

Modeling Retinal Ganglion Cell Dysfunction in Optic Neuropathies

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Abstract

As in glaucoma and other optic neuropathies cellular dysfunction often precedes cell death, sensitive assessment of retinal ganglion cell (RGC) function represents a key outcome measure for neuroprotective strategies aimed at targeting distressed but still viable cells. Here we offer a conceptual framework to identify progressive stages of RGC dysfunction leading to cell death in mouse models of glaucoma and other optic neuropathies based on non-invasive pattern electroretinogram (PERG), to differentiate phenotypic and altered RGC response dynamics, to assess susceptibility to stressors and to assess reversible dysfunction.

Key words: Retinal ganglion cell function; Pattern electroretinogram; Glaucoma; Optic neuropathy

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Introduction.

Neuroprotection in glaucoma and other optic neuropathies is an area of intense investigation in preclinical models [1-8]. Neuroprotective strategies include a large variety of methods (dietary, exercise, environment, pharmacological, molecular, radiation, stem cells, gene therapy) that target specific aspects of neuronal biology or nonspecifically target conditions such as elevated IOP, inflammation, or immune system malfunction that may eventually cause neuronal damage. It is becoming increasingly clear that, in glaucoma and other optic neuropathies, cellular and axonal dysfunction often precede cell death [9-13]. This helps focusing neuroprotective strategies in a time window of opportunity at which distressed but still viable cells can be rescued from irreversible stages of neurodegeneration and perhaps restored to their normal function [14].

Thus, sensitive assessment of retinal ganglion cell (RGC) function in preclinical models and investigation of the effects of stressors and neuroprotectants can provide a powerful tool for early detection of neuronal dysfunction, as well as prediction of disease progression with or without treatment. Here we offer a conceptual framework to model progressive RGC dysfunction in glaucoma and other optic neuropathies based on non-invasive, high-throughput electrophysiological methods suitable for neuroprotection studies.

To be or not to be, this is the question.

Progressive RGC dysfunction and death can be thought to undergo 6 stages that can be defined in lay terms (**Table 1**) from stage 0 (healthy) to stage 6 (effaced). Each stage is identifiable *in vivo* with non-invasive electrophysiology and/or non-invasive imaging. The ideal time window of opportunity for early detection, prediction of progression, and effective neuroprotection appears to be at stages 1-2 (functional tipping point) and perhaps at stage 3 (very sick). RGC dysfunction at stages 1-3 is potentially identifiable with electrophysiology but it is unlikely that it will be detected with imaging. Stage 4 is identifiable with real-time imaging of apoptosing RGCs [15, 16] and stages 4-5 are identifiable with OCT imaging as loss of inner retina thickness. At stages 4-6 electrophysiology is expected to be at floor level and is no longer useful to monitor disease progression.

Table1. Conceptual stages of cell death						
0	1	2	3	4	5	6
Healthy	At Risk	Sick	Very sick	Dying	Dead	Effaced
Non-invasive electrophysiology						
Normal	Perhaps abnormal	Moderately abnormal, Reversible	Very abnormal, Perhaps reversible	Floor, Irreversible	Floor	Floor
Non-invasive imaging						
Normal	Normal	Normal	Perhaps abnormal	Abnormal	Very abnormal	Floor

Table 1. Conceptual stages of retinal ganglion cell (RGC) death in glaucoma and optic neuropathies that can be tested with non-invasive electrophysiology such as pattern electroretinogram (PERG) and imaging such as optical coherence tomography (OCT) and scanning laser ophthalmoscopy (SLO) combined with apoptotic markers.

Non-invasive assessment of RGC function.

Visual function can be assessed with several methods [17] that depend on the activity of both retinal and post retinal pathways such as Visually Evoked Potentials (VEP), reflex-based optomotor response (OMR) and operant training. Here we focus on the Pattern Electroretinogram (PERG), which assesses RGC function directly. While there are other RGC-sensitive ERG methods, the PERG is the best understood and most sensitive technique which specifically depends on the presence of functional RGCs [18]. The PERG amplitude is proportional to the number of RGCs at a given retinal location, and the spectrum of spatial frequencies at which a PERG response can be generated corresponds to the size to RGC receptive field centers [19-21]. The PERG spatial resolution for gratings (acuity) and contrast threshold correspond to corresponding behavioral measurements [22]. Crucially, the PERG is rapidly abolished after optic nerve crush and is early altered in glaucoma, before histological RGCs loss [18]. Important for neuroprotection studies, the PERG signal has a signal-to-noise ratio of about 1 Log unit, which allows meaningful assessment of changes over time and treatment over the entire disease scale. Assessment of PERG changes is simplified by recent developments of PERG technology that allow simultaneous assessment of responses with high signal-to-noise ratio from each eye in mice using a single subcutaneous needle in the snout [23]. This eliminates the necessity of corneal electrodes that may damage the cornea, deteriorate eye optics and alter eye pressure, and also facilitates experiments based on comparison between the responses of the two eyes.

Physiological significance of altered PERG signal.

The PERG has been extensively used in preclinical and clinical studies of glaucoma and optic neuropathies to detect of RGC dysfunction in cross-sectional and longitudinal studies. While these are important applications, they do not provide information on the nature of PERG changes. For example, a reduced PERG signal may be the result of the missing contribution of lost RGCs and dying RGCs, reduced contribution of dysfunctional RGCs, and normal contribution of healthy RGCs in unknown relative proportions. An observable example of this originates from *in vivo*, real-time imaging of human and rodent glaucomatous retinas labeled with the fluorescent biomarker annexin 5, which identifies a subpopulation of apoptosing RGCs coexisting with apparently normal RGCs [24]. **Figure 1A** summarizes a simple model of progressive glaucoma in which, between time zero and time 1, a proportion of RGCs (30%) becomes sick, functions 50% of normal and survives for a limited amount of time. At time 2, sick RGCs start dying while 30% of remaining healthy RGCs becomes sick, with the process repeating over time. These events will be reflected in progressive loss of function (PERG amplitude) and inner retina structure (OCT thinning), with the former expected to anticipate loss compared to the latter. The horizontal distance between the decay curves of function and structure provides an estimate of the lifespan of sick RGCs, which represents a time window of opportunity for

treatment to prevent RGC death. The vertical distance between the decay curves of function and structure provides an estimate of RGC dysfunction that is not accounted for by cell death, which represents an opportunity for treatment to restore RGC function. The function-structure decay model shown in Figure 1 is in good agreement with experimental data in DBA/2J glaucoma [25] and is also in good agreement with longitudinal clinical data in early glaucoma patients [26]. Analogue models may be hypothesized for a variety of conditions impacting the susceptibility and lifespan of RGCs together with their ability to generate electrical signals under a protracted degenerative process.

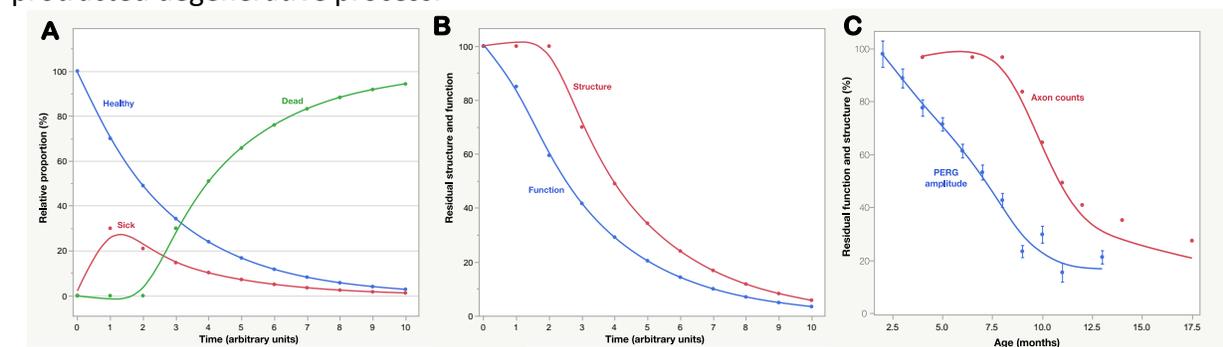


Figure 1. A: Model of progressive optic neuropathy in which a given proportion (30%) of healthy RGCs becomes sick at time 1 and survives until time 2, with the process repeating at each successive time unit. B: Assuming that sick RGCs function 50% of normal, the overall RGC function as measured by PERG decreases exponentially over time, anticipating a similar decay of the mean inner retinal thickness as measured by OCT. C: Natural history of PERG amplitude and optic nerve axon counts in DBA/2J mouse glaucoma. PERG data are replotted from Saleh et al, 2007 and represent the mean \pm SEM of 32 mice; average axon counts are derived from Libby et al 2005 and Anderson et al, 2005. The shaded area represents the 95% confidence interval of the spline regression curve.

PERG dynamics and RGC functional properties

To have a better insight into the significance of PERG changes in optic nerve disorders, it is necessary to isolate the activity of surviving and still functional RGCs. This can be done by investigating how the PERG response changes over a range of conditions. The resulting response dynamics reflects the ability of functional RGCs to detect changing conditions and regulate their activity accordingly. Additional insight into the activity of functional RGCs is provided by the PERG response latency, which reflects the contribution of healthy and sick RGCs but not the missing contribution of dying and dead RGCs. PERG latency is defined as the time that elapses between the onset of the visual stimulus and the peak of response. PERG amplitude and latency dynamics can be investigated for a variety of physiological stimuli and positive/negative stressors, eventually providing a panel of biomarkers that will be helpful to identify the cause of dysfunction and formulate predictions on disease progression with or without treatments. Changes in PERG response dynamics can be used to test a number of neuronal function properties (**Table 2**). These properties are widely used in different contexts with varying semantics but are pragmatically defined here as they provide a framework to test hypotheses of different RGC functional conditions as well as biomarkers for neuroprotection studies.

Table 2. Neuronal processes testable with PERG dynamics	
Homeostasis	Stable function despite perturbation
Gain control	Reduction of sensitivity for high intensity stimuli
Adaptation	Reduction of response for repeated stimuli
Susceptibility	Temporary loss of function upon stress
Resilience	Reduced susceptibility to stress
Enhancement	Nonspecific improvement of function
Restoration	Recovery of lost function
Protection	Prevention of future loss of function
Rescue	Salvage of residual function
Plasticity	Reconfigured function

Table 2. Relevant attributes of RGC function that can be investigated with PERG dynamics before and after neuroprotective treatment

PERG dynamics - Physiological approaches

Physiological approaches such as changes of stimulus intensity/duration or body posture may result in different PERG changes that reflect the ability of functional RGCs to regulate their activity. **Figure 2** summarizes conceptual examples of homeostasis, gain control, and adaptation.

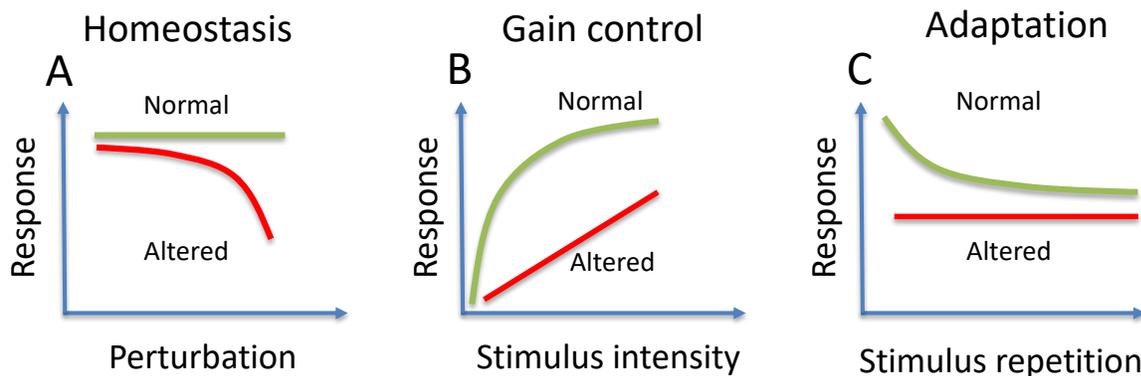


Figure 2. Conceptual examples of normal (green lines) and altered (red lines) homeostasis (A), gain control (B), and adaptation (C).

Homeostasis

Homeostasis (**Figure 2A**) characterizes the ability of RGCs to maintain a constant response over a wide range of physical and biological perturbations. Altered homeostasis perhaps represents the earliest stage of RGC dysfunction that is reflected in the PERG. Detection of altered homeostasis helps formulating an early diagnosis and predicting progression and may represent

the rationale for neuroprotective intervention. Altered RGC homeostasis is thought to occur in pre-glaucomatous DBA/2J mice and patients with suspicion of glaucoma, where a normal PERG may become reduced in susceptible eyes but not in control eyes upon physiological IOP elevation during head down tilt [27, 28]. PERG susceptibility upon head-down tilt in glaucoma suspects predicts thinning of retinal fiber layer thickness after 5 years [29].

Gain control

Gain control (**Figure 2B**) characterizes the ability of RGCs to regulate their activity with increasing stimulus strength. Typically, the relationship between PERG amplitude/latency and stimulus contrast is not linear, implying regulatory mechanisms in the RGCs and/or in the inner retinal circuitry impinging on them to contain RGC response in an optimal dynamic range. Altered PERG contrast gain control means that RGCs function in a different way, which may represent a biomarker of functional remodeling to improve survival rather than heralding cell death. Examples of altered PERG contrast gain control dynamics of amplitude and latency are provided by experiments in different mouse models in which neurotrophic support has been disturbed without causing cell death, including intravitreally injection of Brain Derived Neurotrophic Factor BDNF, anti-BDNF or lesion of the superior colliculus [30]. These results represent a proof of concept that PERG dynamics could be used as a tool for in-vivo monitoring of RGC functional plasticity and for phenotypic screening (see Figure 4).

Adaptation

Adaptation (**Figure 2C**) characterizes the autoregulatory ability of RGCs to reduce their activity to a lower level in response to sustained visual stimuli that cause high energy demand. Visual stimuli that induce strong PERG adaptation are steady-state, high-contrast reversing patterns in human [31] and transient, high-contrast reversing pattern with superimposed flickering light in mouse [32] that are associated to increased metabolic demand and vasodilation. PERG adaptation is reduced in human aging, glaucoma, optic neuritis and non-arteritic-ischemic-neuropathy (NAION) [33, 34] and is also reduced in DBA/2J mouse glaucoma [35]. PERG adaptation can be restored in DBA/2J glaucoma with sustained diet that supports mitochondrial function [35].

PERG dynamics - Interventional approaches

Interventional approaches such as treatment or exposure to stressful conditions may result in a variety of PERG changes that reflect temporary changes of RGC function. **Figure 3** summarizes conceptual examples of enhancement, restoration, susceptibility and resilience that can be applied to both standard PERG responses and PERG response dynamics.

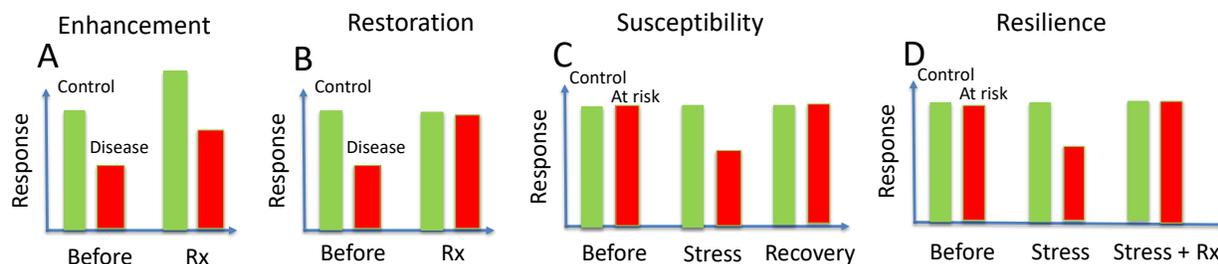


Figure 3. Conceptual examples of enhancement (A), restoration (B), susceptibility (C) and resilience (D) in control eyes (green bars) and diseased eyes (red bars).

Enhancement

Enhancement (**Figure 3A**) characterizes nonspecific improvement of PERG signal after treatment. Both the normal PERG amplitude of the control group and the reduced PERG amplitude of the group with manifest pathology improve, meaning that the treatment boosts function of all RGCs independently of pathology. For example, citicoline (cytidine-5'-diphosphocholine) has been shown to improve the PERG amplitude in glaucoma and NAION patients [36] but has been also shown to induce sustained improvement of PERG amplitude in control subjects [37]. Enhancement of PERG amplitude in control human subjects is also reported to occur after oral administration of Levodopa [38].

Restoration

Restoration (**Figure 3B**) characterizes specific improvement of PERG amplitude after treatment in the manifest pathology group but not in the control group. For example, Ventura and Porciatti [39] treated both controls and glaucoma patients with IOP lowering medications. Comparable reduction of IOP in both groups resulted in PERG amplitude improvement in the glaucoma group but not the control group. In a microbead mouse model of glaucoma resulting in substantial RGC loss and PERG loss, Lu *et al*, 2020 [40] intravitreally injected AAV vectors to deliver transcription factors OCT4, SOX2 and KLF4, which restored PERG loss.

Susceptibility

Intrinsic susceptibility of glaucoma and optic neuropathies to complex trait inheritance and aging is well known [41-44]. Susceptibility to exogenous factors (**Figure 3C**) is less investigated and less understood, and it is used here to characterize reversible reduction of an otherwise normal PERG amplitude in response a variety of stressors (physiological, physical, chemical, molecular, etc.). Inducible, reversible PERG reduction can be used as provocative test to investigate factors that impair RGC defense mechanisms. Examples are temporary IOP elevation in glaucoma [27, 45], Another example of inducible, reversible PERG loss that targets the optic nerve is provided by retrobulbar lidocaine injections in mice, which temporarily block axon transport in the optic nerve [46]. Postconditioning after repeated lidocaine injections results in endogenous upregulation of trophic factors in the retina that have a neuroprotective effect in the DBA/2J mouse glaucoma [47]. Investigation of susceptibility to stressors may represent a very promising field, as susceptibility to specific stressors provides the rationale and a target for neuroprotection.

Acquired resilience

Body tissues have the ability to autoregulate protective or repair mechanisms, including adaptive remodeling, in response to a large variety of everyday exogenous stressors, similarly to the immune system, which make the tissue resilient to future stress of the same nature [48]. Repair mechanisms include adaptive remodeling during the course of the neurodegenerative disease [49]. Acquired resilience is used here to characterize resilience gained after specific treatments that minimize susceptibility to provocative tests (**Figure 3D**). So far, few provocative tests such as head-down posture have been proposed such as head-down posture [27, 28] or water drinking[50], which cause IOP elevation and may result PERG reduction in susceptible eyes. Combined assessment of susceptibility to novel provocative tests and acquired resilience to the same provocative test after treatment may represent a promising field of investigation, as it would provide a predictive index of neuroprotective efficacy of treatments based on baseline assessment.

Functional phenotyping

The need of functional phenotyping increases in parallel with the ever-increasing number of genetically engineered rodent models. Susceptibility and resilience in glaucoma have a strong genetic component [51]. For example, BXD mice (derived from crossing glaucoma-resilient C57BL/6J mice with glaucoma-prone DBA/2J mice) allow investigation of genetic regulation of factors associated with glaucoma risk[9, 52]. One way to determine the RGC functional phenotype of genotypes with different resilience/susceptibility is to investigate the PERG contrast dynamics (**Figure 4**). At low contrast (0.3) PERGs of pre-glaucomatous C57BL/6J and DBA/2J mice have similar amplitude and latency. With increasing contrast, the rate of change of amplitude and latency of the two strains substantially diverge, indicating different contrast gain and integration time [53]. This suggests different functional circuitry in the inner retina that may be related to the larger RGC population in DBA/2J compared to C57BL/6J [54]. Contrast gain and integration time also change in the same phenotype upon genetic and induced changes of neurotrophic support [30], suggesting altered functional circuitry subserving RGC response.

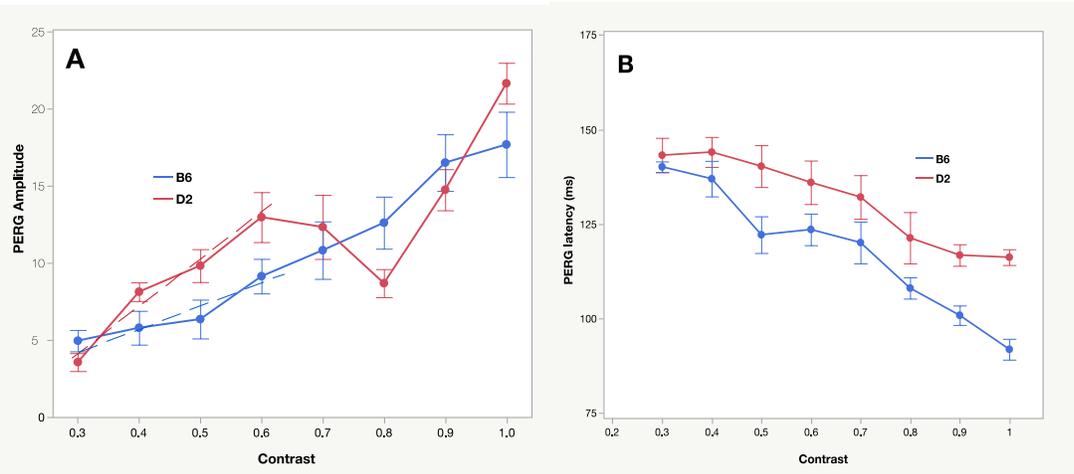


Figure 4. Contrast transfer function of PERG amplitude (A) and latency (B) in different mouse strains. B6: C57BL/6J; D2: DBA/2J. A: Different slopes of dashed lines represent different contrast gains of B6 and D2 PERGs. The dip in response amplitude at 0.8 contrast in the D2 contrast function indicates that generators with different spatio-temporal properties interact resulting in reduction of the summed response. Replotted from Porciatti *et al*, 2010.

Age-related factors

Neuroprotection studies are typically performed over an extended time period. Intrinsic, age-dependent functional changes need to be accounted for to isolate the effect of specific stressors and treatments. For example (Figure 5A), in D2 mice IOP increases with increasing age while the PERG amplitude progressively decreases to a floor at about 11 months [25]. Statistical analysis reveals that while there is a strong inverse correlation between PERG amplitude and IOP ($r^2 = 0.43$, $p < 0.001$), age independently contributes ($p < 0.0001$) to progressive PERG loss. This implies that there are specific age-related factors that could be the target of neuroprotective strategies, in addition to IOP lowering [55]. Williams *et al*, [10] identified mitochondrial abnormalities as major driver of neuronal dysfunction. B2 mice treated with either vitamin B3 rich diet or gene therapy driving *Nmnat1*, a key NAD⁺-producing enzyme, did not develop glaucoma despite IOP elevation [10]. Similar results have been obtained with a diet enriched with pyruvate, which provides bioenergetic support [56]. Age-related factors also alter eye structure independently of IOP [57, 58] (Figure 5 B,C,D). Between 2.5 and 6 months of age, both B6 and D2 mice grow in weight and eye length, although after 6 months eye elongation and anterior chamber depth is considerably larger in D2 in association with increased IOP. Cone *et al*, 2010 [59] induced IOP elevation in B6, D2, and CD1 mice by an average of 4.4 mmHg with intracameral injections of microbeads, resulting in different eye elongation and RGC death in the three strains after 6-12 weeks. Altogether, spontaneous and induced change of eye size may alter biomechanical properties of eye tissues which may also play a role in RGC and axon susceptibility independently of IOP.

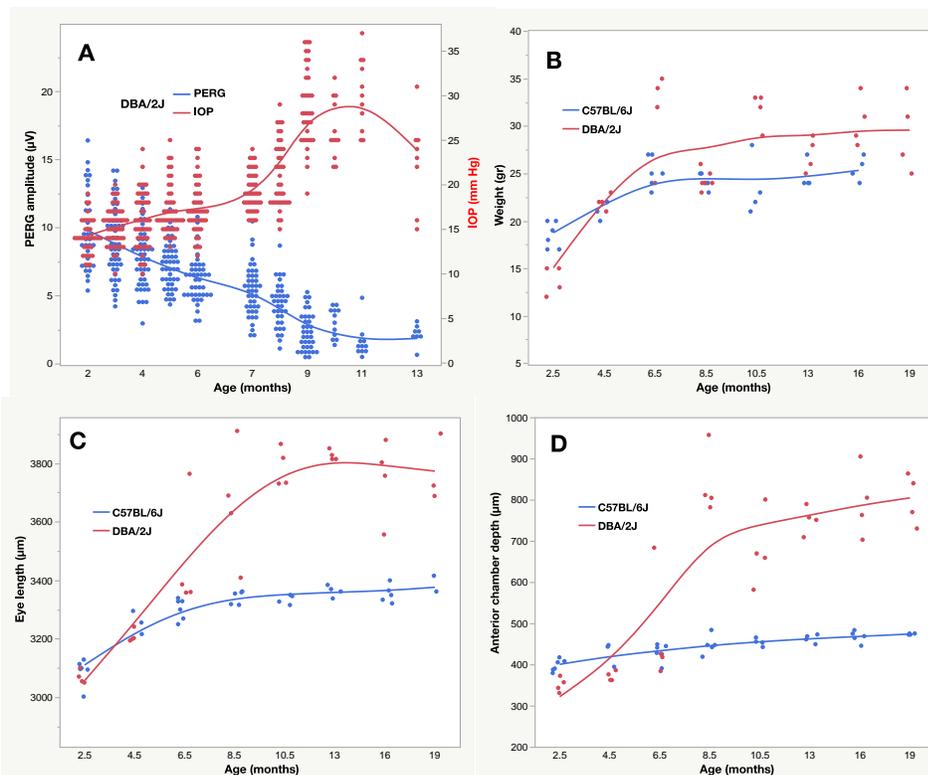


Figure 5. Age-related changes in C57BL/6J and DBA/2J mice. A: PERG amplitude and IOP; B: body weight; C: Eye length; D: anterior chamber depth. Panel A is replotted from Saleh et al, 2007 and panels B, C, D from Chou et al, 2010. The shaded areas represents the 95% confidence interval of the spline regression curves.

Conclusions

The conceptual framework we have discussed is based on the assumptions that 1) in the progression of glaucoma and optic neuropathies, reversible RGC dysfunction precedes RGC death, 2) RGC dysfunction and its dynamics can be assessed non-invasively with PERG, 3) susceptibility to positive/negative stressors is reflected in PERG changes of the opposite sign. Although these assumptions have not been firmly demonstrated, they are supported by a substantial body of literature including the examples shown above. Altogether, the proposed conceptual framework isolates several different RGC physiological conditions that can be characterized with PERG and used as biomarkers for staging the disease, for establishing sensitive functional endpoints at early stages of disease before irreversible RGC death, and for assessing the effect of neuroprotective treatments. PERG dynamics can also be used for phenotyping genetically engineered models with possible alteration of RGC function.

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