consisted of a modified  
204 scoring protocol from previously described literature (17). Briefly, measurements of cyst height were  
205 taken at four points equidistant, 500  $\mu$ m from the optic nerve, two on the superior-inferior line  
206 bisecting the optic nerve, and two on the nasal-temporal line bisecting the optic nerve. Measurements  
207 were then translated to a scoring scale as previously described, in which a score of 1 was assigned to  
208 zero cyst height and a score of 6 was assigned to cyst height  $>100$   $\mu$ m. The score was averaged  
209 across all four points (16). The second method consists of the manual segmentation of cyst area.  
210 Briefly, OCT images were analyzed for cyst area using the Image J software. Center scans, those  
211 which best bisect the optic nerve, were quantified by manually segmenting individual cyst cavities  
212 and recording the total area of cysts in the scan. Similar quantifications were performed on nasal-

213 temporal slices located approximately 500  $\mu$ m superior and inferior of the center scan, and area was  
214 reported as a sum of these measurements.

215 **2.9 Visually Guided Swim Assay**

216 AAV2/4-EF1 $\alpha$ -*RS1* vector-treated mice and mice injected with the hypertonic buffer  
217 underwent a VGSA performed in light and dark conditions to assess functional vision at  
218 approximately 5-6 months of age compared to treatment-naïve controls. The VGSA has been  
219 described in detail elsewhere (18). In brief, the mice undergo four training days in the light, four  
220 testing days in the light, two training days in the dark, and four testing days in the dark, and the  
221 performance of each mouse to 5 different platform locations were measured during each training or  
222 testing day. For each trial, the mice were placed into a plastic pool and were expected to find a  
223 platform. Five of the eight possible platform positions are randomly assigned each day with all  
224 platforms being used approximately the same number of times. A maximum time to platform (TTP)  
225 was set at 60 seconds for each trial to prevent fatigue and undue stress. After the mouse had  
226 completed the trial or after 60 seconds of searching, the mouse was removed from the pool, dried,  
227 and placed in its cage until the next trial. Mice were excluded in rare cases where they could not be  
228 motivated to participate and search for the platform. Data from all trials was excluded if a mouse had  
229 a swim time greater than one standard deviation from the mean of the group and consistent floating  
230 occurred, such as more than three episodes of floating per trial or more than three corrections per  
231 trial. Corrections for floating include snapping fingers or touching the mouse's tail.

232 **2.10 Immunohistochemistry**

233 Eyes were enucleated and a small puncture was made through the cornea with a 27-G needle.  
234 Eyes were placed in 4% paraformaldehyde for 12 hours and transferred to PBS for 24 hours. After  
235 fixation, eyes were embedded in Tissue-Tek O.C.T. compound (VWR, Batavia, IL) and frozen in a  
236 bath of 2-methylbutane cooled with liquid nitrogen. Eyes were sectioned on the superior-inferior axis  
237 at a thickness of 10  $\mu$ m using a Cryostat microtome and stored at -80°C for future use.

238 For immunohistochemistry, sections were permeabilized using 0.3% Triton X-100 in PBS for  
239 10 minutes at room temperature, blocked with a blocking buffer containing 5% BSA, 5% normal  
240 goat serum, and 0.05% Triton X-100 in PBS for 1 hour at room temperature, and then incubated with  
241 primary antibodies in a dilution buffer containing 5% BSA, 1% normal goat serum, and 0.05% Triton  
242 X-100 in PBS at 4 °C overnight. The following day, samples were washed in PBS three times before  
243 incubation with secondary antibodies or streptavidin Alexa Flour-568 conjugate at room temperature  
244 for 1 hour. After washing in PBS an additional three times, samples were mounted with VectaShield  
245 mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were taken using a  
246 fluorescence microscope and color channels were overlayed in Image J. No contrast enhancements or  
247 brightness levels were altered after acquisition.

248 Antibodies used for immunohistochemistry were as follows: anti-CtBP2/RIBEYE antibody  
249 (BD Transduction Laboratories #612044; 1:200 dilution); biotinylated-peanut agglutinin  
250 (biotinylated-PNA) (Vector Laboratories #B-1075; 1:500 dilution).

251 Images were taken directly superior and inferior of the optic nerve head and were gathered at  
252 40x magnification. Three serial images were acquired on either side of the optic nerve leading to six  
253 total images gathered per eye. These images were then independently quantified by three individuals  
254 masked to treatment groups and the number of cones per image was averaged. The quantifications  
255 were averaged and divided by the image width to produce the reported average cones per 100  $\mu$ m.

256 **3 Results**

257 **3.1 Natural history of disease in the *Rs1*-KO mouse compared to WT BL/6 mouse.**

258 Cyst severity was quantified over time in the *Rs1*-KO mouse using a modified cyst severity  
259 scoring system, as previously described by Bush et al. (17). Figure 1 demonstrates typical OCT  
260 findings in the *Rs1*-KO mouse at P15, and 2-, 3-, and 6- months of age, compared to a WT mouse  
261 (Figure 1A). At all-time points, cysts, or schisis, can be observed in the *Rs1*-KO mouse retina. This  
262 compares to the organized, laminar retinal architecture of the WT mouse. Cysts are apparent as early  
263 as P15 with an average severity score of 2.75 (Figure 1C). Cysts appear the most severe at 2- to 4-  
264 month-old, hitting a local maxima at 3 months. Thereafter, severity reduced with maturity.

265 In untreated *Rs1*-KO mice, significant thinning of the ONL occurred from P15 to 1 month of  
266 age (Figure 1B,  $p < 0.0001$ ). After 1 month, the ONL thickness in *Rs1*-KO mice did not significantly  
267 change over time, indicating that the majority of the photoreceptor cell loss occurred shortly after  
268 birth and during eye maturation.

269 ERGs investigate the electrical response of the retina *in vivo* to understand how the disease  
270 affects signal transmission in light and dark conditions. ERGs of *Rs1*-KO mice demonstrated  
271 consistently reduced function of rod-dependent and cone- dependent visual pathways compared to  
272 WT. Figure 1 demonstrates typical ERG waveforms for dark-adapted (3.0 SCR) and light-adapted (5  
273 Hz flicker) metrics (Figure 1D).

274 In summary, the *Rs1*-KO mouse model closely resembles the clinical course in humans by  
275 demonstrating cyst formation, significant ONL thinning, and diminished electrical activity of retinal  
276 photoreceptors and bipolar cells observable on ERG. In our hands, this model has a phenotype  
277 similar to that described by Sieving et al. (12).

278 By treating at 1 month of age, we aimed to determine if we could mitigate the formation of  
279 severe cysts and subsequently improve the electrical signaling of retinal cells in adult mice after the  
280 initial ONL thinning phase. Results from vector-treated and hypertonic buffer-injected eyes were  
281 compared to those of treatment naïve eyes (untreated).

282 **3.2 Cyst severity is significantly reduced in vector-treated and hypertonic buffer-injected  
283 eyes at 1 MPI.**

284 Using a modified cyst severity scoring system, vector-treated *Rs1*-KO eyes (n=10)  
285 demonstrated significantly less cyst severity than untreated eyes ( $p=0.002$ ; Figure 2B) at 1 MPI.  
286 Hypertonic buffer-injected eyes also showed a similar reduction in cyst severity to compared to  
287 untreated eyes at 1 MPI ( $p < 0.0001$ , Figure 2B). The observed reduction in cyst severity occurred at 1  
288 MPI at which point the mice are 2 months of age. As previously described, peak cyst formation in  
289 *Rs1*-KO mice begins around 2 months of age, suggesting injection of the vector or hypertonic buffer  
290 leads to reduction in cysts at a significant time in cyst development.

291 At 2 and 5 MPI no discernible distinction in cyst severity was evident among untreated,  
292 vector-treated, or hypertonic buffer-injected mice. The attenuation of significance observed in these  
293 comparisons can be ascribed to the previously established reduction in cyst severity observed in  
294 untreated *Rs1*-KO mice, per the natural disease progression, rather than an increase in cysts within  
295 the injection groups (12). ONL thickness remained stable from 1 to 5 MPI in all cohorts, and  
296 perceived significance at 5 MPI is likely an anomalous data (Figure 2C).

297 Cysts spatially separate bipolar cells and photoreceptors, and it is hypothesized that the lower  
298 b-wave amplitudes and/or electronegative ERG phenotype associated with XLRS is a consequence of  
299 this spatial separation. To examine the potential influence of reduced cysts on retinal electrical  
300 signaling, we conducted ERGs under light-adapted and dark-adapted conditions.

301 **3.3 Hypertonic buffer outperforms untreated and vector-treated eyes in light-adapted ERGs,**  
302 **but not in dark-adapted ERGs.**

303 Vector-treated *Rs1*-KO eyes (n=10) or hypertonic buffer-injected *Rs1*-KO eyes (n=16) were  
304 compared to untreated *Rs1*-KO eyes.

305 **3.3.1 Light-Adapted**

306 The retinal cone pathway was measured using two light-adapted ERG assays; the light  
307 adapted 5 Hz flicker and 3.0 flash. At 1 MPI, the vector-treated eyes and hypertonic buffer-injected  
308 eyes demonstrated superior cone function to untreated eyes in the 5 Hz flicker assay (*vector-treated*:  
309  $p=0.0003$ ; *hypertonic buffer*  $p<0.0001$ ; Figure 3A). This effect persisted to 5 MPI (*vector-treated*:  
310  $p=0.0255$ ; *hypertonic buffer*:  $p=0.0018$ ; Figure 3A). Although both injection groups were superior to  
311 untreated controls, hypertonic buffer-injected eyes had significant improvement over vector-treated  
312 eyes at the 5 MPI time point ( $p=0.0235$ ), suggesting a more robust cone rescue occurred after  
313 subretinal injection of a hypertonic buffer compared to the low-dose AAV2/4-EF1 $\alpha$ -*RS1* vector.

314 In the 3.0 flash, vector-treated eyes demonstrated superior cone function to untreated eyes till  
315 2 MPI (*vector-treated*:  $p=0.0006$ ; *hypertonic buffer*:  $p=0.0003$ ; Figure 3B). However, hypertonic  
316 buffer-injected eyes had a more robust and long-term improvement in function, demonstrating  
317 superior cone function over untreated eyes out to 5 MPI ( $p=0.006$ ). The robust and long-term  
318 improvement in cone functioning observed in the hypertonic buffer group was a surprising finding  
319 and indicates that the subretinal injection of buffer alone enabled long-term benefits in retinal cone  
320 function. Representative waveforms demonstrate these findings at 5 MPI (Figure 3, A-B).

321 **3.3.2 Dark-Adapted**

322 To measure the electrical function of the eye in dark conditions, isolating the rod response,  
323 the 3.0 SCR and 0.01 dim flash ERG assays were utilized. We found no significance between the  
324 untreated, vector-treated, or hypertonic buffer-injected eyes at 1 MPI in either 3.0 SCR (*vector-*  
325 *treated*:  $p=0.433$ ; *hypertonic buffer*:  $p=0.531$ , Figure 3C), or 0.01 dim flash (*vector-treated*:  $p=0.28$ ,  
326 *hypertonic buffer*:  $p=0.158$ , Figure 3D). No significant effect was observed up to 5 MPI in the dark,  
327 suggesting that subretinal injections of low-dose AAV2/4-EF1 $\alpha$ -*RS1* vector or buffer had minimal  
328 impact on rod-dependent retinal function.

329 Following the observed improvements in cone specific electrical pathways, we investigated  
330 whether these improvements were associated with differences in functional vision.

331 **3.4 Vector-treated and hypertonic buffer-injected mice perform faster in a light-adapted**  
332 **swim assay than untreated *Rs1*-KO mice.**

333 To assess functional vision, mice subretinally injected with the vector or hypertonic buffer  
334 were subjected to a VGSA and compared to untreated *Rs1*-KO mice. Mice swam at 5-6 months of  
335 age, in light- and dark-adapted environments, and were timed until they found a platform in the pool.  
336 In light-adapted swimming conditions, we observed that vector-treated mice (2.92 seconds) were  
337 significantly faster at finding the platform than untreated mice (5.06 seconds) ( $p=0.0002$ ) (Figure  
338 4A). The hypertonic buffer-injected mice (2.51 seconds) were also significantly faster than untreated  
339 mice when swimming in a lighted environment ( $p<0.0001$ ). These results confirm that the subretinal  
340 injection of vector or buffer alone provided a benefit in functional vision in the light, which correlates  
341 with the apparent reduction of cysts, and improved cone pathway ERG signaling. There was no  
342 observable significance between vector-treated, hypertonic buffer-injected, and untreated mice in a  
343 dark-adapted swim environment (Figure 4B).

344 Together, long-term ERG data and functional vision assay suggest that subretinal injections  
345 of a hypertonic buffer solution alone led to reductions in cyst severity and cone-specific

346 improvements in retinal function as well as functional vision. The improvements observed in buffer-  
347 treated eyes were equivalent or sometimes superior to improvements observed in vector-treated  
348 eyes.

349 **3.5 Investigation of the effect of tonicity on cone rescue**

350 Our data suggests that the injection of hypertonic buffer alone leads to significant  
351 improvements in cone-specific ERG amplitudes, light-adapted visual function, and retinal  
352 architecture in *Rs1*-KO mice. Previous studies have demonstrated a temporary reduction in existing  
353 retinal cavities following the subretinal injection of a balanced salt solution; however, the long-term  
354 effects on visual function and electrical signaling of the retina has not been studied (19).  
355 Additionally, cystoid retinal conditions in XLRS patients can be modified by the application of  
356 diuretic ophthalmic solutions such as brinzolamide and acetazolamide, but the connection between  
357 fluid reabsorption and cyst resolution is not well understood. Previous literature has suggested that  
358 the retinoschisin protein physically interacts with Na/K-ATPase and may act to mediate Na/K-  
359 ATPase activity, affecting signaling and ion gradients within the retina (20, 21). We noted that the  
360 buffer solution, used both to store the AAV vector and used in the initial buffer injections, contained  
361 a high salt content achieving a greater than physiologic osmolarity. Therefore, we explored the idea  
362 that the tonicity of the injected buffer played a role in promoting cyst resolution and improving  
363 retinal signaling. To investigate this hypothesis, we repeated experiments with a subretinal injection  
364 of an isotonic buffer. The isotonic buffer has a similar composition to the hypertonic buffer except  
365 possessing a lower concentration of NaCl. The endpoints of these experimental groups also include  
366 ERG to measure retinal electrical function and OCT to measure retinal structure and cyst severity.  
367 The results for these tests are as follows.

368 **3.6 Cyst area is reduced in hypertonic buffer and isotonic buffer-injected eyes, with greater  
369 reduction observed in the hypertonic buffer group.**

370 To determine if structural differences exist when modulating the tonicity of the injection  
371 buffer, OCT was used to image the retinal layers *in vivo*. Using ImageJ software, images were then  
372 assessed for cyst severity using manual segmentation and measurement of visible cyst cavities  
373 reported as total cyst area. Outer nuclear layer (ONL) thickness measurements were taken to assess  
374 photoreceptor cell survival.

375 We observed less cyst severity in the hypertonic buffer and isotonic buffer-injected eyes  
376 compared to untreated eyes at 3 weeks post-injection (WPI) (*hypertonic buffer*:  $p=0.0033$ , *isotonic*  
377 *buffer*:  $p=0.0431$ , Figure 5A). By 1 MPI, hypertonic and isotonic buffer-treated eyes continued to  
378 have less severe cysts than untreated eyes (*hypertonic buffer*:  $p<0.0001$ , *isotonic buffer*:  $p<0.0001$ ).  
379 This effect persisted until 2 MPI for the hypertonic buffer-injected eyes ( $p=0.0380$ ). In summary,  
380 hypertonic buffer-injected eyes demonstrated an early reduction in cyst severity, originating as early  
381 as 3 weeks PI and this effect persisted to 2 MPI. Additionally, hypertonic buffer-injected eyes had a  
382 reduction of severity to a slightly greater magnitude than isotonic buffer-injected eyes at 3 WPI and 1  
383 MPI. Cyst area was decreased across all groups by 5 MPI.

384 Though cysts were decreased at 1 MPI, ONL thickness was not different across time points  
385 between hypertonic buffer-injected, isotonic buffer-injected, and untreated eyes (*hypertonic buffer*:  
386  $p=0.8995$ , *isotonic buffer*:  $p=0.9916$ , *hypertonic buffer vs isotonic buffer*:  $p=0.9921$ , Figure 5B).

387 Having observed a notable decrease in cysts among mice subjected to a subretinal injection of  
388 hypertonic buffer, and to a lesser effect isotonic buffer, we investigated whether cyst reduction  
389 paralleled improvements in electrical functioning of the retina.

390 **3.7 Hypertonic buffer-injected eyes have a more robust cone rescue on light-adapted ERG**  
391 **over untreated and isotonic buffer-injected eyes.**

392 *Rs1*-KO eyes were subretinally injected with hypertonic buffer solution (n=16) or isotonic  
393 buffer solution (n=7) and compared to untreated eyes (n=27).

394 **3.7.1 Light-Adapted**

395 Following an observed reduction in retinal cysts after subretinal injection of a hypertonic  
396 buffer, we investigated whether the improvement in cysts paralleled a similar improvement in  
397 electrical function of the retina.

398 Cone function was measured using two separate light-adapted ERG assays; the light adapted  
399 3.0 flash and 5 Hz flicker.

400 Under the 3.0 flash following light adaptation, both isotonic buffer and hypertonic buffer-  
401 injected eyes displayed superior cone function over untreated eyes, persisting from 1 MPI  
402 (*hypertonic buffer*:  $p < 0.0001$ , *isotonic buffer*:  $p = 0.0192$ , Figure 6A) to 5 MPI (*hypertonic buffer*:  
403  $p = 0.0060$ , *isotonic buffer*:  $p = 0.0089$ ; Figure 6A). Although both buffer groups demonstrated an  
404 improvement in cone pathway signaling over untreated eyes, hypertonic buffer-injected eyes  
405 exhibited a more pronounced improvement.

406 In the 5 Hz flicker protocol, isotonic buffer-injected eyes showed some improved functioning  
407 over untreated eyes from 1 MPI (*isotonic buffer*:  $p = 0.0386$ , Figure 6B) to 5 MPI (*isotonic*:  
408  $p = 0.0077$ , Figure 6B). Though there are observable improvements in cone function in the isotonic  
409 buffer-injected eyes, the magnitude and significance of these improvements are less than the  
410 improvements observed in the hypertonic buffer-injected eyes. Hypertonic buffer-injected eyes  
411 demonstrated a more robust improvement in cone function and were superior to isotonic buffer-  
412 injected and untreated eyes up to 5 MPI (*hypertonic buffer vs untreated*:  $p = 0.0018$ ; *hypertonic buffer*  
413 *vs isotonic buffer*:  $p = 0.0208$ , Figure 6B).

414 **3.7.2 Dark-Adapted**

415 Combined rod-cone function was measured by the 3.0 SCR, and isolated rod function was  
416 measured using the 0.01 dim flash. There was no persistent significant difference between hypertonic  
417 buffer-injected, isotonic buffer-injected, or untreated eyes in this setting, suggesting minimal rod  
418 rescue following injections (Figure 6C-D).

419 These findings suggest that the injection of either isotonic or hypertonic buffer significantly  
420 improves cone-specific signaling pathways measured on ERG, and this beneficial effect persisted  
421 over the course of 5 months. In addition, these data suggest that the tonicity of the buffer introduced  
422 is a significant factor in cyst reduction and in ERG improvement, as more notable improvements  
423 were observed in eyes that received the hypertonic buffer compared to those that received the  
424 isotonic buffer.

425 **3.8 Peak cyst severity on OCT is negatively associated with cone function on ERG at 5 MPI.**

426 Hypertonic buffer-injected eyes had both the lowest cyst severity and the greatest ERG  
427 improvements in cone dominant pathways. To explore this finding, we investigated whether cyst  
428 severity is truly associated with a long-term impairment in electrical signaling of the retina. Peak cyst  
429 severity, the highest cyst area reached by the eye, was graphed against ERG metrics at 5 MPI, and  
430 included hypertonic buffer, isotonic buffer, and untreated eyes. As part of the natural disease course  
431 in XLRS, retinal cysts hit a local maxima of severity between 2 and 4 months of age. The comparison  
432 of peak cyst severity to 5 MPI ERG amplitudes investigates whether the degree of cyst severity  
433 during critical times is correlated with long-term electrical function in the retina. By looking at long-  
434 term outcomes, we are able to see if the reduction of cysts could influence long-term retinal function

435 beyond any short-term effect from bringing the retinal layers closer together. Additionally, at 5 MPI  
436 there are little to no cysts present in uninjected mice based on natural history and could not explain  
437 the variation in ERG amplitudes at that time.

438 In light-adapted, cone-dominant, ERG metrics we found ERG amplitudes are negatively  
439 correlated with peak cyst severity in both the 5 Hz flicker and 3.0 flash (5 Hz flicker:  $p=0.0002$ ;  
440 Figure 7A) (3.0 flash b-wave:  $p=0.016$ ; Figure 7B). These findings suggest greater cyst area has a  
441 more significant impact on impairing cone signaling than rod. Whether or not it is due to improved  
442 cone survival or improved cone functioning was unknown.

443 On dark-adapted, rod-dominant ERG metrics, there is no correlation between peak cyst area  
444 in either the a-wave or b-wave metrics (3.0 SCR b-wave:  $p=0.4482$ ; Figure 7C) (3.0 SCR a-wave:  
445  $p=0.9436$ ; Figure 7D) (0.01 dim flash b-wave:  $p=0.4722$ ; Figure 7E) (0.01 dim flash a-wave:  
446  $p=0.8346$ ; Figure 7F). Cyst severity, or the reduction thereof, does not appear to influence long-term  
447 rod signaling.

448 To determine whether the improved cone amplitudes were associated with an increased  
449 number of cone photoreceptor cells, we used an immunofluorescence assay to quantify cone density  
450 in eyes injected with hypertonic buffer compared to untreated eyes.

### 451 **3.9 Increased cone density at 6 months old suggests potential cone preservation mechanism 452 following injection of hypertonic buffer.**

453 ONL thickness measurements, which are generally used to measure photoreceptor cell  
454 survival, showed no difference in thickness measurements between treated and untreated groups  
455 (Figure 5B). The overall stability in the ONL suggests rods survive long-term, regardless of cyst  
456 severity. In the setting of a low overall percentage of cones in the mouse retina (2.8% of total  
457 photoreceptors), the impact of the injections on cone survival may not be accurately determined by  
458 measuring outer nuclear layer thickness on conventional OCTs (22).

459 To reconcile improved ERG findings with the nonsignificant ONL findings,  
460 immunohistochemistry was performed to more precisely measure cone cell survival. Retinal  
461 cryosections were stained with biotinylated-PNA to quantify cone outer segments. These sections  
462 were then imaged, quantified, and averaged to give the output of cones per 100  $\mu\text{m}$  of the retina.  
463 Hypertonic buffer-injected eyes had a mean of 20.95 cones per 100  $\mu\text{m}$ , a significantly higher cone  
464 density than untreated *Rs1*-KO eyes with a mean of 13.42, suggesting improved cone survival  
465 compared to untreated eyes (*hypertonic buffer vs. Untreated*:  $p=0.0147$ , Figure 8B).

466 Together, this data shows that the subretinal injection of a buffer solution partially  
467 ameliorated intra-retinal cysts and lead to improved amplitudes in cone-specific ERGs as well as in  
468 functional day vision. The beneficial effect of the buffer solution after subretinal administration was  
469 partially mediated by the osmolarity of the buffer solution.

## 470 **4 Discussion**

471 XLRS gene therapy, both in mice and human trials, is currently underway, however, human  
472 trials have faced numerous challenges due to the development of adverse ocular immune reactions in  
473 some participants (9, 10). The primary goal of our study was to investigate whether low-titer gene  
474 therapy could circumvent immune reactions. We found that delivering a  $2 \times 10^9$  vg/mL dose of  
475 AAV2/4-EF1 $\alpha$ -*RS1* subretinally in *Rs1*-KO mice did not cause meaningful rescue of functional  
476 metrics when compared to the metrics of a sham injection group, which received the hypertonic  
477 buffer injection alone. Because higher titers delivered using AAV2/4 under the CMV promoter  
478 subretinally and intravitreally have been shown to rescue the retinal phenotype (23), these results  
479 indicate that sufficient retinoschisin expression is required to achieve the highest efficacy.

480 Considered together with the data from the “sham” hypertonic buffer injections, it suggests that the  
481 efficacy of this very low dose vector may have been due solely to its diluent buffer.

482 Throughout our study, we observed that eyes that received subretinal injections of the  
483 hypertonic buffer consistently had less severe cysts and higher ERG amplitudes compared to the  
484 uninjected eyes of *Rs1*-KO mice. Those eyes that received hypertonic buffer injections performed  
485 comparably to our low dose gene therapy cohort which received the vector in the same buffer, even  
486 exhibiting a more robust effect at later time points when the gene therapy cohort had lost  
487 significance. Reduction of cysts for short periods following subretinal injection of a buffer has been  
488 reported in this model previously, but not explored thoroughly (19). In the prior report, retinal  
489 detachment was hypothesized to reduce cysts. The unexpected long term beneficial effects on XLRs  
490 phenotypes including ERG, VGSA, and photoreceptor survival after the subretinal injection of  
491 hypertonic buffer has not been previously reported.

492 Notably, the injection of hypertonic buffer alone attenuated the severity of cysts at 3 weeks,  
493 and this effect persisted until at least 2 months post injection. An overall reduction of cysts was  
494 apparent at 5 MPI across all groups, consistent with the waning of cysts that occurs during disease  
495 progression.

496 Interestingly, a concomitant improvement in cone-specific ERG amplitudes was observed in  
497 hypertonic buffer-treated eyes compared to those of uninjected eyes. Since the presence of cysts  
498 could interfere with bipolar cell function and impede electrical transduction, the improvements seen  
499 in ERGs could be a consequence of the attenuation of cyst severity in the retina.

500 Paradoxically, the improvements in cone-specific ERGs persisted despite age-related cyst  
501 resolution in both groups, and hypertonic buffer-injected eyes continued to possess higher ERG  
502 amplitudes at 5 MPI. Age-related cyst resolution in this model accompanies a blurring of the  
503 laminations on OCT, and may be associated with a new phase of retinal dysfunction. The thicknesses  
504 of the ONLs were not different between these groups of mice, so the reason behind the functional  
505 improvements seen in hypertonic buffer-injected eyes long after cysts have naturally resolved was  
506 unclear.

507 Since cones are reported to represent only 2.8 percent of photoreceptors in mouse retinas, it is  
508 likely that the effect of the injection on cone cell survival would not be reflected by ONL thickness  
509 measurements (22). We used immunohistochemistry to quantify the cone photoreceptor cells in eyes  
510 that received injections of buffer compared to in uninjected eyes. When we examined cone density  
511 using an immunofluorescent assay, we observed significantly higher cone density per 100  $\mu$ m in  
512 hypertonic buffer-injected eyes than in untreated controls. An increase in overall cone density likely  
513 explains the improvement in ERG amplitudes seen in light-adapted settings and improved visual  
514 function in the light. This finding suggests a potential cone-preserving mechanism is associated with  
515 the subretinal introduction of hypertonic buffer. The relationship between cyst reduction and  
516 enhanced cone survival has not been previously reported and could have significant clinical  
517 implications.

518 Managing XLRs symptoms using topical agents such as brinzolamide has been found to  
519 reduce cysts along with a beneficial increase in visual function (6-8). However, whether managing  
520 cysts in XLRs could have long-term benefits is not clear. Our study provides compelling evidence  
521 that the attenuation of cysts may promote the survival of cones in the retina, and lead to better  
522 electrical cone signaling and functional vision in the long-term.

523 The effects of a hypertonic buffer subretinal-injection on improving the function and structure  
524 of the retina have not been previously reported. Previous studies propose that retinoschisin 1 (*RS1*)  
525 might have additional functions outside of its structural function and play a role in governing fluid  
526 distribution across retinal layers, potentially by engaging with Na/K-ATPase channels via a binding  
527 mechanism (20, 21). In the absence of a functional protein, this pathway has the potential to become  
528 dysregulated, resulting in the formation of fluid filled retinal cysts and creating a spatial separation of

529 bipolar and photoreceptor cells. Despite the incomplete understanding of the mechanism, this  
530 phenomenon could provide an explanation for the observed benefits of carbonic anhydrase inhibitors  
531 (CAIs) in mitigating cyst development and enhancing visual function among XLRS patients (2).  
532 These data provided the rationale behind our investigation whether tonicity of the buffer could affect  
533 fluid movement out of cysts and resolve schisis.

534 ERG and OCT experiments were repeated with an isotonic buffer to determine if this effect  
535 was influenced by the tonicity of the solution versus the mechanical action of the subretinal injection.  
536 We found that the isotonic buffer-injected cohorts did not perform as well as the hypertonic buffer-  
537 injected eyes. Though isotonic buffer-treated eyes had reduced cysts, similar but slightly greater than  
538 hypertonic buffer-injected eyes, they experienced lower b-wave amplitudes in light-adapted ERGs.

539 These experiments indicate that the tonicity of the injected solution plays a role in the  
540 reduction of cysts and function of the retina after injection.

541 Currently, our findings suggest that the injection of a buffer that is hypertonic to the  
542 physiological environment alleviates intraretinal cysts, promotes the survival of cone photoreceptors,  
543 and preserves retinal function in a mouse model of XLRS. Furthermore, these data suggested that  
544 managing the severity of cysts can have a long-term impact on the health of the retina. To examine  
545 this notion, we analyzed the relationship between peak cyst severity of each animal with their long-  
546 term functional outcome. When we measured peak cyst severity against endpoint ERG amplitudes,  
547 we observed a negative correlation between the peak severity of cysts and ERG amplitudes in light-  
548 adapted ERG settings. This correlation was seen regardless of the treatment cohort (untreated,  
549 hypertonic buffer-, and isotonic buffer-injected), suggesting that the light-adapted metrics, and thus  
550 cone function and or survival, are closely related to severity of cysts. In the setting of greater cyst  
551 severity, cone function is more attenuated than rod function. This also suggests that long term cone  
552 function can be predicted by the degree of cyst reduction achieved by treatment. These results  
553 suggest the successful management of cysts can impact the long-term health and function of the  
554 retina.

555 This study has provided evidence that the management of cysts through an osmolarity-related  
556 mechanism can have beneficial effects. Improvements in cone function and survival, cyst severity,  
557 and light-adapted visual function after hypertonic buffer-injection suggest tonicity is a mediator of  
558 this effect. We speculate that this effect is due to the shifting of fluid out of the retinal space, where  
559 cysts form, and into the subretinal space where the choroidal vasculature can reabsorb the cystic  
560 fluid.

561 Aquaporins (AQP) represent a family of water-transporting channels with a pivotal role in  
562 upholding water homeostasis across various organ systems. They have been previously identified in  
563 the inner nuclear layer of the retina, where studies suggest they have an involvement in regulating  
564 retinal water balance (24). When AQP was knocked out in mouse models, electroretinograms (ERGs)  
565 demonstrated decreased b-wave amplitudes and delayed latency in a series of ERGs with increasing  
566 light intensity (24). These findings are parallel to our observations in the *Rs1*-KO mouse natural  
567 history and underscore the connection between the electrical function of the eye and the fluid  
568 homeostasis of the retina. Systemic infusions of hyperosmolar fluid have been shown to reduce  
569 edema in other organs, like the brain, in the setting of acute cerebral injury (25). With this precedent,  
570 strategies leveraging fluid exchange to promote the resolution of cysts in edematous retinal  
571 conditions merit further exploration.

572 Although the exact mechanism is unknown, we have shown that injection of a buffer into the  
573 subretinal space leads to reduced cyst severity in the *Rs1*-KO mouse and that lower peak cyst severity  
574 is associated with improved electrical function, improvements in light-adapted visual acuity, and  
575 greater preservation of cones. This effect is partially modulated by the tonicity of the buffer and is  
576 most prominent in eyes injected with a hypertonic buffer. These findings have the potential to open

577 new doors in the development of therapies for patients with XLRS and could shine a light on how  
578 fluid balance influences cyst resolution and disease severity.

579 **5 Tables**

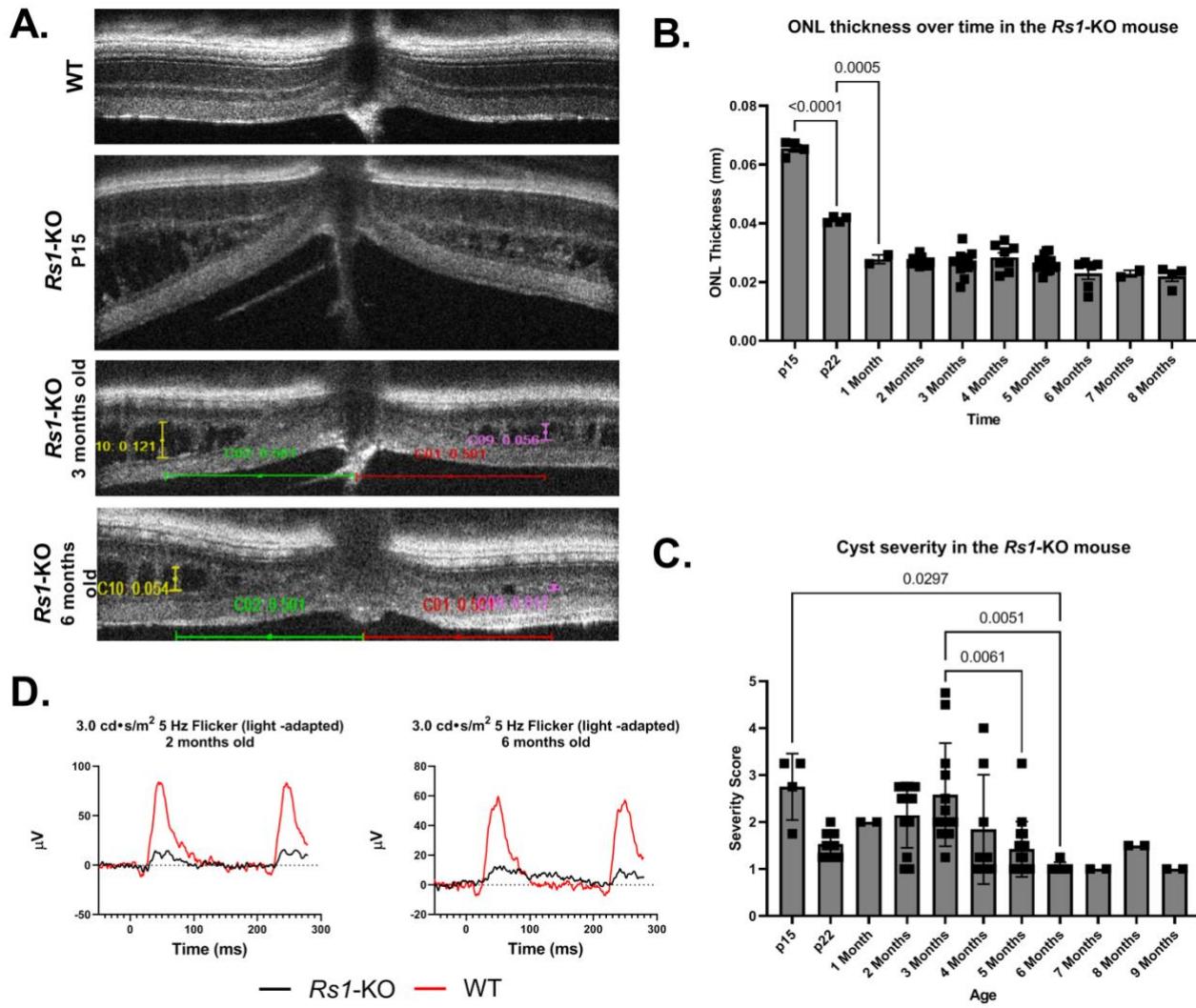
580 **Table 1: Primers**

<i>RS1</i> Promoter F2 33%	5'-TAGGGGCCACATCTTCCAAC-3'
PLA2 33%	5'-GTTCTCGGACGCCTCGTCAACAC-3'
RSWT2-R 33%	5'-GTGACAAAGAGCCACACAACAGTGACC-3'

581 **Table 2: Buffer solutions**

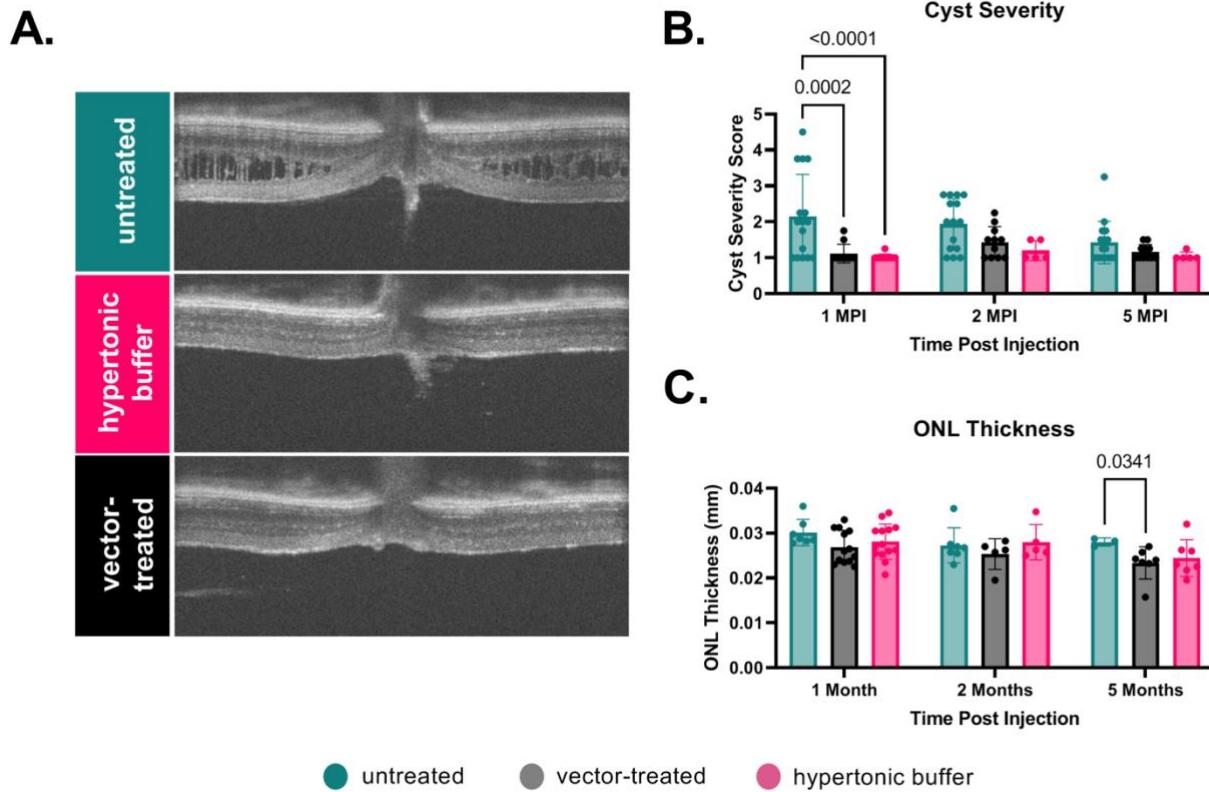
AAV storage buffer	<ul style="list-style-type: none"><li>• 180 mM NaCl</li><li>• 10 mM Na<sub>3</sub>PO<sub>4</sub></li><li>• UltraPure water</li><li>• pH 7.2-7.4</li></ul>
hypertonic injection buffer	<ul style="list-style-type: none"><li>• 0.001% Gibco Pluronic F-68</li><li>• Gibco dPBS (-/-)(Catalog #14190144) [Contains 155.2 mM NaCl]</li><li>• Additional 24.8 mM NaCl (for 180 mM NaCl total)</li><li>• pH 7.4</li></ul>
isotonic injection buffer	<ul style="list-style-type: none"><li>• 0.001% Gibco Pluronic F-68</li><li>• Gibco dPBS (-/-)(Catalog #14190144) [Contains 155.2 mM NaCl]</li><li>• pH 7.4</li></ul>

582 6 Figure Legends



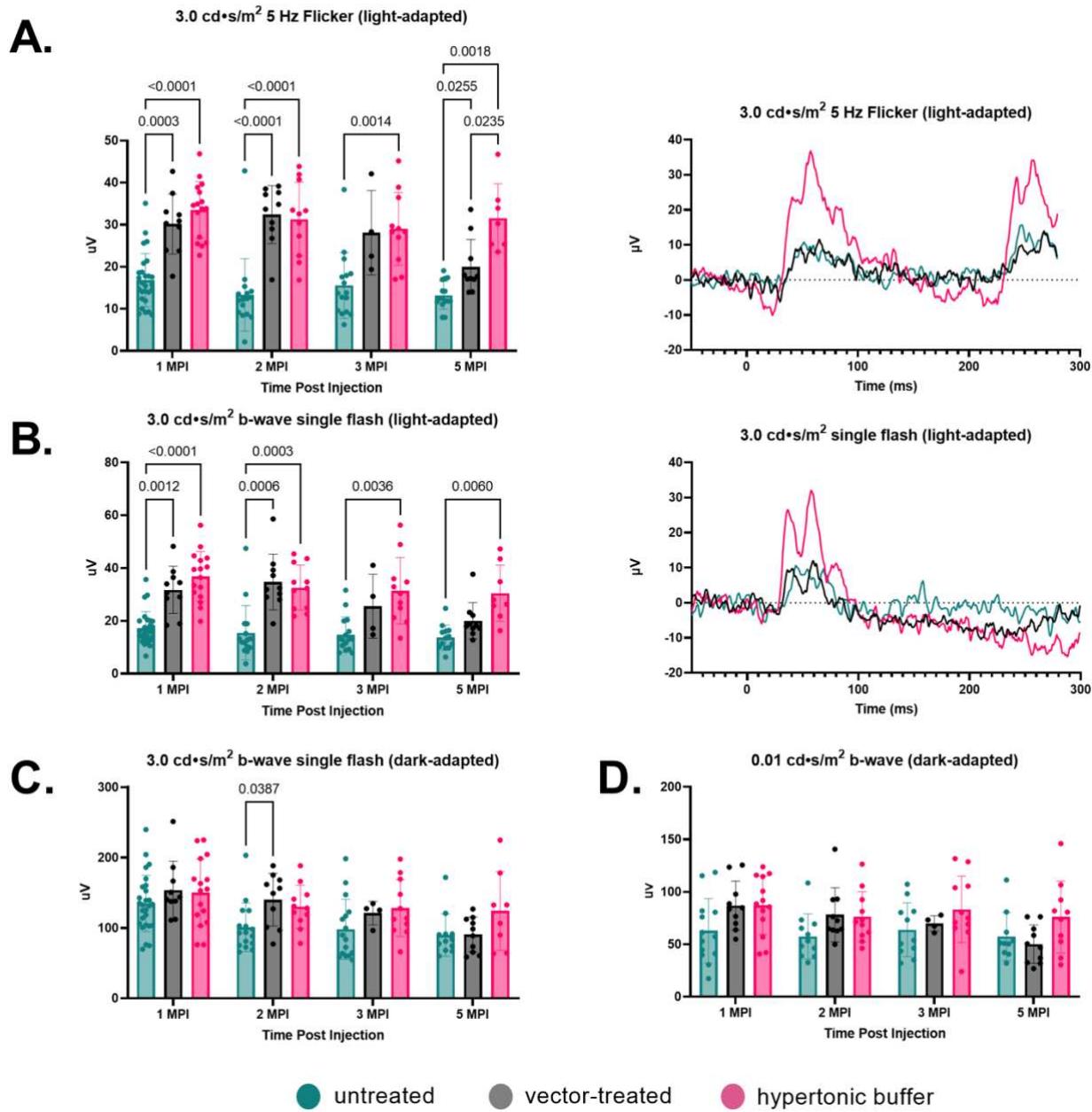
583 **Figure 1 (A-D): Natural history of disease in the *Rs1*-KO mouse compared to WT BL/6  
584 mouse.**

585 OCT images were collected from p15 to 9 months of age in *Rs1*-KO mice, and ONL thickness (B)  
586 and cyst severity (C) were measured at each age. Measurements of cyst height were taken at four  
587 points equidistant, 500  $\mu$ m, from the optic nerve, and the averaged values for each eye were reported.  
588 For cyst severity, cyst height was measured and translated to a scoring scale of 1 to 6, with 1  
589 representing a cyst height of 0. Representative OCT images demonstrate retinoschisis in *Rs1*-KO  
590 mice at P15, 3-, and 6 months old, compared to a WT mouse without retinoschisis (A). ERGs of *Rs1*-  
591 KO mice and WT mice were collected at 2 and 6 months of age. As early as 2 months post-injection,  
592 *Rs1*-KO mice show reduced ERG B wave amplitudes and delayed latency of the b wave peak,  
593 compared to the robust WT waveform (D).



594 **Figure 2 (A-B): Cyst severity is significantly reduced in vector-treated and hypertonic buffer-  
595 injected eyes at 1 MPI.**

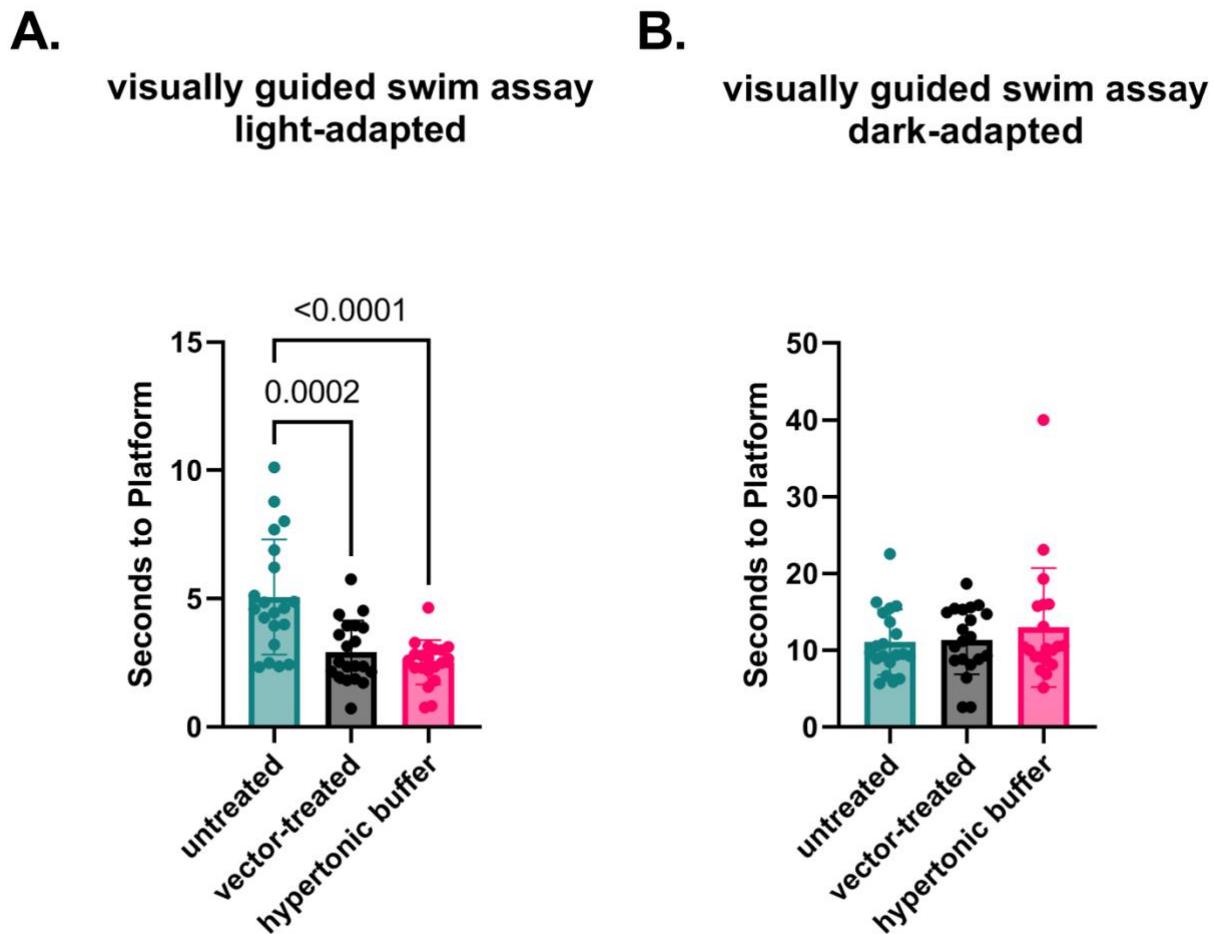
596 OCT images of vector-treated, hypertonic buffer-injected, and untreated eyes were collected at 1, 2,  
597 and 5 MPI. Measurements of cyst severity (B) and ONL thickness (C) were taken at four points  
598 equidistant, 500 um, from the optic nerve, and each datapoint is the averaged value of an eye. For  
599 cyst severity, cyst height was translated to a modified scoring scale of 1 to 6, and averaged for that  
600 eye. Both vector-treated ( $p=0.002$ ) and hypertonic buffer-injected eyes ( $p<0.0001$ ) had significantly  
601 less cysts than untreated eyes at 1 MPI, and continued to have few to no cysts over time.  
602 Representative OCT findings at 1 MPI (A).



603 **Figure 3 (A-D): Hypertonic buffer outperforms untreated and vector-treated eyes in light-  
604 adapted ERGs, but not in dark-adapted ERGs.**

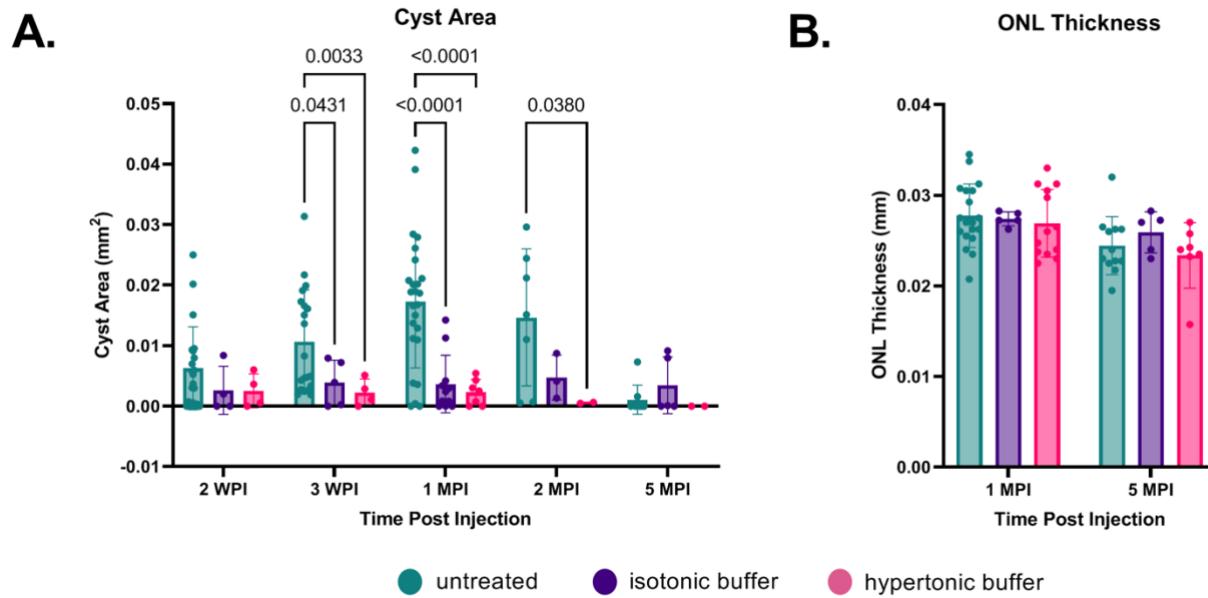
605 Cone function was measured using two separate light-adapted ERG assays - the 5 Hz Flicker and 3.0  
606 cd·s/m<sup>2</sup> bright flash. In response to the 5 Hz flicker stimulus, vector-treated eyes and hypertonic  
607 buffer-injected eyes had higher amplitudes than untreated eyes at 1, 2, 3, and 5 MPI (5 MPI: *vector-  
608 treated*:  $p=0.0255$ ; *hypertonic buffer*:  $p=0.018$ ) (A). Hypertonic buffer-injected eyes were also  
609 superior over vector-treated eyes at the 5 MPI time point ( $p=0.0235$ ) (A). Under the 3.0 cd·s/m<sup>2</sup>  
610 bright flash assay, an additional measurement of cone-pathway function, vector-treated eyes  
611 demonstrated superior cone function to untreated eyes till 2 MPI ( $p=0.0006$ ), whereas the hypertonic  
612 buffer-injected eyes were superior till 5 MPI ( $p=0.006$ ) (B). (In summary, hypertonic buffer-injected  
613 eyes had a more robust and long-term improvement in cone-dominant pathways.) Representative  
614 waveforms demonstrate these findings at 5 MPI (A-B). Combined rod-cone function was measured  
615 by the 3.0 cd·s/m<sup>2</sup> bright flash under dark-adapted conditions (C). At 1, 3, and 5- MPI, there was no

616 difference between vector-treated, hypertonic buffer-injected, or untreated eyes. At 2 MPI, vector-  
617 treated eyes did demonstrate superior function over untreated eyes, but this effect did not persist.  
618 Isolated rod function was measured using 0.01 cd•s/m<sup>2</sup> dim flash after dark adaptation (D). There  
619 was no persistent significant difference between hypertonic buffer-injected, isotonic buffer-injected,  
620 or untreated eyes in rod dominant ERG settings, suggesting no or minimal rod rescue following  
621 injections.



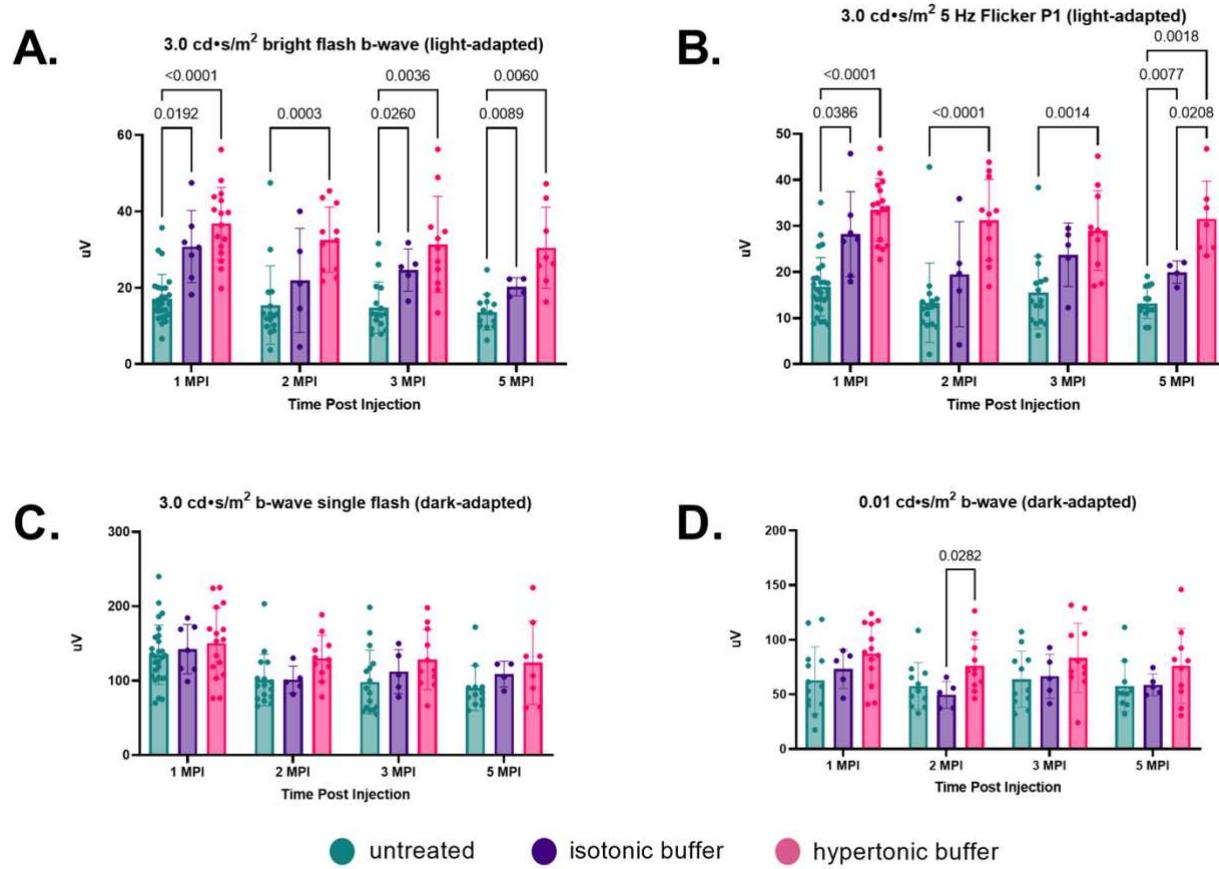
622 **Figure 4 (A-B): Vector-treated and hypertonic buffer-injected mice perform faster in a light-  
623 adapted swim maze than untreated *Rs1*-KO mice.**

624 Mice were placed in a swim maze and testing was performed under dark or light adaptation  
625 conditions. Swim time to a platform was recorded. Each data point is representative of the average  
626 time-to-platform for a randomly selected platform position. In a light-adapted environment, vector-  
627 treated and hypertonic buffer-injected mice are significantly faster at finding swim platforms than  
628 untreated *Rs1*-KO mice (*vector-treated*:  $p=0.0002$ ; *hypertonic buffer*:  $p<0.0001$ ), suggesting  
629 improvement in light-adapted visual acuity (A). In a dark-adapted environment, there is no  
630 significant difference in time-to-platform between vector-treated, hypertonic buffer-injected, and  
631 untreated eyes, suggesting no effect on visual acuity in the dark (B).



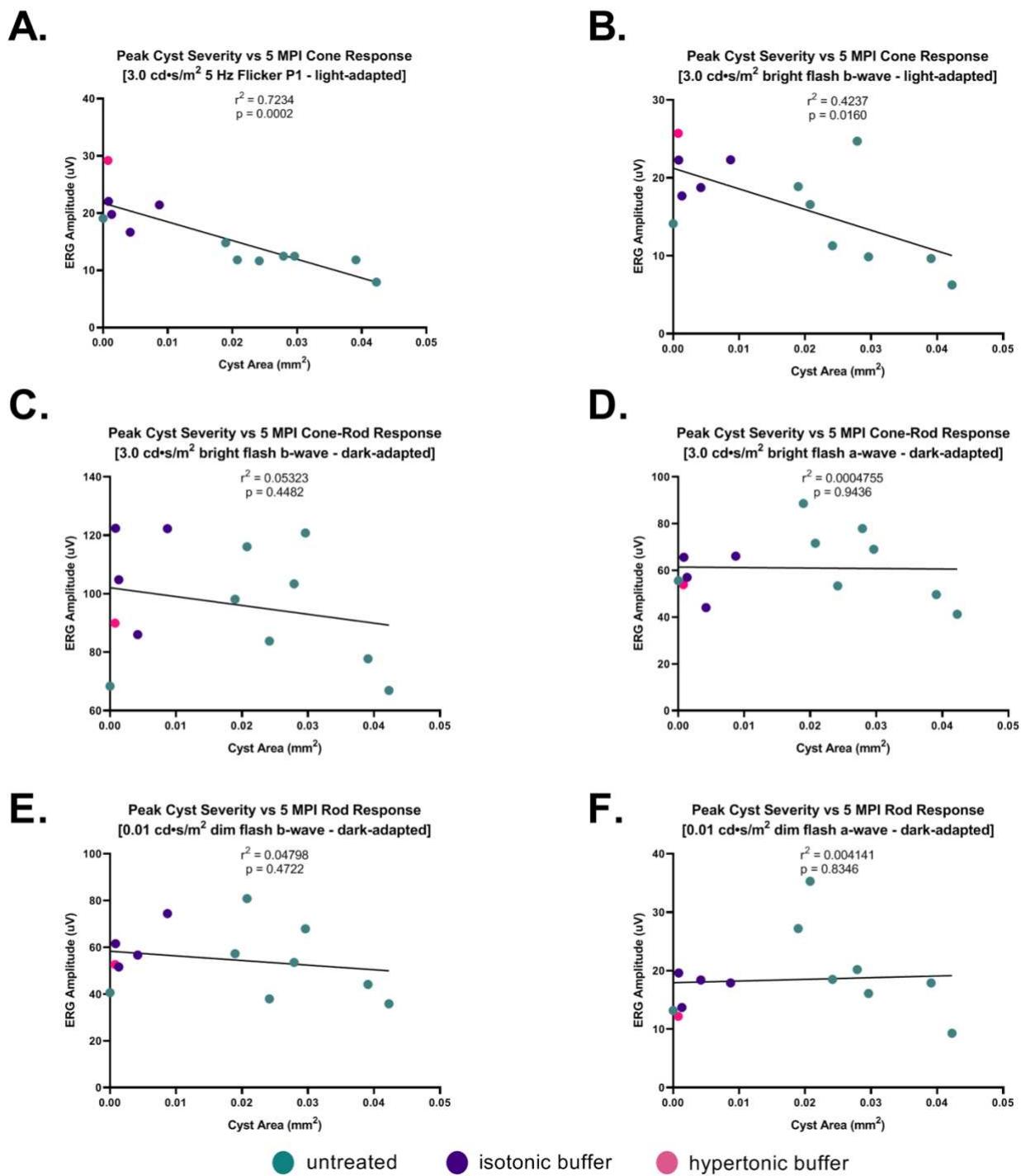
632 **Figure 5 (A-B): Cyst area is reduced in hypertonic buffer and isotonic buffer-injected eyes,  
633 with greater reduction observed in the hypertonic buffer group.**

634 OCT images were collected at 2- and 3- weeks post injection (WPI), 1 MPI, and 2 MPI. Cyst area  
635 quantification and outer nuclear layer (ONL) thickness measurements were taken to assess  
636 photoreceptor cell survival. Each data point is representative of one eye. Starting at 3 WPI, cyst area  
637 is significantly reduced in hypertonic buffer and isotonic buffer-injected eyes compared to untreated  
638 eyes (3 WPI: *hypertonic buffer*:  $p=0.0033$ ; *isotonic buffer*:  $p=0.0431$ ). For the hypertonic-buffer  
639 injected eyes, this effect persisted to 2 MPI (2 MPI: *hypertonic buffer*:  $p=0.0380$ ) (A). Cyst area is  
640 decreased across all groups at 5 MPI (A). ONL thickness remains stable to 5 MPI and does not  
641 significantly differ between injection groups and untreated controls (B).



642 **Figure 6 (A-D): Hypertonic buffer-injected eyes have a more robust cone rescue on light-  
643 adapted ERG over untreated and isotonic buffer-injected eyes.**

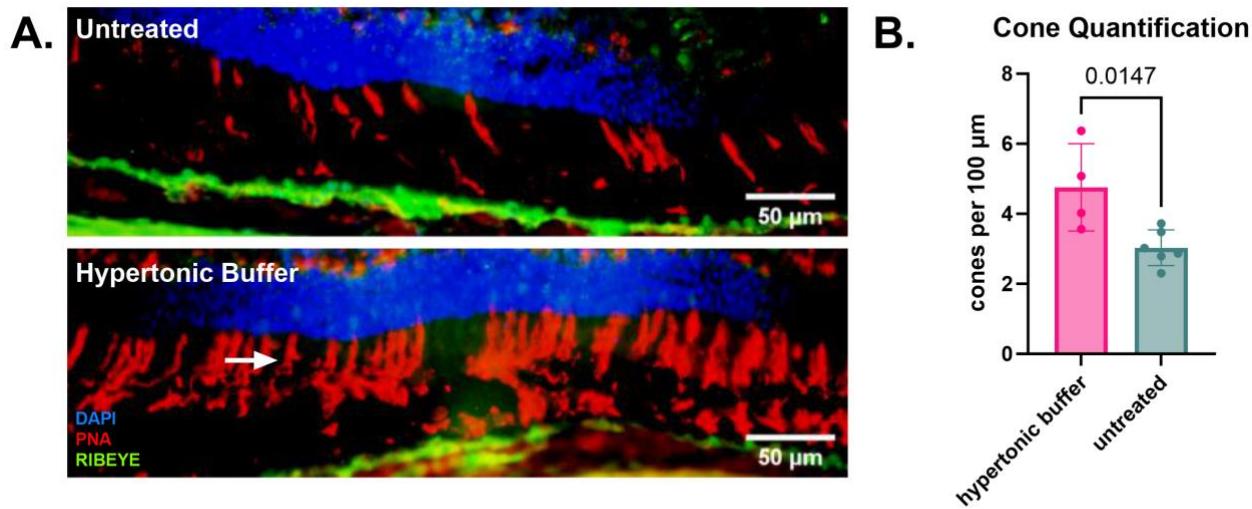
644 To determine if tonicity had a potential effect on improving electrical functioning of the retina, an  
645 isotonic buffer was subretinally introduced into the eyes of *Rs1*-KO mice. Cone function was  
646 measured using two separate light-adapted ERG assays - the 5 Hz Flicker and 3.0 cd•s/m<sup>2</sup> bright  
647 flash. Each data point is representative of one eye. In response to the 3.0 cd•s/m<sup>2</sup> bright flash, both  
648 isotonic buffer and hypertonic buffer-injected eyes displayed superior cone pathway functioning over  
649 untreated eyes, persisting to 5 MPI (5 MPI: *hypertonic buffer*:  $p=0.0060$ ; *isotonic buffer*:  $p=0.0089$ )  
650 (A). However, hypertonic buffer-injected eyes exhibited a more pronounced improvement over  
651 untreated controls compared to isotonic buffer-injected eyes. Under the 5 Hz flicker assay, isotonic  
652 buffer and hypertonic buffer-injected show improved functioning over untreated eyes to 5 MPI (5  
653 MPI: *hypertonic buffer*:  $p=0.0018$ ; *isotonic buffer*:  $p=0.0077$ ). However, hypertonic buffer-injected  
654 eyes were superior to isotonic buffer-injected eyes at 5 MPI ( $p=0.0208$ ). (In summary, hypertonic  
655 buffer-injected eyes had a more robust and long-term improvement in cone-dominant pathways). The  
656 combined rod-cone function was measured by the 3.0 cd•s/m<sup>2</sup> bright flash under dark-adapted  
657 conditions (C). Isolated Rod function was measured using 0.01 cd•s/m<sup>2</sup> dim flash after dark  
658 adaptation (D). There was no persistent significant difference between hypertonic buffer-injected,  
659 isotonic buffer-injected, or untreated eyes in rod dominant ERG settings, suggesting no or minimal  
660 rod rescue following injections.



661 **Figure 7 (A-F): Peak cyst severity on OCT is negatively associated with cone function on ERG**  
662 **at 5 MPI.**

663 Peak cyst severity of hypertonic buffer-injected, isotonic buffer-injected, and untreated eyes was  
664 determined by comparing the cyst area measurements of each eye at 1, 2, and 5 MPI. The cyst area of  
665 each eye representing their peak severity was correlated with its ERG amplitudes at 5 MPI. Each dot  
666 is representative of one eye. A negative correlation was found between cyst area and ERG  
667 amplitudes in the light-adapted 5 Hz Flicker ( $p=0.0002$ ) and 3.0 cd•s/m<sup>2</sup> bright flash ( $p=$  (A, B)).  
668 Little to no correlation was found between peak cyst area and ERG amplitudes in dark-adapted 0.01

669 cd•s/m<sup>2</sup> or 3.0 cd•s/m<sup>2</sup> tests (C, D, E, F). These findings suggest increasing cyst area has greater  
670 impact on impairing cone electrical function, than rod photoreceptors.



671 **Figure 8 (A-B): Increased cone density at 6 months old suggests potential cone preservation**  
672 **mechanism following injection of hypertonic buffer.**

673 Representative immunofluorescent staining (RED) of cone outer segments, demonstrating increased  
674 density of cones in hypertonic buffer-injected eyes compared to untreated eyes with sparse cone  
675 staining (A). Retinal sections were collected from untreated *Rs1*-KO eyes and hypertonic buffer-  
676 injected eyes at approximately 6 months of age. Sections were stained by PNA to visualize cone  
677 outer segments. The number of cone outer segments per 100 μm of the retina was quantified by three  
678 masked participants, and averaged (cone outer segment indicated by white arrow). Green channel for  
679 RIBEYE staining of synapses. Each data point is representative of one eye. Hypertonic buffer-  
680 injected eyes show significantly higher cone density per 100 μm than untreated *Rs1*-KO eyes  
681 ( $p=0.0147$ ) (B).

682 **7 Acknowledgments and Funding**

683 We would like to thank Dalyz Ochoa and Budd Tucker Ph.D. for providing the AAV2/4-EF1 $\alpha$ -*RS1*  
684 vector for this study. We would also like to thank Paul Sieving M.D., Ph.D. for providing us with the  
685 *Rs1*-KO mouse line.

686 This work was generously supported by the Chakraborty Family Foundation and the Keech  
687 Professorship.

688 **8 References**

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