Increased susceptibility to injury in older eyes

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1 **Abstract**

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3 **Purpose:** To determine whether there is an age-dependent susceptibility in retinal function in
4 response to repeated anterior chamber cannulation with or without intraocular pressure (IOP)
5 elevation.

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7 **Methods:** Baseline electroretinograms (ERGs) were measured in 3- and 18-month-old Sprague-
8 Dawley rats (n=16 each group). Following baseline assessment, eyes were randomly assigned to
9 undergo a 60 minute anterior chamber cannulation with IOP either left at baseline (sham, 15
10 mmHg) or elevated to 60 mmHg. This was repeated three additional times with each episode
11 separated by one week. At weeks 1 to 3 dark-adapted retinal function was assessed immediately
12 prior to cannulation with final functional assessment at week 4.

13

14 **Results:** Both sham and IOP elevated eyes of older rats showed retinal dysfunction, which
15 became more pronounced with the number of repeated insults. This effect was largest for
16 responses arising from the inner retina. Repeated insult in younger eyes did not produce a
17 change in amplitude, but an increase in the sensitivity to light of photoreceptor and bipolar cell
18 components of the ERG.

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20 **Conclusion:** Repeated trauma, not IOP, produces permanent retinal dysfunction in older eyes.
21 Younger eyes appear to be able to withstand this type of injury by upregulating sensitivity of outer
22 and middle retinal responses to maintain normal inner retinal function.

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1 Introduction

2 The incidence and prevalence of glaucoma increases exponentially with age.¹ However, the most
3 well defined risk factor for glaucoma, elevated intraocular pressure (IOP) shows only a modest
4 increase with age.² This suggests ageing influences IOP-independent pathways to increase the
5 risk of neuronal injury. Studies in the area of stroke provide evidence that older brains are less
6 capable of recovery from ischemic insult than younger ones.^{3, 4} DiNapoli and colleagues⁵ have
7 shown that older rats exhibit a deterioration in the blood-brain-barrier, which they propose could
8 account for the greater neuronal damage and slower recovery of older brains following stroke.

9
10 Consistent with these studies, Kawai et al⁶ have shown that there is an age-related decline in
11 retinal ganglion cell (RGC) numbers (2 month versus 24 month Fischer rats). They demonstrated
12 that the remaining RGCs in 2 year old rats were more sensitive to ischemia reperfusion injury
13 induced by IOP elevation than the 2-month-old rats. Katano and colleagues⁷ considered the effect
14 of a short period of acute IOP elevation on retinal function in young and old Wistar rats and found
15 that 18-month-old rats failed to fully recover from an IOP insult of 80 mmHg for 120 minutes,
16 whereas 4-month-old rats showed complete recovery. These studies suggest that older eyes are
17 more susceptible to severe IOP elevation. What is not known is whether older eyes show
18 increased sensitivity and impaired recovery to more moderate levels of IOP elevation, and
19 whether older eyes are able to recover from repeated episodes of IOP elevation.

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21 This study will investigate the susceptibility of 3 and 18-month-old rats to repeated IOP elevation
22 (60 mmHg for 1 hour). This combination of this IOP magnitude and duration has been shown to
23 produce the largest separation between attenuation of inner and outer retinal responses.⁸ Data
24 from Zhi et al⁹ suggest that inner retinal blood flow is compromised at 60 mmHg, but choroidal
25 flow is not, which may account for the observations of Bui et al.⁸ Retinal function in young rats
26 can completely recover from one episode of IOP elevation as high as 70 mmHg^{10, 11} but it is
27 unclear whether multiple episodes of a similar magnitude will produce a cumulative retinal
28 dysfunction. Given that studies have demonstrated that older neurons are more susceptible to

stress, we will investigate whether repeated episodes of IOP challenge produces greater functional deficits in older eyes.

Methods

Animals

All experimental procedures abide by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approval was obtained from our facility's Animal Ethics Committee. Two cohorts of Sprague-Dawley (SD) rats (three-month-old, n = 16 and eighteen-month-old, n = 16) were housed in a 12:12 hr light:dark cycle with a maximum illuminance of 50 lux. Food and water were available *ad libitum*.

Prior to experimentation, animals were anaesthetized via intramuscular injection of ketamine:xylazine (60:5 mg/kg, Troy Laboratories Pty Ltd., NSW, Australia) followed by topical application of proxymetacaine (0.5%, Alcon Laboratories, NSW, Australia) for corneal anaesthesia. Mydriasis was achieved by application of tropicamide (0.5%, Alcon Laboratories) and phenylephrine hydrochloride (2.5%, Chauvin Pharmaceuticals Ltd, UK). Repeated cannulation appeared to have little effect on pupil size in 3 or 18-month old rats.

Experimental protocol

All animals underwent overnight dark adaptation to allow for ERG measurement (see later), immediately after which animals underwent anterior chamber cannulation. This was repeated for the next three weeks for a total of four insults, with 7 days of recovery between each IOP challenge. At the fifth week only dark-adapted ERG was measured, followed by animal termination. Thus, each functional assessment allowed 7 days recovery after the previous IOP challenge. Repeated cannulation appeared to have little effect on pupil size in 3 or 18-month old rats.

Each animal had one eye randomly assigned to either IOP elevation (60 mmHg) or sham (15 mmHg) for one hour. Throughout the treatment body temperature was maintained by a circulating water pad. Core temperature and pulse were monitored continuously, and blood pressure was measured every 15 minutes (tail cuff sphygmomanometer; ML 125, ADInstruments Pty Ltd. NSW, Australia).

Repeated IOP elevations

IOP elevation was achieved by cannulating the anterior chamber using beveled glass pipettes (75 µm tip, Harvard apparatus, Kent, U.K.). We have previously calibrated our glass pipettes and shown that resistance across the tip is likely to have negligible effect at 60 mmHg.¹² Each glass pipette was attached via polyethylene tubing (1.27×0.97 mm, Microtube Extrusions, NSW, Australia) to a Hanks balanced salt solution reservoir (JRH Biosciences, Kansas City, KS). A pressure transducer was placed in series to continuously monitor IOP (Transpac, Abbot Critical Care Systems, Sligo, Ireland). The height of the reservoir was pre-calibrated to give a pressure of either 15 (sham baseline) or 60 mmHg (IOP challenge). The beveled glass pipette was then inserted with the aid of micromanipulators (World Precision Instruments, KITE-R, Florida, USA) into the rodent's anterior chamber, perpendicular to the corneal centre avoiding contact with iris and lens.

Electroretinography

Retinal function was assessed with the full field ERG, following dark-adaptation (>12 hrs). Electrode configuration and flash characteristics have been described previously.¹⁰ Briefly, purpose-built chlorided silver electrodes were used, where the active (0.8 mm diameter) was positioned on the corneal apex and the ring-shaped reference placed around the sclera near the equator. The ground electrode (F-E2-30 Grass Telefactor, West Warwick, RI) was inserted subcutaneously into the tail. Electrical contact and corneal hydration was maintained with carmellose sodium 10 mg/mL (Celluvisc, Allergan, Irvine, CA, USA). The ERG stimulus was

generated by light emitting diodes (5 Watt, 5500 K, Luxeon, Calgary, Alberta, Canada) in a Ganzfeld sphere (Photometric Solutions International, Huntingdale, VIC, Aust). ERGs were collected over a range of luminous energies (-6.53 to 2.07 log scotopic cd.s.m⁻²). Noise was reduced by averaging 20 signals at the dimmest light levels and progressively fewer with increasing luminous energy, whilst the interstimulus interval was lengthened from 2 to 120 seconds to allow total recovery between successive flashes.

ERG analysis

Photoreceptor response

The leading edge of scotopic a-wave is modeled using a delayed Gaussian equation.^{13,14}

$$P3(i, t) = Rm_{P3} \cdot \left[1 - \exp \left(-i \cdot S \cdot (t - t_d)^2 \right) \right], t > t_d \quad \text{Equation 1}$$

In Equation 1, the P3 (μV) response for a given luminous energy (i , log (cd.s.m⁻²)) is expressed as a function of time (t , s), saturated amplitude (Rm_{P3}) and sensitivity (S , m².cd⁻¹.s⁻³) after a brief delay (t_d , s). All parameters were optimized by minimizing the sum-of-square merit function with the Solver module in Excel (Microsoft™, Redmond, WA) across an ensemble of waveforms returned from the top three luminous energies (1.78, 1.95, 2.07 log cd.s.m⁻²), which elicits saturated a-wave amplitude.¹⁵

Bipolar cell response

To isolate the bipolar cell component (P2) of the ERG, the modeled P3 (Equation 1) is subtracted from the raw ERG.¹⁶ The amplitude of the isolated putative P2 as a function of luminous energy is modeled with a saturating hyperbolic function (Equation 2).¹⁷

$$V(i) = V_{\max} \frac{i}{i + k} \quad \text{Equation 2}$$

The P2 amplitude (V , μV) is defined as a function of luminous energy (i , log cd.s.m⁻²), $V_{\max}$ (μV) a saturated amplitude and the semi-saturation constant (k , log cd.s.m⁻²), the inverse of which gives

sensitivity ($K = 1/k$). Using the Solver module of an Excel spreadsheet (MicrosoftTM) V_{max} and k were optimised to minimise the sum-of-square merit function.

Retinal ganglion cell (RGC) response

The amplitude of the ganglion cell dominated scotopic threshold response (STR)⁸ is measured at fixed time of 130 ms (positive STR, pSTR) after stimulus onset. Due to the small amplitude of STR components, data were averaged across three luminous energies (-5.33, -5.14 and -5.00 log cd.s.m⁻²).

Data analysis and statistics

Data for each eye (i.e. sham 15 mmHg or IOP treated 60 mmHg), is expressed as a percentage relative to its own baseline recorded immediately prior to the first cannulation. A repeated measures two-way ANOVA established that there was no interaction between time (weeks, nested factor) and treatment (15/60 mmHg, between factor), thus data are collapsed across the four weeks. Data across ages could then be compared with a repeated measures two-way ANOVA (between factor age, within factor 15/60 mmHg). A separate two-way ANOVA was used to compare treatment effect across ERG parameters within an age group (between factor ERG parameters, within factor 15/60 mmHg).

Results

Figures 1A and C show that 4 repeated treatments (either 15 or 60 mmHg) produce minimal functional change in 3-month-old rats. After four episodes of cannulation the 18 month rats show a reduction in the b-wave (Figure 1B) and in particular the positive component of STR (Figure 1D) for both sham and IOP elevated eyes.

Insert Figure 1 here

Figure 2 illustrates the effect of treatment (sham and IOP elevation) by expressing ERG parameters for 3 and 18-month-old rats as a percentage change relative to each eye's own baseline. For the 3-month-old rats, repeated measures ANOVA reveals that there was no treatment effect (sham versus IOP elevation) for photoreceptor amplitude (Rm_{P3} , Figure 2A $F_{1,19} = 0.02$, $p = 0.90$), Bipolar cell P2 amplitude (V_{max} , Figure 2C $F_{1,19} = 0.12$, $p = 0.73$), nor ganglion cell amplitude (pSTR, Figure 2E pSTR $F_{1,18} = 0.01$, $p = 0.92$). Furthermore, there was no effect across the number of cannulations (Rm_{P3} $F_{1,19} = 0.85$, $p = 0.47$; V_{max} $F_{1,19} = 1.88$, $p = 0.14$; pSTR $F_{1,18} = 2.70$, $p = 0.05$), indicating that repeated anterior chamber insults (cannulation and/or IOP elevation) do not affect the ERG output in 3-month-old rats. In contrast, Figure 2G shows that in 3-month-old rats phototransduction sensitivity (S) was significantly greater in IOP elevated eyes than the sham treated eyes ($F_{1,18} = 4.58$, $p < 0.05$). This increase in sensitivity was not constant across the number of cannulations ($F_{1,18} = 16.41$, $p < 0.0001$). Analysis of raw phototransduction sensitivity in 3 month-old rats shows that there was a significant increase from baseline in both sham ($F_4 = 2.9$, $p = 0.03$) and IOP treated ($F_4 = 5.2$ $p = 0.002$) eyes.

Bipolar sensitivity (K, Figure 2I) for 3 month sham and IOP elevated eyes were both increased but there was no difference between the two groups ($F_{1,15} = 0.81$, $p = 0.38$). A significant effect across the number of cannulations was also observed ($F_{1,15} = 4.83$, $p < 0.01$), with bipolar sensitivity increasing significantly from baseline after cannulations 2 to 4.

In the older rats, Figure 2B shows that there was a decrease in Rm_{P3} for both sham and IOP elevated eyes across all cannulations when compared to baseline. This relative decrease was the same for sham and IOP elevated groups ($F_{1,23} = 0.31$, $p = 0.58$). However, there was a significant effect across the number of cannulations ($F_{1,23} = 7.04$, $p < 0.001$). This finding is similar for the bipolar cell V_{max} , which declines with the number of cannulations (Figure 2D, IOP effect $F_{1,23} = 1.12$, $p = 0.30$, cannulation effect $F_{1,23} = 20.13$, $p < 0.0001$). In Figure 2F, there was also no difference between IOP elevated and sham eyes ($F_{1,21} = 0.02$, $p = 0.90$), however the pSTR did decline significantly after the first cannulation ($F_{1,21} = 6.99$, $p < 0.001$).

Unlike the improvement in sensitivity seen in 3-month-old rats, photoreceptor sensitivity in older rats (Figure 2H) decreases after the fourth repeated cannulation ($F_{1,21} = 3.23$, $p < 0.05$) in both sham and IOP treated cohorts ($F_{1,21} = 0.15$, $p = 0.71$). Bipolar cell sensitivity (K, Figure 2J) in older rats also show a significant decline with repeated cannulation ($F_{1,21} = 12.89$, $p < 0.0001$) for both sham and IOP elevated eyes ($F_{1,21} = 0.01$, $p = 0.94$).

Insert Figure 2 here

Figure 3 shows the overall effect of repeated injury by averaging across the four cannulations. Two-way ANOVA between age group and treatments revealed a significant age effect for all ERG parameters (Rm_{P3} $F_{1,1} = 6.02$, $p < 0.05$; V_{max} $F_{1,1} = 3.05$, $p < 0.0001$; pSTR $F_{1,1} = 12.03$, $p < 0.01$; S $F_{1,1} = 8.80$, $p < 0.01$; K $F_{1,1} = 47.62$, $p < 0.0001$). There was no IOP elevation effect in either 3 or 18-month-old rats (Rm_{P3} $F_{1,1} = 0.09$, $p = 0.76$; V_{max} $F_{1,1} = 0.50$, $p = 0.48$; pSTR $F_{1,1} = 0.27$, $p = 0.61$; S $F_{1,1} = 0.47$, $p = 0.49$; K $F_{1,1} = 0.20$, $p = 0.65$).

To investigate whether an ERG component amplitude was more affected than others, a two-way ANOVA comparing relative changes in Rm_{P3} , V_{max} and pSTR across sham and IOP treated eyes was performed for both ages. In the 3-month-old rats, there was no significant difference between ERG components ($F_{1,2} = 1.31$, $p = 0.28$). However, the decrease in amplitude across Rm_{P3} , V_{max} and pSTR was not uniform in the older rats ($F_{1,2} = 15.16$, $p < 0.0001$), as a Bonferroni post-hoc test reveals that the ganglion cell dominated pSTR was significantly more attenuated than the photoreceptor Rm_{P3} .

In 3-month-old rats bipolar cell sensitivity showed a larger increase with repeated cannulation than did photoreceptor sensitivity ($F_{1,1} = 12.00$, $p < 0.01$, comparison across Figures 3D and 3E). In contrast, older rats showed a greater reduction in bipolar cell sensitivity when compared

with the photoreptoral sensitivity ($F_{1,1} = 106.00$, $p < 0.0001$, comparison across Figures 3D and 3E).

Insert Figure 3 here

Discussion

This study shows that repeated injury has little effect on the retinal function of three-month-old albino rats, but produces dysfunction in eighteen-month-old rats. The observation that there was no dysfunction in younger animals is consistent with previous findings that younger rats can completely recover from a single episode of IOP challenge similar in magnitude to that employed here (70 mmHg for 1 hour).^{10,11,18} Our study extends previous reports, to show that younger rats are able to resist repeated moderate IOP insults of short duration separated by one week.

Our data provides a possible explanation for the functional resilience of the inner retina in 3-month-old rats. In particular, ganglion cell dysfunction arising from repeated injury could be masked if the upstream input to the ganglion cells were to be increased. One way greater input could be achieved is via an improvement in photoreceptor and/or bipolar cell sensitivity to light, as is observed in our data (Figure 2). This means that, at dim light levels, the same number of photons captured would produce a larger ganglion cell response (leftward shift of the luminance energy response function). The mechanism underlying this observed improvement in sensitivity arises is unclear. Studies in the brain show that new neurons can proliferate at the site of ischemic injury.¹⁹ However, an increase in the number of photoreceptors should produce larger photoreptoral amplitude without a change in sensitivity, which was not the case in our data. Therefore the upregulation of sensitivity in 3-month-old rats reflect other mechanisms, possibly alterations in the efficiency of phototransduction proteins.²⁰

Our data also show that in 3-month-old rats bipolar cell sensitivity ($52.8 \pm 16.3\%$, $F_{1,1} = 12.00$, $p < 0.01$) increased even more than phototransduction sensitivity ($14.0 \pm 4.0\%$). This is consistent with the suggestion of Aleman et al²¹ that rod-bipolar cell synapses can increase following retinal injury. A consequence of a greater number of bipolar cell dendrites with the same number of photoreceptors (same phototransduction amplitude) would be increased convergence, which could manifest as an improved sensitivity to light in the rod pathway. We propose that upregulation of sensitivity within the phototransduction cascade and between the photoreceptor and bipolar cell synapses in 3-month-old rats reflect a compensatory mechanism to maintain normal retinal output in response to repeated injury. The data from 18-month-old rats suggests that such compensatory mechanisms are insufficient. Our previous study²² showed that aging itself in 18-month-old compared with 3-month-old rats causes increased sensitivity. This is consistent with a previous study report that in older mice there is evidence of proliferation of rod bipolar and horizontal cell dendrites towards rod photoreceptor spherules.²³ It is possible that 18-month-old rats have already reached their maximal capacity to increase sensitivity and hence in response to repeated stress cannot further compensate. This manifests as loss in ganglion cell output with repeated insult in the older cohort.

It is important to note that in the older eyes both repeated sham cannulation and IOP elevation produced similar functional deficits. Based on this observation, we believe that there is a common mechanism of injury in both sham and IOP elevated eyes. While the exact mechanism of injury in the older eyes from cannulation is unclear, based on previous reports we speculate inflammation may be involved. Hoyng et al²⁴ have reported that cannulation of the rabbit anterior chamber resulted in classic signs of inflammation, which were observable on the anterior ocular surface as well as in the aqueous humour. More recently, Chinnery et al²⁵ showed that corneal trauma followed by topical application of a lipopolysaccharide in mice produced retinal inflammation. It is also well established that basal inflammation increases with age²⁶⁻²⁸ as demonstrated by accumulations of macrophages²⁹ and microglia²⁵ in subretinal space of older mice. Thus it is

possible that inflammatory processes contribute to the non-specific functional losses arising from repeated insults to older eyes.

Conclusion

In 3 month animals, four repeated cannulations with or without IOP elevation did not decrease the amplitude of retinal responses. There was a significant improvement in the sensitivity of outer retinal components, which we believe represents a compensatory mechanism to account for the lack of inner retinal dysfunction in 3-month-old rats. In older eyes repeated trauma (both sham treatment and IOP elevation) produced a cumulative loss of retinal function with the number of cannulations, with the exact mechanism for injury unknown. However, we propose that as the older eyes have already upregulated retinal sensitivity to counteract age-related cell loss, there is no longer a buffer to protect against additional injury.

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1 **Figure Legends**

2 **Figure 1:** **Panel A** shows group average dark-adapted responses in 3-month-old rats after four
 3 sham (dashed) or IOP elevations (solid line). The grey line shows ERG response recorded at
 4 baseline. **Panel B** shows group average dark-adapted responses in 18-month-old rats, other
 5 details as per Panel A. **Panel C** shows group average scotopic threshold responses in 3 month
 6 rats, other details as per Panel A. **Panel D** shows group average scotopic threshold responses in
 7 18 month rats, other details as per Panel A.

8
 9 **Figure 2:** Effect of repeated sham (unfilled circles) or IOP elevation (filled circles) on retinal
 10 function in 3 and 18-month-old rats. All data are expressed as a change relative to baseline
 11 (average $\pm$ SEM). ERG parameters from 3-month-old rats are shown in Panels **A** (R_{mP3} ,
 12 phototransduction amplitude), **C** (V_{max} , bipolar cell P2 amplitude), **E** (pSTR, ganglion cell
 13 amplitude), **G** (S, phototransduction sensitivity) and **I** (K, bipolar cell sensitivity). ERG parameters
 14 from older eyes are shown in Panels **B** (R_{mP3}), **D** (V_{max}), **F** (pSTR), **H** (S) and **J** (K).

15
 16 **Figure 3:** Average effect of repeated sham (unfilled) versus repeated IOP (filled) in 3 and 18-
 17 month-old rats. All data expressed as a change relative to baseline (average $\pm$ SEM) across all
 18 time points. ERG parameters shown in panels **A** (R_{mP3}), **B** (V_{max}), **C** (pSTR), **D** (S), **E** (K).

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