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Head-up tilt lowers IOP and improves RGC dysfunction in glaucomatous DBA/2J mice

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Abstract

The inbred DBA/2J (D2) mouse strain is a well established model of spontaneously elevated intraocular pressure (IOP), progressive glaucomatous loss of retinal ganglion cells (RGCs), and early damage of RGC axons at the level of optic nerve head. Pattern electroretinogram (PERG) studies have shown that surviving RGCs in mice 6–12 month old may be dysfunctional. RGC dysfunction seems to be IOP-dependent, since it may be exacerbated by means of acute IOP elevation with head-down body tilt. Here we test the hypothesis that head-up body posture lowers IOP, resulting in improvement of PERG amplitude in aged D2 mice with glaucoma. We show that head-up body tilt induces age-independent IOP lowering whose magnitude increases with the angle of tilt. For a fixed angle (-60°) of head-up tilt, IOP progressively decreases with a time constant of about 5 min and stabilizes at a value lower by about 5–6 mm Hg compared to the baseline. Head-up tilt also results in an improvement of PERG amplitude in older D2 mice with glaucoma but not in younger D2 mice without glaucoma. Improvement of PERG amplitude in aged D2 mice upon head-up-induced IOP lowering is consistent with the idea that RGCs undergo a stage of IOP-dependent, reversible dysfunction before death. The head-up IOP/PERG protocol may represent a non-invasive way to probe the potential for recovery of RGC dysfunction in D2 mice.

Keywords

Intraocular pressure; retinal ganglion cells; glaucoma; pattern electroretinogram; mouse; DBA/2J

Introduction

The inbred DBA/2J (D2) mouse strain is a well established model of spontaneously elevated intraocular pressure (IOP), progressive glaucomatous loss of retinal ganglion cells (RGCs), and early damage of RGC axons at the level of optic nerve head, which represent the hallmarks of glaucoma (Howell et al., 2007; John, 2005; John et al., 1998; Libby et al., 2005; Sheldon et al., 1995). Photoreceptors, horizontal, and bipolar cells remain histologically intact (Jakobs et al., 2005; Moon et al., 2005). D2 mice develop iris atrophy and pigment dispersion due to mutations of two genes, *Gpnmb* and *Tyrp1* (Anderson et al., 2002). Pigment dispersion impairs drainage of aqueous humor and results in progressively elevated IOP. The iris disease is clearly visible at ~ 6 months and progresses with age, and is associated with increased IOP (Inman et

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al., 2006; Libby et al., 2005; Zhou et al., 2005). Young (2–3 month-old) D2 mice have normal iris, normal IOP, and normal histological appearance of RGCs and optic nerve (John et al., 1998; Libby et al., 2005). RGC and optic nerve degeneration is first apparent between 8 and 9 months of age (Libby et al., 2005; Schlamp et al., 2006). By 18 months of age about 90% of optic nerves show advanced degeneration (Libby et al., 2005).

Surviving RGCs may be dysfunctional. Both cross-sectional (Howell et al., 2007; Saleh et al., 2007) and longitudinal (Saleh et al., 2007) studies of D2 mice showed that the pattern electroretinogram (PERG) — a measure of RGC function in mammals (Berardi et al., 1990; Maffei and Fiorentini, 1981; Maffei et al., 1985; Zrenner, 1990) including mice (Miura et al., 2009; Porciatti, 2007; Porciatti et al., 1996) — approaches the noise level at about 10–11 months, whereas the cone-flash ERG — an index of outer retina function — has still normal amplitude. Histological analyses of RGC axons in eyes with severely reduced PERG show only moderate damage (Howell et al., 2007; Saleh et al., 2007), suggesting that loss of PERG signal in D2 mice younger than one year reflects primarily RGC dysfunction rather than cell death. Progressive loss of PERG signal is significantly correlated with progressive IOP elevation after accounting for the effect of age as independently-contributing factor (Saleh et al., 2007).

Understanding causal relationships between IOP and RGC function/death is one of the key issues in glaucoma (Quigley, 1999; Whitmore et al., 2005; Yücel et al., 2003). However, correlation between IOP and PERG amplitude (Saleh et al., 2007) does not necessarily imply causation. One of the possible ways to address this issue is that of probing the effects of acute IOP modulation on RGC function. If IOP causes (directly or indirectly) insult to RGCs, then RGC dysfunction may be exacerbated by acute IOP elevation and may be improved by acute IOP lowering.

It is known that head-down body posture results in IOP elevation in human (Bill, 1975) (Friberg et al., 1987) as well as in mice (Aihara et al., 2003; Nagaraju et al., 2007). In a previous study we have demonstrated that head-down body tilt induces physiological IOP elevation in D2 mice of different ages (Nagaraju et al., 2007). Head-down tilt causes reduction of PERG amplitude in eyes of older D2 mice but not in eyes of younger mice, suggesting age-dependent susceptibility of RGC function.

The effects of head-up tilt on IOP and PERG are not known. Here we show that head-up body tilt induces age-independent IOP lowering whose magnitude increases with the angle of tilt. We also show that for a fixed amount of head-up tilt (-60°), the IOP progressively decreases to a plateau lower by about 5.5 mm Hg compared to the baseline. Posture-induced IOP lowering is associated with an improvement of PERG amplitude compared to baseline in older D2 mice but not in younger D2. Preliminary results for these studies have been previously reported in abstract form (Nagaraju and Porciatti, 2008; Porciatti et al., 2008).

Methods

Animals and husbandry

A total of 45 DBA/2J mice (Jackson Labs, Bar Harbor, Maine) of different ages ranging between 2 and 15 months were tested. Mice were maintained in a cyclic light environment (12-h 50 lux, 12-h dark) and fed *ad libitum*. For both IOP and PERG measurements, mice were weighed and anesthetized with intraperitoneal injections (0.5–0.7 ml/Kg) of a mixture of Ketamine, 42.8 mg/ml, Xylazine, 8.6 mg/ml and Acepromazine, 1.4 mg/ml. All procedures were performed in compliance with the ARVO statement for use of animals in ophthalmic and vision research. The experimental protocol was approved by the Animal Care and Use Committee of the University of Miami.

Mouse restraining and body tilting

Anesthetized mice were gently restrained in a custom-made goniometric holder that allowed continuous orientation (± 90 deg) of the body posture (Fig. 1). The head was longitudinally restrained using a mouth bite bar and a nose holder that allowed unobstructed vision, and laterally restrained using smoothed ear bars (Porciatti et al., 1999; Porciatti et al., 2007). The tail was oriented along the longitudinal axis, and its tip restrained with tape. The body of the animal was kept at a constant body temperature of 37.0 °C using a feedback-controlled heating pad (T CAT-2LV, Physitemp Instruments, Inc. Clifton, NJ). The goniometric holder allowed IOP and PERG measurement for any angle of body position.

IOP measurement

IOP was measured with an induction/impact tonometer (Tonolab Colonial Medical Supply, Franconia, NH) (Danias et al., 2003). The tonometer was fixed in a vertical position to a support stand by means of clamps. The probe tip was aligned with the optical axis of the eye at 1–2 mm distance using a magnifier lamp. Three consecutive IOP measures of 5 readings each were taken and then averaged (15 readings averaged) (Nagaraju et al., 2007; Saleh et al., 2007; Wang et al., 2005). Local corneal anesthesia was not used since the impact of the Tonolab probe on the cornea is expected to be minimal; it has been previously shown that repeated IOP measurements do not cause obvious corneal damage at biomicroscopic examination. IOP readings obtained with Tonolab have been proven to be accurate and reproducible in different mouse strains including DBA/2J (Haddadin et al., 2009; Nagaraju et al., 2007; Pease et al., 2006; Saeki et al., 2008; Saleh et al., 2007; Wang et al., 2005).

PERG recording

Detailed description of the PERG technique is reported elsewhere (Porciatti, 2007; Porciatti et al., 2007). The eyes of anesthetized mice were typically wide open and in a stable position, with optical axes pointing laterally and upwardly. The PERG electrode (0.25 mm diameter silver wire configured to a semi-circular loop of 2 mm radius) was placed on the corneal surface by means of a micromanipulator and positioned in such a way as to encircle the undilated pupil without limiting the field of view. Reference and ground electrodes were stainless steel needles inserted under the skin the scalp and tail, respectively. A small drop of balanced saline maintained the cornea and lens in excellent conditions for the duration of recording. A visual stimulus of contrast-reversing bars (field area: 50×58 deg; mean luminance 50 cd/m²; spatial frequency: 0.05 cycles/deg, contrast: 98%; temporal frequency: 1Hz) was aligned with the projection of the undilated pupil at 20 cm distance. At 0.05 cycles/deg spatial frequency, the PERG amplitude is maximal (Porciatti, 2007; Porciatti et al., 1996) and the bar size corresponds to the average receptive field size of mouse retinal ganglion cells (Stone and Pinto, 1993). Eyes were not refracted for the viewing distance since the mouse eye has a large depth of focus due to the small pupil size (Remtulla and Hallett, 1985; Schmucker and Schaeffel, 2004). This is also true even when pupils are artificially dilated (Artal et al., 1998). Retinal signals were amplified (10,000 fold) and band-pass filtered (1–30 Hz). Three consecutive responses to 600 contrast reversals each were recorded. The responses were superimposed to check for consistency and then averaged (1800 sweeps). Robust averaging coupled with band-pass filtering was used as best compromise to isolate small PERG signals from uncorrelated noise (Porciatti et al., 2007) and improve the ability to detect changes of PERG signal upon tilting. A noise response to an occluded PERG stimulus was also recorded. The PERG is a light-adapted response. To have a corresponding index of outer retina function, a light-adapted ERG (FERG) was also recorded (band-pass 1–100 Hz, 3 consecutive responses of 25 sweeps each) with undilated pupils in response to strobe flashes of (20 cd/m²/s) superimposed on a steady background light (12 cd/m²) and presented within a Ganzfeld bowl. Under these conditions

rod activity is largely suppressed while cone activity is minimally suppressed (Lyubarsky et al., 2004).

FERG changes after IOP reduction associated with head-up tilt are not expected. In contrast to the PERG, age-related changes of FERG amplitude in the range 2–12 months are relatively small. (Porciatti et al., 2007) (Saleh et al., 2007) In addition, in older D2 mice the FERG does not change after either IOP elevation with head-down body tilting or IOP reduction with mannitol (Nagaraju et al., 2007). The main reason for FERG recording was to have a control that the tilting procedure did not induce artifactual changes of an age- and IOP resilient signal.

PERGs and FERGs were recorded under baseline condition (horizontal), 10–25 minutes after head-up tilt, and 10–25 minutes after horizontal repositioning. For each condition the duration of PERG/FERG recording (one eye only tested) was about 10 minutes. Averaged PERGs and FERGs typically consisted of a major positive peak followed by a slower negative wave (see examples in Figure 5 and in previous studies, (Porciatti et al., 2007; Saleh et al., 2007). PERG and FERG waveforms were automatically analyzed to detect maximal and minimal voltages (i. e., the peak-to-trough amplitude) in the time window 50–250 ms, a time bin where PERG/FERG signals are expected. Automatic analysis was performed in order not to introduce operator bias in response evaluation. Specifically, waveforms were exported in a Sigmaplot worksheet (Systat Software, inc. Richmond, CA, USA) as a string of 512 data points; a custom-made macro written in Sigmaplot language was then run to detect the maximum and minimum amplitudes in the 5–250 ms time window. The response amplitude was defined as difference between maximum and minimum amplitude (peak-to-trough amplitude). The same automated analysis was used to evaluate the amplitude of a “noise” response to an occluded stimulus. In the present study, the relevant comparison is between baseline PERG amplitude (RGC-baseline signal plus noise) and tilted PERG amplitude (RGC-tilted signal plus noise). In a given individual, the difference between tilted and baseline PERG amplitude represents tilt-induced changes of RGC function independently from the absolute noise level of that particular animal. Since PERG amplitudes of older animals were close to or in the range of noise level of the mice population, peak latency measurements were not considered meaningful and have not been included in the analysis.

Statistics

Significant differences between horizontal baseline and tilted position (deltas) of IOP and PERG amplitude in different age groups were tested by 2-way ANOVA and Holm-Sidak post-hoc multiple comparisons with family-wise alpha error set at $P < 0.05$ (Sigmastat 3.5, Systat software Inc. Chicago, IL).

Results

Effect of angle of tilt on posture-induced IOP changes

We systematically investigated the relationship between IOP and positive/negative angle of tilt (ie, head-down and head-up, respectively) in D2 mice of different ages (4 months, $n=7$; 5.5 months, $n=6$; 10–12 months, $n=6$). Starting from horizontal position, body posture was adjusted in incremental steps of $+20^\circ$ (head-down) every 5 minutes, while IOP was measured approximately every minute. After 15 min recovery in horizontal position, the procedure was repeated in the same session for incremental steps of -20° (head-up). Figure 2 (upper panels) displays representative examples of IOP changes (mean of three consecutive measures of 5 readings each) in individual D2 mice of different ages upon incremental steps of tilt angle. Figure 2 (lower panels) also displays progressive IOP mean changes as a function of tilt angle for all mice that completed the entire procedure. It can be noted that in all age groups, progressive increase of $\pm$ tilt angle resulted in progressive IOP change of the same sign as the

tilt angle. Also note that IOP changes had approximately similar magnitude for both head-down and head-up tilt in all age groups.

Tilt-induced changes in IOPs (deltas) were calculated. Delta IOP was defined as the IOP taken 5 minutes after a new angle of tilt minus the average IOP taken at horizontal baseline (3 measurements over 10 minutes). Delta IOP in horizontal position is by definition zero. Two-way ANOVA on two independent factors (age: three groups; tilt angle: 8 angles between -80° and $+80^\circ$) and one dependent variable (delta IOP) indicated a strong effect of tilt angle ($P<0.001$) and an insignificant ($P=0.8$) effect of age. For a given tilt angle, the mean delta IOP was significantly different from that obtained at any other tilt angle (Holm-Sidak post-hoc test, $P<0.05$). Being age-independent, delta IOPs were combined in a single group as a scattergram expressing delta IOP as a function of tilt angle (Fig. 3). Delta IOPs were well described ($R^2 = 0.92$) by a sigmoid function (3rd order polynomial) of the angle of tilt. If the x-axis was expressed as sine of angle of tilt, then data could be even better fitted ($R^2=0.95$) with a linear function having a slope of 5.55 mm Hg (SE 0.877) for unit of sine of angle (90° of body tilt).

Time course of IOP changes upon head-up tilt

To study the effects of posture-induced IOP changes on the PERG, it is necessary that the tilted IOP be reasonably stable with time. We systematically characterized the time course of IOP changes for a fixed angle of head-up body tilt (-60°) in D2 mice of different ages. This tilt angle was chosen as best compromise between magnitude of IOP lowering and simplicity of the procedure. Based on data shown in Fig. 3, IOP reduction with a tilt angle of -60° was close to the maximum attainable at -90° . In addition, the experimental procedure (eg, aligning the PERG stimulus with eye axis) was simpler at -60° than at -90° tilt.

The time course of IOP changes for -60° of tilt was determined in D2 mice aged 2–12 months (2 month old, $n=7$; 6 month old, $n=7$, 10 month old, $n=7$, 12 month old, $n=7$) (Fig. 4). Note in Fig. 4 that baseline IOP values differed among age groups. Upon -60° tilting, IOP progressively diminished and tended to plateau at a lower level in all age groups. Upon horizontal repositioning, IOP recovered to baseline conditions with an approximately mirror-symmetric time course. For both head-up and horizontal repositioning the time constant of the IOP change (time required to reach 63% of its plateau value) was of the order of 5–6 minutes. Baseline IOPs (average of three consecutive measures over 10 minutes) and tilted IOPs (average of 4 consecutive measures 10–25 minutes after tilt) for each age group are reported in Table I.

Two-way ANOVA on two independent factors (age: 4 groups; tilt: 2 positions) and one dependent variable (IOP) indicated a strong effect ($P<0.001$) of both tilt (IOP decreases with head-up) and age (IOP increases with age). Post-hoc multiple comparisons, indicated that difference of means (delta IOPs) were not significantly different from each other. In summary, IOP changes induced by a fixed amount of head-up (-60°) tilt appeared rather stereotyped (ranging between about 5 and 6 mm of Hg) and were age- and baseline IOP independent.

Effect of -60° head-up tilt on RGC function

Approximately one week after IOP measurements, PERGs and FERGs were recorded before, during, and after head-up (-60°) body tilt from the right eyes of D2 mice of different ages (2–3 month old, $n=7$; 6 month old, $n=7$, 10 month old, $n=6$, 12 month old, $n=6$). Tilted PERGs and FERGs were recorded in the 10–25 min time window after onset of tilt. Recovery PERGs/ FERGS were recorded in the 10–25 min time window after horizontal repositioning. Individual PERG and FERG waveforms for representative age groups at different body postures are reported in the first 3 columns of Figure 5. To provide a summary characterization of PERG and FERG changes across conditions, grand-average waveforms were calculated together with their SEM and displayed in the fourth column of Fig. 5. Statistical analysis was performed on

peak-to-trough amplitudes calculated from individual waveforms (see methods). In 2 month-old D2 (first row), the baseline average PERG amplitude was about of 9 μ V and did not change upon head-up tilt. In 10 month-old D2 (second row) as well in 12 month-old D2 (third row) the average baseline PERG amplitude (maximum-to-minimum voltage in the waveform) was about 2 μ V; no obvious waveform comparable to those displayed in the upper panels was apparent in the signal. Upon head-up tilt, the average PERG amplitude substantially increased in both 10 month and 12 month-old D2, and then returned to the baseline magnitude upon horizontal repositioning. In 10 month-old D2 the baseline FERG had peak-to-trough amplitude larger than 60 μ V, which did not change upon head-up body tilt. Comparable FERG waveforms were obtained at all ages (not shown).

Mean PERG amplitudes for different age groups and postures are summarized in Figure 6, together with corresponding SDs. It is apparent that the baseline PERG amplitude decreased with increasing age; by 10–12 months it approached the average noise level of the mouse population. Head-up tilt did not appear to induce PERG amplitude changes in 2 month-old D2. However, it seemed to induce substantial improvement of PERG amplitude in older mice. A two-way ANOVA on two independent factors (age: 4 groups; tilt: 2 positions) and one dependent variable (PERG amplitude) indicated a strong effect ($P < 0.001$) of both age (PERG amplitude decreased with age) and tilt (PERG amplitude increased with head-up). In addition, there was a significant ($P = 0.012$) interaction between age and tilt (PERG changes with tilt depended on age). Post-hoc comparisons indicated that the tilt effect on PERG amplitude was significant in age groups 6 months, 10 months and 12 months but not in 2–3 months ($P < 0.05$, Holm Sidak). Paired t-tests significance levels were, 2–3 months, $P = 0.7$; 6 months, $P = 0.03$; 10 months, $P < 0.001$; 12 months, $P = 0.017$. As an additional statistical control, we "jackknifed" PERG amplitude data by systematically recomputing the averages leaving out one observation at a time. For each jackknifed average, the baseline amplitude was significantly ($P < 0.05$) lower than the tilted amplitude in age groups 6, 10, and 12 months but not different from the recovery amplitude.

Figure 6 also summarizes FERG amplitudes for different age groups and postures. A two-way ANOVA analysis on FERG amplitude data did not reveal significant main effects (age, tilt) or interaction effects (age $\times$ tilt).

Discussion

IOP represents the only treatable risk factor for glaucoma. While several studies in patients with different type and stages of glaucoma have shown that IOP lowering is associated with reduced risk of disease progression (Musch et al., 2009; Parrish et al., 2009) little is known on causal relationships between IOP and RGC function (John, 2005; Mackenzie and Cioffi, 2008). Acute IOP effects on RGC function may provide an opportunity to explore pressure-function relationships. In this study we investigated whether head-up posture in D2 mice results in IOP lowering. We then investigated whether acute IOP lowering upon head-up body tilt results in an improvement of RGC dysfunction in glaucomatous D2 mice as previously reported for pharmacological IOP lowering (Nagaraju et al., 2007).

IOP elevation induced by head-down body inversion is thought to reflect changes in episcleral venous pressure (EVP) according to the Goldmann equation [$IOP = F/C + EVP$], where F is the rate of aqueous production in μ l/min and C is the outflow facility in μ l/min/mm Hg. During head-down tilt, F and C factors in the equation are not expected to change; however the gravitational effects of the venous blood column will cause elevation of EVP and consequently IOP (Bill, 1975; Friberg et al., 1987; Henderson and Haggard, 1918). The magnitude of IOP elevation will increase with increasing angle of tilt. Since the relevant variable for gravitational effects is the vertical height of the column of venous blood, the magnitude of EVP/IOP

elevation is expected to be linearly proportional to the sine of the angle of tilt (vertical height = length of venous blood column · sine of tilt angle), unless factors other than hydrodynamic variables play a role.

Head-down body inversion also induces IOP elevation in mice (Aihara et al., 2003; Nagaraju et al., 2007). The primary posture of mouse is horizontal. It is therefore theoretically possible that a head-up posture results in a reduction of EVP and IOP. Our results show that this is indeed the case. The relationship between IOP change and sine of angle of tilt was approximately linear for both positive and negative angles, with a slope of 5.54 mm Hg per unit of sine angle (90° of body tilt). A relation of this kind is consistent with the simple hydrodynamic model described above (Bill, 1975; Henderson and Haggard, 1918; Nagaraju et al., 2007). While this simple hydrodynamic model largely accounts for experimental data, other factors may contribute to absolute IOP changes, such as the elastic properties of the eye (Silver and Geyer, 2000) as well as ketamine-xylazine anesthesia (Aihara et al., 2008; Avila et al., 2001).

In order to use acute IOP modulation to systematically probe modifiability of RGC function with PERG, we have chosen a fixed angle of head-up tilt (−60°) as compromise between maximum achievable IOP reduction and simplicity of the procedure (see methods). Baseline IOP values increased with increasing age, consistently with previous reports (Libby et al., 2005; Nagaraju et al., 2007; Zhou et al., 2005). IOP changes upon −60° head-up tilt were very consistent, both in terms of magnitude of IOP lowering (~ 5–6 mm Hg) and time course of IOP changes, and were independent of age and baseline IOP. Baseline PERG amplitudes decreased with increasing age and approached the noise level at 10–12 months, in agreement with previous reports (Howell et al., 2007; Saleh et al., 2007). Different from IOP, PERG amplitude changes with tilt were strongly age-dependent. No significant changes occurred in younger (2 month old) mice with normal baseline PERG amplitude; however, improvements of the PERG signal occurred in older mice with abnormal baseline amplitude. Remarkably, some improvement of PERG amplitude also occurred in 12 month-old mice in which baseline amplitudes were in the range of noise. We cannot exclude that improvement of PERG signal upon head-up tilt might occur in mice older than 12 months, even though this seems unlikely because in that age range RGCs and axons display major structural loss (Howell et al., 2007; Libby et al., 2005). We intend to address this issue in future studies.

The FERG had a similar baseline amplitude at all tested ages as previously described (Porciatti et al., 2007; Saleh et al., 2007) and did not change upon head-up tilt. Thus, the effects of acute IOP lowering on PERG signal appear to be specific for some ages in the 2–12 months range at which the baseline PERG signal has reduced amplitude. Since the amplitude of the FERG signal is invariant with age and tilt, we conclude that the acute effect of head-up selectively affect PERG generators, but not FERG generators. Experimental evidence (Miura et al., 2009; Porciatti et al., 1996) indicates that RGCs are the main generators of the mouse PERG. Namely, anatomical section/crush of the optic nerve resulting in RGC retrograde degeneration abolishes the PERG but leaves the FERG unaffected.

It is unlikely that the acute effects of head-up tilt on PERG improvement are the result of artifactual changes in recording conditions during tilt. As described in the method section, a goniometric stereotaxical holder was specifically designed to allow changes of body posture without changing electrode geometry. The visual stimulus was carefully aligned with the pupil projection at different postures. Comparable levels of IOP lowering associated with PERG amplitude improvement (but not FERG amplitude improvement) have been previously obtained in 11 month old — not tilted — D2 mice treated with mannitol, a common pharmacological mean to osmotically reduce IOP. (Nagaraju et al., 2007) Finally, the FERG signal did not change during head-up tilt.

The present results support previous reports of loss of PERG signal in older (6–12 month old) D2 mice associated with IOP elevation (Howell et al., 2007; Saleh et al., 2007). Importantly, improvement of PERG amplitude after IOP lowering strongly suggests that loss of PERG signal in 6–12 month old D2 mice is due, at least in part, to IOP-dependent, reversible RGC dysfunction.

The magnitude of PERG amplitude improvement after acute IOP lowering does not seem to fully account for the excess loss of RGC function compared to loss of RGC axons (fig. 4 of (Saleh et al., 2007) and fig. S5 of (Howell et al., 2007). This may mean that part of cell dysfunction is not recoverable with IOP lowering (Scholz et al., 2008). Alternatively, the magnitude of IOP lowering may have been insufficient to remove the IOP-dependent stress. We intend to address this issue by combining head-up tilt and pharmacological IOP lowering to more drastically reduce IOP.

The mechanisms by which acute IOP changes influence RGC function are matter of speculation. That the effects described in this study occur over a short time scale (minutes) may indicate a biomechanical effect. Indeed, IOP-induced biomechanical changes at the level of the optic nerve head have been described in primates (Burgoyne and Downs, 2008). In bovine eyecups as well as RGC cultures, IOP-induced mechanical strain on RGCs results in excessive release of intracellular ATP in the extracellular space via pannexin hemichannels. Extracellular ATP acts on RGC purinergic P2X7 receptors in autocrine fashion resulting in alteration of the membrane potential, calcium entry and eventually cell death (Reigada et al., 2008; Xia et al., 2009). In RGC cultures, exposure to hydrostatic pressure activates mechano-sensitive transient receptor potential vanilloid 1 (TRPV1), resulting in calcium influx, membrane depolarization, and eventually cell death (Sappington et al., 2009).

Short-term vascular effects may also be possible. In particular, the ocular perfusion pressure (OPP = Mean arterial blood pressure minus IOP) may decrease in head-down and increase in head-up posture. Changes in OPP may be associated to changes in PERG amplitude (Kothe and Lovasik, 1990; Siliprandi et al., 1988). OPP is also expected to decrease with age-related spontaneous IOP elevation, and to increase with pharmacological IOP-lowering treatment. We plan to address posture-dependent OPP and PERG changes in D2 mice in a future study.

Improvement of impaired RGC function after acute IOP lowering does not necessarily mean that chronic IOP lowering is able to keep the cells at a better level of function over time and delay cell death. We are currently addressing this question in a longitudinal study. Should acute effects of IOP lowering have predictive value on long-term structural/functional outcome, then the head-up/PERG paradigm would represent a powerful tool in glaucoma studies using mouse models.

Interestingly, the head-up tilt induced IOP/PERG effects described in this study are approximately mirror-symmetric with corresponding IOP/PERG changes after head-down tilt in D2 mice of similar age range (Nagaraju et al., 2007). Namely, after 60° head-down tilt, IOP increases by about 5 mm Hg in all age groups, whereas the PERG signal decreases in older, but not younger, mice. The head-down IOP/PERG protocol may therefore be used to test RGC susceptibility to additional stress. IOP-dependent RGC susceptibility is likely to have a genetic component. C57BL/6J-*Tyrrp1b*. *GpnmbR150X* mice develop the same iris disease and IOP elevation as DBA/2J mice, but do not develop glaucoma (Anderson et al., 2006). In these mice, the PERG is within the normal range both prior to iris disease and when iris disease is severe and IOP is elevated (Howell et al., 2007).

While the DBA/2J mouse displays some key hallmarks of human glaucoma, it may not represent a valid experimental model for understanding the human condition (Levkovitch-Verbin, 2004). However, the model can be used to test the mechanisms of RGC death in

glaucoma as well as to evaluate new treatment modalities. The effects of acute IOP modulation on PERG signal in D2 mice may have an important translational value. Corresponding studies in human patients with suspicion of glaucoma or glaucoma in the early stages indicate that the effects described here have a counterpart in the human model. In particular, loss of PERG signal exceeds loss of optic nerve structure, suggesting dysfunction of anatomically present neurons (Ventura and Porciatti, 2005); abnormal PERG signal may be improved with IOP lowering, suggesting reversibility of RGC dysfunction (Colotto et al., 1995; Falsini et al., 1992; Nesher et al., 1990; Ventura et al., 2006). Moderate IOP elevation by means of a suction cup impairs the PERG signal in patients with ocular hypertension (Colotto et al., 1996); head-down body tilt may impair the PERG signal in patients with suspicion of glaucoma or glaucoma in the early stages, suggesting susceptibility of RGC function to additional stress (Ventura et al., 2008). Finally, the present results support the role of PERG to aid early detection of RGC dysfunction and its progression in ocular hypertension and early glaucoma (Bach and Hoffmann, 2008; Bach et al., 2006; Bowd et al., 2009; Parisi et al., 2006; Ventura and Porciatti, 2006).

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Figure 1.
Side view of the goniometric holder showing electrode carriers (1), mouth bite and nose holder (2), ear bars (3), and heating plate/thermocouple (4).

Figure 2.

IOP levels as function of progressive change of $\pm$ tilt angle in D2 mice of different ages. Tilt angle was changed in 20 deg increment steps every 5 minutes. IOP was measured approximately every minute (grey symbols and lines: head down; black symbols and lines: head-up). Upper panels: representative examples of IOP changes with progressive tilt in individual D2 mice of different ages. Lower panels: Mean IOP at given angles of body tilt for all mice tested.

Figure 3.

IOP changes (IOP measured 5 minutes after each 20° increment tilt minus horizontal baseline IOP) as a function of angle of tilt (A) or sine of angle of tilt (B) in D2 mice ranging in age 2–12 months. In panel A data have been fitted with a 3rd order polynomial; in panel B data have been fitted with a linear regression (black lines: regressions; grey lines: 95% confidence intervals).

Figure 4.

Time course of IOP changes (mean $\pm$ SD) for a fixed amount of head-up body tilt (-60° for 30 minutes followed by horizontal re positioning) in groups of D2 mice with different ages. Different symbols represent different age groups.

Figure 5.

Effect of -60° head-up body tilt on PERG and FERG of DBA/2J mice of different ages. First column: individual baseline responses; second column: individual responses during tilt; third column: individual responses after horizontal repositioning; fourth column: grand-average waveforms $\pm$ SEM (continuous and dashed lines, respectively) at different body postures. Top row: PERGs of 2 month-old mice; second row: PERGs of 10 month-old mice; third row: PERGs of 12 month-old mice; bottom row: FERGs of 10 month-old mice. Note that at 2 months of age (upper row), the PERG is not affected by head-up tilt, whereas at 10 and 12 months of age (second and third row, respectively) an initially reduced PERG signal improves after tilt and returns to baseline range after horizontal repositioning. Also note that in 10 month-old mice

(bottom row) a normal baseline FERG signal is not affected by body tilt. Comparable FERG responses are obtained in all age groups (2, 6, 10, 12 months).

Figure 6.

Effect of -60° head-up body tilt on PERG (A) and FERG (B) amplitude in groups of DBA/2J mice with different age. Different symbols represent the mean $\pm$ SD PERG amplitude for different age groups. The grey horizontal dashed line (noise) represents the mean amplitude of a response to an occluded stimulus. A: Note that the baseline PERG amplitude decreases with increasing age and is in the range of noise in 12 month-old mice. Also note that during tilt the PERG amplitude does not change in 2 month-old mice compared to baseline, but displays different amounts of improvement in older age groups. After horizontal repositioning (recovery) the PERG amplitude is similar to baseline. B: Note that the FERG amplitude is similar in all age groups and does not change during tilt.

Age	Mean IOP (mm Hg)				
	Baseline	SD	Tilted	SD	Delta
2 months	16.4	0.8	11.1	0.7	-5.3
6 months	21.0	1.4	15.4	0.8	-5.6
10 months	28.1	0.9	22.0	0.6	-6.1
12 months	29.0	1.7	24.1	1.0	-4.9