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Functional characterization of retina and optic nerve after acute and chronic elevation of the intraocular pressure in rats

Sinisa Grozdanic
Iowa State University

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Functional characterization of retina and optic nerve after acute and chronic elevation of the intraocular pressure in rats

by

Sinisa Grozdanic

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

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Program of Study Committee
Ioana M. Sonea (Co-major Professor)
Donald S. Sakaguchi (Co-major Professor)
Daniel M. Betts
Anumantha Kanthasamy
Young H. Kwon

Iowa State University
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Co-major Professor

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Co-major Professor

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For the Co-major Program

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For the Co-major Program

DEDICATION

THIS DISSERTATION IS DEDICATED TO

THE MEMORY OF MY FATHER

DUSAN   GROZDANIC

(1941-1999)

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CHAPTER 1 – GENERAL INTRODUCTION

Dissertation organization

This dissertation is written in an alternative thesis format and contains five chapters: a general introduction (Chapter I), three research papers (Chapters II, III and IV) and a general discussion (Chapter V). The general introduction includes an introduction to the problem with literature review concerning rat models for studying glaucoma. Chapter II contains the article "Characterization of the pupil light reflex, electroretinogram and tonometric parameters in healthy rat eyes" which was recently submitted to the journal Current Eye Research, Chapter III contains the article "Functional characterization of retina and optic nerve after acute ocular ischemia in rats" which was submitted to the journal Investigative Ophthalmology and Visual Sciences and Chapter IV contains the article "Functional characterization of retina and optic nerve after chronic elevation of the intraocular pressure in rats" which is in preparation for submission to the journal Experimental Eye Research.

INTRODUCTION

Background and Significance

"A crucial feature of glaucoma is the progressive death of retinal ganglion cells. Ganglion cell death is also a feature of a number of other optic neuropathies and retinopathies, and, as with glaucoma, some of these disorders have an ischemic cause" (Osborne[A] et al, 1999). Glaucoma is thought to affect over 67 million people worldwide (Quigley, 1996). Glaucoma is characterized as a progressive optic neuropathy with characteristic optic disc changes and progressive visual field defects (Quigley, 1999). The elevated intraocular pressure (IOP) is considered a primary risk factor for the initiation and progression of glaucomatous neuropathy (Leske MC, 1983).

Currently, all registered glaucoma drugs target regulation of the intraocular pressure by decreasing production or increasing outflow of the aqueous humor (Allen, 2000). However, in many patients, despite adequate control of the IOP, the loss of vision further progresses, which dictates the necessity for the development of new drug generations which should potentially have neuroprotective properties (Goldblum and Mittag, 2002; Allen, 2000). The exact mechanisms behind the continuous RGCs loss in glaucoma are not known. However different theories have been proposed as potential explanations of the RGCs death in glaucoma as recently reviewed by Craig and McKay (1999), Osborne et al (2001) and Farkas and Grosskreutz (2001). In the following pages, we review the current hypotheses of the neuronal death in glaucoma and present data about genetic factors, effects of the mechanic stress due to elevated IOP, ischemic changes caused by vascular deficiency and most frequently used animal models in glaucoma studies.

1. Genetic factors

Glaucoma is a genetically heterogeneous disease. At least fifteen loci have been linked to the disorder, and 6 genes have been identified (Stone et al, 1997; Craig and Mackey, 1999; Rezaie et al, 2002). The protein products of two (myocilin/TIGR and optineurin) of six identified genes are expressed in the anterior segment (trabecular meshwork; Karali et al, 2000; Rezaie et al, 2002) but also in the posterior segment of the eye (retina and optic nerve; Karali et al, 2000; Clark et al (2001[A], 2000[B]); Rezaie et al, 2002).

a) TIGR/Myocilin gene (TIGR/MYOC).

It has been suggested that the TIGR/MYOC gene transcribes a protein which might play an important role in controlling aqueous humor outflow (Nguyen et al, 1998). Recent data also showed the presence of the protein in astrocytes of the optic nerve head (Clark[A] et al, 2001). It has been (Caballero et al, 2000; Caballero and Borras, 2001) shown that the mutated TIGR /MYOC protein which is lacking the olfactomedin domain, has an altered secretion from the cell. The exact role of the TIGR/MYOC protein is not known, but it is possible that TIGR/MYOC protein has

some vital physiological role in hypertensive eyes since its messenger RNA is up-regulated in chronic hypertensive rat eyes (Ahmed et al, 2001).

b) *Optineurin (OPTN)*

Optineurin is expressed in trabecular meshwork, non-pigmented ciliary epithelium, retina and brain (Rezaie et al, 2002). The OPTN gene codes a 66 kD protein of unknown function that has been implicated in the tumor necrosis-α signaling pathway (Li et al, 1998) and interacts with diverse proteins including Huntingtin (Hattula and Peranen, 2000) and Ras-associated protein RAB8 (Hattula and Peranen, 2000). The exact role of the optineurin is still not known (Rezaie et al, 2002).

2. Mechanical theory for the retinal ganglion cell death in glaucoma

According to the mechanical theory the elevation of IOP might lead to the compression of neuronal axons, blockade of the axonal transport, activation of the glial cells, remodeling of the extracellular matrix and subsequent neuronal degeneration (Nickelis, 1996; Quigley, 1999).

a) Blockade of the axonal transport

It has been hypothesized that withdrawal of trophic factors is potential cause of the ganglion cell death in glaucoma (Nickelis, 1996; Farkas and Grooskreutz, 2001). In the adult mammalian retina, RGCs might be dependent on the presence of the brain derived neurotrophic factor (BDNF) in order to survive (Nickelis, 1996; Farkas and Grosskreutz, 2001). Thus, any event which might interfere with the transport of growth factors and/or their receptors could have fatal consequences on the RGC survival (Nickelis, 1996; Farkas and Grosskreutz, 2001). Results of short-term studies measuring anterograde axoplasmatic flow with tritiated leucine in normal dogs in which the IOP was artificially elevated (intraocular perfusion pressures of 12 to 100mm Hg) revealed a pressure-related accumulation of the label at the scleral lamina cribrosa (Gelatt and Brooks, 1999). Slowed axoplasmatic flow was also detected in the colony of dogs (Beagles) with inherited glaucoma (Gelatt and Brooks, 1999). Pease et al (2000) and Quigley et al (2000) showed that the retrograde axonal transport deficit can diminish transport of the brain derived neurotrophic factor (BDNF)

and its receptor TrkB in the rat model of acute elevation of the IOP and monkey model of the glaucoma. These data suggested that mechanical effects of the elevated IOP on RGC axons might have a role in the development of neurodegeneration. Even in normal tension glaucoma, lowering of IOP decreases the progression of the disease (Sheegeda et al, 2002). Thus, IOP is still a risk factor in normal tension glaucoma and lowering of IOP is the principal goal, even in those patients (Allan, 2000; Sheegeda et al, 2002).

b) Glial cell activation and matrix remodeling due to elevated IOP

It has been previously noticed that many glaucomatous optic nerve heads (ONH) are characterized by the cupping of the optic disc and compression, stretching and remodeling of the cribiform plates of the lamina cribrossa in humans (Quigley et al, 1983). The hypothesis was that reactive changes in the glaucomatous ONH occur as a response to elevated IOP (Quigley et al, 1983). Astrocytes participate in the remodeling process during development and after neuronal tissue injuries (Hernandez[A], 2000). Due to the structure of the lamina cribrosa, the sensitive balance between astrocytes and fibroelastic extracellular matrix might be seriously disrupted as a result of the elevated IOP in glaucomatous eyes (Hernandez[A], 2000). Recent data showed that hydrostatic pressure stimulates synthesis of elastin (Hernandez[B] et al, 2000;) in cultured optic nerve astrocytes from glaucomatous patients. Pena et al. (2001) showed that elastin is over expressed in the optic nerve heads of monkeys with experimentally induced glaucoma. Furthermore, Agapova et al (2001) showed that cultured human optic nerve head astrocytes and optic nerve heads from glaucomatous patients have upregulated specific matrix metaloproteinases, which might have remodeling activity in glaucomatous optic nerve heads (Hernandez[A], 2000). These remodeling events might lead to the lack of the mechanical support for the axons which might result in the compression and axonal damage (Hernandez[A], 2000, Hernandez[B] et al, 2000).

3. Vascular theory for the retinal ganglion cell death in glaucoma

A lot of evidence has accumulated to suggest that vascular insufficiency and ischemia of the optic nerve might play important role in the pathogenesis of glaucoma

(Hayreh, 1999). Fluorescein angiography studies from glaucomatous patients revealed reduced total retinal blood flow and dye leakage from optic nerve head capillaries, suggesting that oxygenation and nutritive transport to the RGC might be seriously diminished (Nickelis, 1996). In addition, a decrease of choroidal (Duijm, 1997) and retrobulbar blood flow (Yamazaki and Drance, 1997) were correlated with the severe visual loss in glaucoma, and authors suggested that deficits in the ocular blood supply might be responsible for the severity of visual loss in glaucomatous patients (Duijm, 1997; Yamazaki and Drance, 1997). There is a large amount of evidence linking ischemia with the retinal ganglion cell death in different experimental models (Goldblum and Mittag, 2002). The appearance of glaucomatous abnormalities of the optic nerve head (cupping and hemorrhages) suggests that primary damage occurs at the level of the optic nerve head which might result in selective damage of the RGCs (Levin, 1999). The effect of hypoxia can be observed on neurons and non-neuronal cells (astrocytes, Muller cells, microglia and blood vessel cells), but probably the simultaneous deficits in the physiological activity of these cells are responsible for the RGC death during glaucoma (Osborne et al, 2001).

a) Glutamate-induced excitotoxicity

Glutamate excitotoxicity develops when extracellular glutamate levels are increased, due to decreased uptake (Trotti et al, 1998) or increased release (Schwartz et al, 1996) from the neuronal and non-neuronal (glial) cells. These conditions develop during hypoxic or traumatic episodes in the nervous system (Farkas and Grosskreutz, 2001). Excessive activation of the glutamate receptors (primarily NMDA receptors) leads to the entry of the calcium ions in the cell and calcium overload (Mattson, 2000). It has been postulated that calcium overload causes mitochondrial failure, inappropriate formation of free oxidative radicals and activation of complex cascades of proteases, nucleases and lipases which may result in the apoptotic cell death (Mattson, 2000).

Data from animal models suggest that glutamate toxicity may contribute to ganglion cell death during hypoxic events (Lam et al, 1995) by activating ionotropic glutamate receptors. Furthermore, elevated concentrations of the glutamate have

been reported in the vitreous of glaucomatous dogs (Brooks et al, 1997) as well as humans and monkeys (Dreyer et al, 1996). However, excess vitreous glutamate has not been confirmed in recent studies in human (Honkanen et al, 2001) or primate glaucoma (Wamsley et al, 2002), so the validity of the hypothesis of glutamate-mediated neurotoxicity in glaucoma still has to be investigated.

b) *Reactive response of the glial cells (astrocytes, Muller cells and microglia)*

After an ischemic insult at the level of the retina and/or optic nerve head, glial cells might lose their trophic and supportive role and become reactive. It has been shown that reactive microglia might actively secrete potentially neurotoxic substances such as glutamate (Bezzi et al, 2001), TNF-α (Yan et al, 2000) and up-regulate production of nitric oxide (Liu and Neufeld, 2000). The lack of the physiological supportive role of glial cells might seriously compromise capacity of the neurons to resist to neurotoxic insults (Hernandez[A], 2000). Martin et al (2002) showed decrease of the astrocytic glutamate transporter (GLT1 and GLAST) proteins in chronic hypertensive rat eyes but not in the rat eyes in which optic nerves were transected. "Glutamate transporters are thought to play important roles in the termination of glutamatergic synaptic transmission. The glutamate transporters are able to concentrate glutamate several thousand fold across the cell membrane and are responsible for maintaining low resting levels (<1uM) of the transmitter extracellularly, thereby protecting neurons against harmful over stimulation of excitatory amino acids" (Trotti et al, 1998). The microglial response and or lack of supportive astrocyte function might be responsible for the progression of neurodegenerative changes (Bezzi et al, 2001; Liu and Neufeld, 2000; Trotti et al, 1998).

While different theories have been proposed to explain the possible mechanism behind RGC death in glaucoma, the exact mechanism is still not clear. However, there is possibility that each of the above mentioned mechanisms have partial role in the development of the glaucomatous pathology.

In order to achieve a better understanding of the different pathological events during glaucoma, and eventually develop new strategies for the neuroprotection of the

ischemic retina and optic nerve, it is essential to develop animal models which should be easily reproduced, predictable and easy to maintain in a laboratory environment.

In the following pages we will describe most commonly used models for glaucoma studies: monkey, dog and rodent models (rat and mice).

1. Monkey model of glaucoma

Probably, the best model for the human glaucoma studies is the monkey model of glaucoma since morphological and electrophysiological changes in this model closely resemble glaucomatous pathology in humans (Jonas and Hayreh, 1999; Marx et al, 1986; Hare et al, 2001; Yucel et al, 1999; Burgoyne et al, 1995). Glaucoma in monkeys is usually induced by argon or diode laser photocoagulation of the trabecular meshwork, which results in the obstruction of the aqueous humor outflow and chronic elevation of the IOP (Gaasterland and Kupfer, 1974; Wang et al, 1998). Changes which occur in the experimental monkey glaucoma are very similar to human glaucoma and they include: lack of standard flash ERG deficits despite significant RGCs loss (Marx et al, 1986; Hare et al, 2001), focal loss of the large optic nerve axons (Yucel et al, 1999), characteristic thinning of the retinal nervous fiber layer (Jonas and Hayreh, 1999) and gradual development of the optic nerve cupping (Burgoyne et al, 1995).

2. Dog model of glaucoma

Another extremely valuable model for glaucoma studies is a spontaneously occurring dog model of glaucoma. Primary glaucoma has been reported in at least 45 different dog breeds (Gelatt and Brooks, 1999). The best characterized model of dog glaucoma is primary open-angle glaucoma in Beagles (Gelatt and Brooks, 1999). "It has been characterized by open iridocorneal angles in early glaucoma, elevated intraocular pressure and decreased aqueous humor outflow. With moderate glaucoma, the iridocorneal angles are typically open; intraocular pressure is elevated, facility of aqueous outflow is decreased; and variable optic disc cupping and focal disinsertions of the zonules from the lens are developed. Advanced glaucoma exhibits

narrow to closed iridocorneal angles, decreased facility of aqueous outflow, elevated intraocular pressure, lens dislocation, optic disc atrophy, and finally phthisis bulbi" (Gelatt et al, 1977). While morphological degeneration of the optic nerve structures (Brooks[A] et al, 1989; 1989[B], 1995), the alteration of aqueous humor outflow pathways (Brooks[c] et al, 1989; Samuelson et al, 1989; Barrie et al, 1985, 1985[B]) and electrophysiological function deficits (Ofri et al, 1993; 1994) have been reported, data about molecular changes in this model are sparse (Kallberg et al, 2002). A dog model of glaucoma holds a great promise for the better understanding of the RGC death in hypertensive eyes, however more molecular approaches must be developed to better understand glaucomatous changes in dogs and make a correlation with the changes described in human glaucoma. Furthermore, tissue from this dog colony was not accessible for researchers out of University of Florida in the past, so all published data came from the Gelatt's group, which probably affected more extensive use of the dog model.

3. Rodent models of glaucoma

Understanding of the glaucoma pathophysiology depends on the precise correlation of molecular events and *in vivo* changes of the retina and optic nerve function during the progress of the disease. Rodents (rats and mice) became the preferred models for studying glaucoma due to availability of genomic and proteomic data, potential for use of transgenic animals, cost and ease of maintenance (Goldblum and Mittag, 2002). Morphological and molecular data from different rodent models which mimic some of the glaucomatous changes significantly improved the amount of information about potential causes of the RGC death (Levkovitch-Verbin et al, 2000; Mittag et al, 2000; Johnson et al, 2000; Garcia-Valenzuela, 1995).

In the following pages, the major characteristics of the different rodent models commonly used in glaucoma studies are presented.

3.1. Rat models with the open-angle glaucoma phenotype

Currently, there are two rat models which mimic open-angle glaucoma changes:

a) Rat model of chronic ocular hypertension induced by cauterization of the vortex and episcleral veins

This model was initially described by Sharma and coworkers in 1995 (Shareef et al, 1995). The principle for the induction of the chronic elevation of the IOP is cauterization of the episcleral and vortex veins which are the major outflow pathways for the aqueous humor. The initial procedure described in 1995 was performed by the cauterization of one to four vortex veins (Shareef et al, 1995). Rats which received cauterization of a single vortex vein did not developed elevation of the IOP up to 30 days after surgery. Immediately, following cauterization of two, three or four episcleral veins operated rats developed significant elevation of the IOP – for 2 cauterized veins control eye values were 13.3 mmHg while operated eyes had value of 28 mmHg (elevation of the IOP lasted for 42 days). Rats which received cauterization of 3 vortex veins had IOP value for the control eyes of 13.2 mmHg and in operated eyes of 53 mmHg for initial 4 days postoperatively. However, 4 days after surgery, IOP in operated eyes declined to 26 mmHg and was sustained at similar levels for the length of the testing period (9 weeks). Rats which received cauterization of 4 vortex veins developed elevated IOP of 60 mmHg in operated eyes for 10 days (length of the testing period).

In a later study from the same group (Laquis et al, 1998) analysis of the RGC counts after cauterization of 2 vortex veins revealed the loss of 8, 8.4 and 8% cells in the central, middle and peripheral retina respectively two weeks after surgery. After 4 weeks of increased IOP, the loss was 14, 14.5 and 23% (central, middle and peripheral retina) of the total cell count from the control, non-operated eyes. After six weeks of the elevated IOP, there was decrease of 24% in the central retina, 22.2% in the middle retina and 32.2% in the peripheral retina when compared to the total number of RGCs. Eight weeks after surgery, the cell counts indicated a loss of 27% for the central retina, 28.2 for the middle retina and 46.8 % for the peripheral retina, which closely resembles the position of visual field and structural optic nerve deficits from glaucomatous human patients (Lundy and Choplin, 1998). This study showed the continuous loss of RGCs in eyes with chronically elevated IOP, which

predominantly affected peripheral retina. Sawada and Neufeld (1999) reproduced the same model in 1999 by cauterizing 3 vortex veins. In their hands the success rate of the IOP elevation after surgery was 80% and they achieved stable 1.6 fold elevation of the IOP for 6 months. The cell count from this study revealed similar dynamics and rate of RGC loss as in the Laquis et al. (1998) study (around 20% loss of RGCs after 6 weeks of elevated IOP). Authors concluded (Sawada and Neufeld, 1999) that cauterization of vortex and episcleral veins "provide a reproducible, quantitative and low cost model for pharmacological experiments on neuroprotective agents for the treatment of the optic neuropathy associated with glaucoma".

While the morphological characterization of the changes in chronic hypertensive rat eyes is well established, data about potential molecular mechanisms involved in the development of pathology are rare. Recently, the up-regulation of the inducible nitrous oxide synthetase in optic nerve head of human glaucomatous eyes was described (Neufeld et al, 1997). The authors hypothesized, that the up-regulated iNOS might be partially responsible for the neuronal loss in hypertensive eyes (Neufeld et al, 1997). They showed that treatment with aminoguanidine, a substance with iNOS inhibitory activity, dramatically reduced neuronal death in rats with chronically induced elevation of the IOP (Neufeld et al, 1999). Aminoguanidine treated animals had 9.6 $\pm$ 3.5% of RGC loss compared to 35.9 $\pm$ 5.7% in non-treated hypertensive rats (Neufeld et al, 1999).

Mittag et al. (2000) hypothesized that rat neuronal death in rat glaucomatous retinas occurs by apoptotic death due to the disruption of mitochondrial membrane potentials. They showed the presence of focal damage in hypertensive eyes surrounded by cells stained with a specific dye which detects condensed nuclei of apoptotic cells (YOYO-1) (Mittag et al, 2000). Furthermore, when they performed specific staining for the *in vivo* detection of the mitochondrial potential (CMTMR dye), they found that glaucomatous eyes had significantly disrupted potentials of the mitochondrial membranes compared to control (non-operated) eyes (Mittag et al, 2000).

The major disadvantage of vortex-vein cauterization model is that venous circulation might be seriously disrupted, which might cause the disturbance of choroidal circulation and potential photoreceptor function (Bayer[A] et al, 2001). A functional study from Bayer[A] et al. (2001) showed changes of the retinal electrophysiological activity. They showed that in operated rats which developed chronic elevation of the IOP and were followed sequentially, all recorded ERG parameters showed significant time-dependent changes in glaucomatous eyes relative to their contra-lateral normal eyes, with oscillatory potentials (OPs) showing the earliest significant difference after only 3 weeks of high IOP. When they compared different groups of unilateral glaucomatous rats, beyond 8 weeks of elevated IOP, only the OPs showed a continued decrease with time. However, it is not clear whether this change was induced by chronic elevation of the IOP, disruption of the choroidal circulation or both.

The second disadvantage of this model is low rate of surgical success in terms of the elevation of the IOP and development of morphological damage (Goldblum and Mittag, 2002). Contrary to reports from Sharma's and Neufeld's groups, Mittag et al (2000) described only a temporary elevation of the IOP for 15 days in the same model, and they modified the procedure by repetitive subconjunctival injections of 5-fluorouracil (5-FU, an inhibitor of cell proliferation), with the objective of preventing recanalization of the cauterized blood vessels. It is still not clear why Mittag et al. (2000) noticed such a dramatic difference in the duration of IOP elevation in this model. McKinnon et al. (1999) described relatively low surgical success for this model: 49% of operated animals developed chronic elevation of the IOP, but only 20% of animals had detectable morphological damage of the optic nerve after vortex vein cauterization.

b) Rat model of chronic ocular hypertension induced by injection of hypertonic saline into limbal veins

This model was initially described by Morrison and coworkers (Morrison et al, 1997). The elevation of the IOP is induced by injection of the hypertonic saline (1.65 – 1.75M) into limbal aqueous humor collecting veins, which causes sclerosis of aqueous

humor outflow pathways (Morrison et al, 1997). When authors injected lower concentrations of the saline (0.5M, 1M and 1M) they did not notice significant elevation of the IOP (Morrison et al, 1997). However, with saline concentration above 1.85M they noticed development of a strong inflammatory response and uveitis (Morrison et al, 1997).

The major pathophysiological characteristics of this model are: focal degeneration of the RGC axons (Morrison et al, 1997), optic nerve head loss of neurothropins (Johnson et al, 2000), loss of gap junctional connexin43 labeling (Johnson et al, 2000) and astrocytic proliferation at the optic nerve head level (Johnson et al, 2000).

The major advantages of this model are: mild to moderate elevation of the IOP, which can be titrated by the concentration of the injected saline (Morrison et al, 1997; Goldblum and Mittag, 2002). However, potential disadvantages of this model are the technically demanding procedure, multiple saline injections can be required to sustain the elevation of the IOP (McKinnon et al, 1999; Goldblum and Mittag, 2002).

3.2. Rat models with the closed-angle glaucoma phenotype

a) S-antigen induced closed-angle uveitic glaucoma phenotype

This is a rat model of uveitic, secondary closed-angle glaucoma, which was described by Mermoud et al in 1994. Bovine S-antigen (a protein isolated from the retina fraction of different animal species (Gregerson et al, 1987; Doekes et al, 1988)), which is known to induce chronic uveitis (Mermoud et al, 1994; Gregerson et al, 1987), was used in the study to induce uveitis and secondary uveitic glaucoma. In this model, the mechanism behind the IOP elevation is considered to be uveitis–induced obstruction of the trabecular meshwork, development of the anterior synechia and secondary aqueous humor outflow blockade after S-antigen injection.

This model is characterized by a decrease of the IOP to a mean of 16.5 +/- 4.3 mmHg on days 2-5 after S-antigen injections from a mean pre-experimental value of 20.5 +/- 5.4 mmHg (Mermoud et al, 1994). Aqueous humor production was decreased and outflow facility (the ability of the trabecular meshwork to drain aqueous fluid)

increased at day 3 after S-antigen injection which resulted in initial decrease of the IOP (Mermoud et al, 1994). The IOP levels in this model initially increased from days 6 to 20 (35.8+/-9.1mmHg) due to the increase in aqueous humor production and normal or decreased outflow facility (Mermoud et al, 1994). Histopathological study revealed inflammation of the anterior and posterior segments from days 9 to 21 after S-antigen injection (Mermoud et al, 1994). Aqueous humor production was decreased and outflow facility (outflow facility is ability of trabecular meshwork to drain aqueous fluid) increased at day 3 after S-antigen injection.

The major disadvantage of this model is presence of the strong inflammatory response which affects anterior and posterior segment of the eye, so it is very difficult to determine whether damage to the optic nerve is induced by the inflammatory response, rather then elevation of the IOP (Mermoud et al, 1994). This model has not been extensively described (Mermoud et al 1994; Mermoud et al, 1995) so data about its validity are lacking (Goldblum and Mittag, 2002).

b) Laser-induced chronic ocular hypertension

Recently, several groups described successful chronic elevation of the IOP in rat eyes after cauterization of the trabecular meshwork with a laser (Ueda et al, 1998; WoldeMussie et al, 2001; Levkovitch-Verbin et al, 2002). Laser induced cauterization of the trabecular meshwork has been a standard model for glaucoma induction in monkey eyes (Jonas and Hayreh, 1999; Marx et al, 1986; Hare et al, 2001; Yucel et al, 1999; Burgoyne et al, 1995). The first description of the laser-induced chronic elevation of the IOP in rats came from Ueda and coworkers in 1998. They injected India ink in the anterior chamber of albino rats and after 7 days applied argon laser energy to the trabecular meshwork enriched with dye particles.

This procedure has been modified recently (WoldeMussie et al, 2001 and Levkovitch-Verbin et al, 2002) by application of the argon laser energy to the episcleral veins posterior to the limbus. While the intention was to induce the cauterization of limbal vessels, histological analysis revealed development of the anterior synechia secondary to the laser treatment (Levkovitch-Verbin et al, 2002;

Quigley - personal communication), which puts this model in the category of closed-angle glaucomatous changes (Quigley - personal communication).

The major advantage of this procedure is reliable elevation of the IOP, high surgical success (between 80-100% treated animals developed elevation of the IOP) and reproducible damage to the optic nerve (Quigley - personal communication). The major disadvantage is need for repeated laser sessions to sustain the elevation of IOP and uveitis occurrence after laser procedures (Goldblum and Mittag, 2002, Quigley – personal communication).

3.3. Experimental models which mimic acute glaucomatous changes

a) Rat model of acute ocular ischemia

One of the most frequently used models for the investigation of molecular mechanisms, and potential therapeutic strategies for retina and optic nerve ischemia has been a rat model of acute elevation of the intraocular pressure (to 110 mmHg for 60 minutes by cannulation of the anterior chamber with a needle connected to a raised saline reservoir), characterized by an ischemia-reperfusion injury (Goldblum and Mittag, 2002). Previous studies using this model have identified molecular mediators with neurotoxic properties during ischemia-reperfusion injury of the rat retina and optic nerve, such as excitatory amino acids (Lagreze et al, 1998), free oxidative radicals (Augustin et al, 1998; Shibuki et al, 2000) and cytokines (Hangai et al, 1996; Yoneda et al, 2001). Traditionally, quantitative analysis of the damage has been achieved only by end stage counting of the cells in different retinal layers (Hughes WF, 1991) or retrograde fluorescent labeling of retinal ganglion cells (Selles-Navarro et al, 1996). Most frequently, the functional analysis of retina after ischemia-reperfusion injury using electroretinography was performed for a period of time not longer than 7 days postoperatively (Donello et al, 2001; Chao and Osborne, 2001; Singh et al, 2001; Osborne et al, 2002), while one study observed ERG function in rats after ischemia-reperfusion injury for 28 days (Shibuki et al, 2002). Majority of studies showed significantly decreased b-wave amplitudes (Donello et al, 2001;

Shibuki et al 2002; Chao and Osborne, 2001; Osborne et al, 2002) and oscillatory potentials amplitudes (Shibuki et al 2002), while a-wave amplitudes were relatively non-affected (Donello et al, 2001; Shibuki et al 2002). However, Chao and Osborne (2001) and Singh et al (2001) demonstrated a significant reduction of both a- and b-wave amplitudes in rat eyes exposed to acute ocular ischemia.

To our knowledge, there is only one abstract which documented a temporary decrease of the optic nerve and retina function estimated by the quality of the pupil light reflex (PLR) in rats after ischemia-reperfusion injury (Clarke and Gamlin, 1998). Clark and Gamlin demonstrated complete loss of the consensual PLR function in 3 of 5 operated rats in the first two days after surgery. Two rats had partial PLR deficits in the first 2 days postoperatively, and they completely recovered their PLR responses by 10 days postoperatively. Rats which completely lost PLR in the first 2 days, partially recovered the PLR function between 3 and 10 days postoperatively. When authors performed RGCs count they found decreased number of RGCs in operated eyes which had sustained PLR deficits.

The major advantage of this model is easy induction and reliable reproduction of the RGCs damage (Selles-Navarro et al, 1996). However, the major disadvantage of this model is development of the acute ocular ischemia that produces outer retina necrosis (Buchi, 1992), which is not a characteristic feature of glaucoma.

b) Rat model of the glutamate induced neurotoxicity

Amino acids glutamate and aspartate are major excitatory neurotransmitters in the retina (Chen and Diamond, 2002). Glutamate has been identified as one of the major causes of cytotoxicity in brain injury and brain disorders, leading to secondary neuronal degeneration (Choi, 1988) and believed by some to be a potential cause of the RGC degeneration in glaucomatous patients (Sucher et al, 1997)

Reports which described retinotoxic properties of the glutamate (Sisk et al, 1984; Sisk and Kuwabara, 1985), showed that intravitreal injection of the monosodium L-glutamate causes intracellular swelling, necrosis, and disappearance of most inner retinal neurons in rats (Sisk et al, 1984;1985). Sisk and Kuwabara demonstrated that

the adult rat retina experienced severe degenerative changes characterized by massive intracellular swelling and secondary necrosis following intravitreal glutamate injection. In their study, the degeneration first involved retinal ganglion cell and inner nuclear layers, but later involved the outer retina (photoreceptors). Different groups (Vorwerk et al, 1996; Osborne et al, 1999; Lam et al, 1999; Honjo et al, 2000; Kwong and Lam, 2000; Laabich and Cooper, 2000; Kido et al, 2000; Mittag et al, 2000; Sun et al, 2001) demonstrated that an intravitreal injection of the glutamate or glutamate analogs (NMDA was most frequently used) can damage the ganglion cell layer and the inner plexiform layer. Akaike et al. (1998) detected the appearance of internucleosomal fragmentation of retinal DNA and terminal transferase dUTP nick-end labeling (TUNEL)—positive nuclei in the inner retina of rats intravitreally injected with NMDA (glutamate agonist) which implied that retinal neurons are dying primarily by apoptosis.

While intravitreal glutamate (NMDA) injection represents model of the retinal ganglion cell and inner retinal layers damage, it does not provide the complex picture of the pathology which is usually seen in acute and chronic models of the ocular hypertension, which seriously diminishes value of this model as a studying tool of the real glaucomatous changes (Goldblum and Mittag, 2002). Furthermore, validity of the glutamate-toxicity hypothesis was seriously questioned by results of recent studies which did not confirm excess vitreous glutamate in human (Honkanen et al, 2001) or primate glaucoma (Wamsley et al, 2002).

c) Optic nerve crush/axotomy injury model

The primary goal for the use of this model is quantification of the primary RGCs damage and assessment of the secondary degeneration independently of elevated IOP (Schwartz and Yoles, 1999; Yoles et al, 1999). It has been shown that direct damage to the optic nerve fibers leads to their degeneration and the cell death by apoptosis (Garcia-Valenzuela et al, 1994). The optic nerve crush injury is usually followed by the massive infiltration of inflammatory cells (Frank and Wolburg, 1996) which is not a characteristic of the glaucomatous optic nerve damage. The supporters

of the crush model suggested that the secondary neuronal degeneration which is usually seen in this model may underlie the spread of damage seen in glaucoma after the primary cause of the disease has been treated (Schwartz and Yoles, 1999).

The optic nerve crush/axotomy injury model is probably the least representative model of the glaucomatous damage compared to already mentioned models (Goldblum and Mittag, 2002).

4. Mouse models of the glaucoma phenotype

DBA/2NNia and DBA/2J inbred mouse strains have been found to spontaneously develop a form of secondary glaucoma phenotype (pigment dispersion) in almost 100% of the individuals (Anderson et al., 2001; John et al., 1998; Sheldon, Warbritton, Bucci, & Turturro, 1995). The exact genetic defect responsible for DBA/2J (D2) phenotype was recently identified (Anderson et al, 2002). Using high-resolution mapping techniques, sequencing and functional genetic tests, Anderson et al (2002) showed that iris pigment dispersion (IPD) and iris stroma atrophy (ISA) result from mutations in related genes encoding melanosomal proteins.

IPD is caused by a premature stop codon mutation in the Gpnmb (GpnmbR150X) gene, as proved by the occurrence of IPD only in D2 mice that are homozygous with respect to GpnmbR150X; otherwise, similar D2 mice that are not homozygous for GpnmbR150X do not develop IPD. ISA is caused by the recessive Tyrp1b mutant allele and rescued by the transgenic introduction of wild type Tyrp1 (Anderson et al, 2002).

Anderson et al. (2002) hypothesized that IPD and ISA alter melanosomes, allowing toxic intermediates of pigment production to leak from melanosomes, causing iris disease and subsequent pigmentary glaucoma. Pathologic studies indicate that the changes in these animals start at about 4 months of age with iris pigmented epithelium loss and subsequent iris atrophy at 7 months leading to progressive closure of the angle and IOP elevation at 8-9 months of age (Pang et al., 1999). This leads to a significant reduction of the RGC number and inner plexiform layer thickness at 14 months of age followed by optic nerve damage and electroretinographic deficits (Bayer[B] et al., 2001)

The mouse, as a model for studying glaucoma pathophysiology, might provide a powerful experimental tool for drug studies, hypothesis testing, and genetic molecular dissection of the glaucoma pathogenesis (Goldblum and Mittag, 2002). So far there have not been independent reports which actually confirmed that these lines of mice have elevated IOP.

The main characteristic feature of glaucoma is the progressive and relatively slow damage/death of RGCs (Quigley, 1999). As ganglion cell death is also a feature of a number of other optic neuropathies and retinopathies, the acute induced models which are useful in some aspects for glaucoma research can resemble more closely these other diseases than they do glaucoma (Goldblum and Mittag, 2002). Examples of such a resemblance are the optic nerve crush model and central artery occlusion in the case of the ischemia/reperfusion model (Goldblum and Mittag, 2002). However, the most relevant rodent glaucoma model should include chronic elevation of IOP as one of the most frequent features which characterize glaucoma.

We decided to use the vortex-vein cauterization model because it represents a model with chronic elevation of the IOP and the induction of the model is relatively easy to perform, while the model of acute elevation of the IOP (110mmHg/60 min) was primarily selected due to the reproducible outcome of the retina and optic nerve damage.

Currently, estimation of the damage in the experimental rat models of the chronic ocular hypertension, is usually evaluated by histological or immunocytochemical analysis once when the experimental animal is euthanized. Only three recent studies described some of the electroretinographic properties of chronically hypertensive rodent eyes (Mittag et al, 2000; Bayer et al, 2001[A,B]) and there are no studies which tried to evaluate function of the optic nerve in rodent models off the chronic ocular hypertension. Unfortunately, the morphological approach alone does not provide any information about dynamics of the disease in different time points. Furthermore, neuroprotective strategies cannot be completely evaluated, since

a normal morphological appearance after a neurotoxic insult does not confirm that all cells are functional.

The principal goal of this study was to describe functional responses (ERG and PLR) of rat eyes after acute and chronic elevation of the IOP and correlate functional with morphological changes. Since monitoring of the pupil light reflex reflects the combined function of the retina and optic nerve, we were interested to use electroretinography as an additional method to evaluate if damage occurred in different retinal layers beside RGC layer. The functional monitoring of the retina and optic nerve might provide significant amount of information about changes observed in different experimental models of chronic ocular hypertension.

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CHAPTER 2 – CHARACTERIZATION OF THE PUPIL LIGHT REFLEX, ELECTRORETINOGRAM AND TONOMETRIC PARAMETERS IN HEALTHY RAT EYES

Sinisa Grozdanic, Donald S. Sakaguchi, Young H.Kwon, Randy H.Kardon and Ioana M. Sonea

Abstract

Purpose: To characterize the pupil light reflex (PLR), electroretinographic (ERG) and tonometric parameters in healthy rat eyes.

Methods: Brown Norway rats were used for experiments. The PLR was evaluated with a computerized pupillometer (n=27), ERGs were recorded simultaneously from both eyes (n=27) and IOP was measured with a Tonopen (n=15).

Results: The analysis of the PLR parameters confirmed the consensual PLR was significantly smaller in amplitude (p=0.03) and increased latency time (p=0.001) compared to the direct PLR. Electroretinography (1600+200 cd/m^2) revealed a-wave amplitude of 207.2±13 μV and the b-wave 554.3±24.5 μV. The flicker ERG recording revealed amplitudes of 40.6±2.4 μV. Tonometry measurements revealed that isoflurane, but not halothane, anesthesia suppressed the IOP (non-anesthetized: 25.3±1.0 mmHg; 1% halothane+30% NO: 26.2±1.1 (p>0.05); 1% isoflurane+30% NO: 20.1+1.6 (p<0.05)).

Conclusions: Consensual PLR in rats has a relative deficit comparing to the direct PLR. Isoflurane anesthesia has suppressive effect on the IOP in healthy rat eyes.

Introduction

Development of easily induced and reproducible animal models is essential to gain a better understanding of pathological processes during glaucoma, which is one of the major blinding diseases. The laboratory rat has become a model system for studying glaucoma (1,2,3). Multiple studies have described methods for the non-invasive monitoring of the intraocular pressure (IOP) in conscious (4,5) or anesthetized rats (6,7,8,9). Moore et al. (6) introduced use of the hand held applanation tonometer (Tonopen XL) as a useful device for the monitoring of the IOP in healthy rats. Sharma et al. (1) used a pneumotonometer to measure IOP in anesthetized rats in which chronic ocular hypertension was induced by cauterization of two vortex veins. A recent report (5) described use of the modified Goldman applanation tonometer, while Kontiola et al (9), reported use of the induction/impact tonometer for the non-invasive monitoring of the IOP in rats. While precise estimation of the IOP is essential for the evaluation of the rat glaucoma models, correlative data describing retina and optic nerve functional deficits are lacking, with the exception of the two recent studies which described some of the electrophysiological properties of chronically hypertensive rat eyes (10, 11). It has been known for some time that elevated IOP represents a risk factor for developing visual field defects characteristic for human glaucomatous patients. Due to the fact that humans with glaucoma may develop visual defects even without ocular hypertension (normotensive glaucoma), it became important to develop diagnostic strategies which will detect glaucomatous changes in addition to the IOP status. An objective estimation of optic nerve function can be achieved by recording visual evoked potentials (VEPs) or the pupil light reflex (PLR). Recording of VEPs, due to the primary representation of central retina fields in the visual cortex, is a method primarily used for the estimation of central retinal damage. In glaucoma the peripheral visual fields are predominantly affected and it is essential to use techniques that will reliably permit the objective monitoring of peripheral retina and optic nerve function. Characterization of the PLR is a sensitive method for determining retina and optic nerve status during ophthalmic diseases and, when combined with electroretinography (ERG) can offer objective information about

the status of the retina and optic nerve. However, while standard flash electroretinography provides information about the status of photoreceptors and neurons in the inner nuclear layer, retinal ganglion cell activity is usually undetected unless a patterned visual stimulus is used. Previous reports demonstrated that the estimation of the PLR quality is an objective parameter of optic nerve function, especially in rodents, since status of the photoreceptors does not correlate completely with the quality of the PLR (12,13,14). Careful characterization and correlation of the electrophysiological and tonometric parameters are likely to provide novel information about the dynamics of developing injuries during chronic ocular hypertension in rats. In this study we were interested to characterize parameters in healthy rats which might be of importance for the *in vivo* characterization of rat models of acute and chronic ocular hypertension such as: pupil light reflex (PLR), electroretinographic responses (flash and flicker ERG) and IOP.

Materials and methods

All animal studies were conducted in accordance with the Declaration of Helsinki and The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23) and procedures were approved by the Iowa State University Committee on Animal Care. Brown Norway rats (4-6 months of age, n=42) were used for experiments. Rats were kept under 12 hours light and 12 hours dark regime.

Computerized pupillometry

The pupil light reflex was evaluated with a custom-made computerized pupillometer (University of Iowa, Iowa City, IA). Rats (n=27) were anesthetized initially with 3% halothane, 30% NO and 70% O_2. A light anesthesia was maintained with 1% halothane, 30% NO and 70% O_2 to avoid suppression of the PLR response detected with the use of higher dose of anesthetics. The computerized pupillometer was attached to 2 infrared sensitive CCTV video cameras for simultaneous visual monitoring of both pupils. However, a one channel computerized pupillometer was used to record the movement of only one pupil while the stimulus light was alternated

between the right and left eye. For this study, the pupil recording was randomly selected (right or left) for each animal. Four different intensities of light stimulus luminance were delivered: $45.8cd/m^2$; 82 cd/m^2; $153cd/m^2$; 300 cd/m^2. The stimuli were delivered through a mask equipped with 4 green (light stimulus delivering) and 3 infrared (iris illuminating) diodes per eye. Optimal positioning of the light stimulus with respect to the eye was obtained by inserting calibrated photosensitive diodes into the orbits of a preserved rat head. The surface of each diode corresponded approximately to the surface of the dilated rat pupil ($50mm^2$); alternating light stimuli were delivered to each diode and the system adjusted until the light stimuli measured by both diodes were identical. This ensured that the alternated light stimulus used in animals was equal in luminance for the right eye and left eye stimulation. Each camera was equipped with cutoff filters, to avoid detection of the corneal reflection of the stimulus light produced by green diodes. Custom made software routines (Winnana software, University of Iowa) were used to analyze the recorded tracings of the pupil movements in response to light stimuli and to objectively determine the timing and amplitude of the pupil reflex responses (Figure 1)

Electroretinography

Electroretinography was used to determine physiological amplitudes and latency times of the retinal responses in 27 rats. Animals were dark adapted for at least 6h prior to each experiment. The animals were anaesthetized as previously described and the pupils dilated with 1% tropicamide and 2.5% phenylephrine. Rats were placed in a specially designed dome whose interior was completely covered with aluminum foil to obtain a Ganzfeld effect. Body temperature was maintained using a microwave-heated thermal pad. A light stimulus was delivered through the ceiling of the dome using a PS-22 stimulator (Grass-Telefactor, West Warwick, RI). To avoid any possibility of direct illumination of the eyes from the light source, the ceiling port was protected by a foil-wrapped baffle, which prevented direct dispersion of the light to the animal eyes which would result in the unequal illumination of the whole retinal field. The homogeneity of the light field at the level of the rat eyes was examined by

using a cadmium-sulphide photo-sensitive diode with a 50 mm^2 surface area (approximate surface area of the dilated rat pupil). The luminance of the Ganzfeld dome surface was measured with a J17 LumaColor TM photometer equipped with a J1803 luminance head (Tektronix, Willsonville, OR). Five different quadrants of the dome positioned in front of, dorsally, ventrally and laterally (right and left) to the rat head were evaluated. Measured luminance in all quadrants was 1600± 200 cd/m^2. Two cotton-wick electrodes, containing Ag-AgCl cells immersed in saline, were used to simultaneously record electroretinograms from both eyes. The reference electrode was positioned in the ear, while the ground electrode was placed on the back (subcutaneous). A Neuropack-MEB 7102 Evoked Potential Measuring System (Nihon-Kohden America, Foothill Ranch, CA) was used to deliver a triggered output to the flash stimulator and collect signals from both eyes. A flash ERG routine was delivered at a 0.2 Hz frequency (20 averaged signals per recording session). Isolated cone responses were recorded from previously light adapted eyes by delivering stimuli at 20 Hz (100 averaged signals per recording session). To avoid potential bias due to electrode differences, recordings were repeated with electrodes switched to the opposite eyes. The difference between right and left eyes was never above 20% of recorded amplitudes.

Intraocular pressure monitoring

Intraocular pressure was measured with a hand-held tonometer (Tonopen XL, Mentor, Norwell, MA). A calibration of the Tonopen was performed by comparing IOP results measured using invasive manometry and tonometry in 5 rats. Briefly, animals were anesthetized with 3% isoflurane, 30% nitrous oxide (NO) and 70% oxygen (O$_2$). Anesthesia was maintained with 2.0% isoflurane, 30% NO and 70% O$_2$ and body temperature was maintained using a heating pad. A cannula connected to a manometer (VR-6, Electronics for Medicine Inc., White Plains, NY) was placed in the anterior chamber of the eye. At the same time a 25-gauge needle connected to a reservoir containing 0.9% NaCl was carefully inserted in the anterior chamber. The IOP was then elevated by elevating the reservoir. Readings from the manometer and

Tonopen were obtained simultaneously and a regression line was calculated $(y=7.3029 \pm 0.7015x,\ r^2=0.78)$. To evaluate the quality of the Tonopen handling procedure we compared the IOP of the right and left eyes of all animals using a Paired t-test. Intraocular pressures were compared in awake, non-anesthetized rats, and under isoflurane or halothane anesthesia. To obtain data from awake, non-anesthetized animals we trained young rats to be calm and cooperative during handling, starting at 21 days of age. Before measuring the IOP, the corneas were anesthetized with 0.5% proparacaine hydrochloride. The Tonopen was held perpendicular to, and applied toward the center of the cornea. All valid readings which were obtained after contact with the cornea were used for the calculation of mean values for each eye. To investigate the possible influence of circadian rhythms on the IOP, measurements were obtained 6-8 hours after the start of the light cycle and 6-8 hours after the start of the dark cycle (non-anesthetized animals). To evaluate the effect of anesthesia on the IOP, 6-8 hours after start of the dark cycle animals were anesthetized with 3% isoflurane (or halothane) and 30% nitrous oxide (NO) with 70% oxygen. After rats were immobilized, the concentration of the anesthetic was decreased to 1% of isoflurane (or halothane) for 10 minutes before obtaining the IOP recordings.

Statistical analysis

Statistical analysis was performed by using Student's t-test, Paired t-test and Repeated measurement ANOVA as appropriate, with GraphPad (GraphPad, San Diego, CA) software.

Results

Evaluation of the pupil light reflex (PLR)

The reflex contraction of the pupil to a light stimulus provides an objective measure of the afferent conduction of the visual system. The amplitude, time and pattern of the PLR depend on afferent activity by the retina, conduction of the signal by the optic nerve and the properties of the pupillomotor system. Since the motor

output of the neuronal reflex of pupil contraction to light is distributed to both pupils in mammals, monitoring the pupil from just one eye is sufficient to assess any asymmetry of light input between eyes.

Computerized pupillometry confirmed existence of the consensual PLR in rats (n=27). The consensual PLR was significantly smaller for all measured parameters and at all tested light intensities when compared to the direct PLR. The constriction diameter at highest light intensity (300 cd/m^2) for direct pupillary response (light stimulus to the monitored eye) was 3.63 ± 0.15 mm (mean ± SEM) and for consensual (light stimulus opposite to the monitored eye) 3.74 ± 0.16 mm (p=0.03, Paired t-test, Fig. 2a). The maximal percent change in diameter was observed at the highest light intensity and was 13.8 ± 0.9 % (mean ± SEM) for direct PLR and 10.4 ± 0.9% for consensual PLR (Fig. 2b). The ratio (direct/consensual) was 76 ± 4 %.

The time that elapses between the onset of light and beginning of pupillary contraction is the latency time. The latency time, velocity and acceleration of the PLR depend on the functional status of the sphincter muscle and the condition of neural elements which are driving the PLR. We were interested in these parameters since our recent work showed that these parameters are dramatically affected in rat eyes after acute elevation of the intraocular pressure (15). At the highest tested light intensity maximal velocity was 3.4 ± 0.1 mm/s (mean ± SEM) for direct PLR and 3.1 ± 0.1 mm/s for the consensual PLR (p=0.0003, Paired t-test, Fig. 2c), maximal acceleration for the direct response was 2.8 ± 0.1 mm/s^2 (mean ± SEM) and for the consensual was 2.5 ± 0.1 mm/s^2 (p=0.0007, Paired t-test). The latency time for the direct PLR was 298.8 ± 5.2 ms (mean ± SEM) and for the consensual 316.1 ± 5.4 ms (p=0.0012, Paired t-test, Fig 2d).

Electroretinography

The electroretinogram is an extracellular response, which arises during retinal activity because cell membranes become hyperpolarized or depolarized. Any pathological event, which may affect electrophysiological properties of the retinal cells as sinks or sources of the current, will be detected by a change in the amplitude

and/or latency time. However, to register changes due to retinal pathology it is essential to establish standard values from healthy animals.

Electroretinographic responses (n=27) were recorded to determine physiological values of amplitudes and latency times (Fig. 3 and 4) under our experimental conditions. Under Ganzfeld illumination of 1600 ± 200 cd/m² a-wave amplitude was 207.2 ± 13 μV (mean ± SEM), with a latency of 25.6 ± 0.7 ms (mean ± SEM). The average recorded amplitude value for the b-wave under the same conditions was 554.3 ± 24.6 μV with a latency time of 21.4 ± 1.8 ms (Fig. 4a and b). Although, the rat photoreceptor layer is predominantly composed of rods, we recorded isolated cone responses (flicker ERG, 20 Hz frequency of the light stimulus, Fig.4c and d) from light adapted eyes. The flicker ERG recording (n=27) revealed averaged amplitudes of 40.6 ± 2.4 μV (mean ± SEM) with the latency time of 53 ± 1.2 ms (mean ± SEM). The usual strategy when inducing acute or chronic ocular hypertension in laboratory models is to operate on one eye, and use the opposite eye as a control. We investigated if it was possible to simultaneously record electroretinographic signals from both eyes which would be useful for the objective evaluation of a deficit after operating only on one eye. We also determined whether ERGs recorded from left and right eye differed. The simultaneous recording of the flash and flicker ERG responses did not reveal significant differences between right and left eyes in (Fig 3 and 4) in all tested parameters (amplitudes and latency times for a- and b-waves of the flash ERG, amplitudes and latency times for flicker ERG responses).

Tonometry

An estimation of the elevated IOP in rats is the only parameter for an evaluation of the success of the experimentally-induced chronic ocular hypertension. To evaluate physiological characteristics of the IOP we recorded values in 10 animals and analyzed different factors which might have significant effect on the objective evaluation of the IOP. Consistent with previous reports (4) we determined that Tonopen values must be corrected using an equation from the invasive manometry recordings since the Tonopen has a tendency to underestimate high values, and

overestimate low values of the IOP. We determined the linear regression which was subsequently used to correct Tonopen values ($y_{corrected\ value}=7.3029\pm0.7015x_{Tonopen\ value}$; Fig 5). Comparison of IOP measurements revealed no statistical difference (p>0.05, Student's t-test) between male (25.5 $\pm$ 2.2 mmHg, n=5) and female rats (24.9 $\pm$ 0.6 mmHg, n=5, Fig 6a). To confirm quality of the tonometric values we compared values obtained between left and right eyes and did not find significant differences (left eye: 25.2 $\pm$ 1.0 mmHg (mean $\pm$ SEM); right eye: 24.3 $\pm$ 1.0 mmHg; p>0.05, Paired t-test, Fig 6b). To further characterize the IOP in healthy rats we evaluated potential effect of the circadian rhythm and effect of general anesthesia (isoflurane or halothane anesthesia combined with NO). We found minor elevation of the IOP during night period, however this difference was not statistically significant (night period: 24.3 $\pm$ 1.0 mmHg; day period: 22.3 $\pm$ 1.4 mmHg; p>0.05, Paired t-test, n=10). We discovered that the same percent of halothane anesthesia had no suppressive effect on the IOP (non-anesthetized: 25.3 $\pm$ 1.0 (mean $\pm$ SEM, mmHg); 1% halothane+30% NO: 26.2 $\pm$ 1.1 (p>0.05); 1% isoflurane+30% NO: 20.1 $\pm$ 1.6 (p<0.05); Repeated measurement ANOVA, n=8, Fig 6c).

Discussion

The principal goal of this study was to characterize the pupil light reflex, electroretinogram and intraocular pressure in the healthy Brown Norway rats.

Pupillometry

We have shown that the consensual pupil response in rats is well developed but exhibits significantly different amplitudes, velocity, acceleration and latency times when compared to the direct pupil response. The PLR has been used clinically and experimentally to assess retinal and optic nerve function (16,17) and our intention is to use these techniques as non-invasive methods for continuous *in vivo* characterization of possible damage in acute and chronic hypertensive rat eyes. We recently demonstrated time-dependent decreases in the quality of the PLR in acute and chronic hypertensive rat eyes and also in mouse eyes whose episcleral veins and

trabecular meshwork were laser cauterized (15,18,19). Whiteley et al (20) described the use of the PLR monitoring as an excellent non-invasive method for the evaluation of regenerative mechanisms of retinal ganglion axons through peripheral nerve grafts connected to the pretectal olivary nucleus. Since strategies for the induction of ischemic damage due to elevated intraocular pressure usually involves surgery of one eye, monitoring of the pupil parameters of the control, non-operated eye might provide very precise information about retinal and optic nerve integrity. The velocity, acceleration and latency time of the PLR in the operated eye also reflects the status of the ischemic damage of the pupil sphincter and dilator muscles. However, in the cases where paradigm of one-operated vs. one-control eye is used, these parameters might provide information about the status of neural transmission pathways and be of the great importance for the evaluation of potential success of pharmacological or transplant-based therapeutic strategies. We confirmed presence of the consensual pupillary response in pigmented rats which was significantly smaller comparing to the direct pupillary response (76 $\pm$ 4 % of direct PLR). These results are in agreement with data from previous study (21) where the consensual PLR was reported to be 78% of the direct. Contrary to that study, Chan et al (22) described no difference between direct and consensual PLR in pigmented rats. However, threshold sensitivity data and original pupil tracings from Chan et al. study implicated difference between direct and consensual response, but statistical analysis did not reveal significance.

Electroretinography

In the present study we simultaneously monitored electroretinographic function from both eyes. The characterization of the ERG amplitudes and latency times are likely to have significant importance in the evaluation of retinal damage due to acute or chronic ocular hypertension in rat eyes. Recent reports implicated that the monitoring of the PLR is a more valuable method for estimation of optic nerve status due to presence of the PLR in mice with destroyed photoreceptors (12). Identification of the melanopsin-containing retinal ganglion cells which are light sensitive and project to pretectal olivary nucleus and suprachiasmatic nucleus (SCN) further support that

hypothesis (13,14). These studies exemplify the importance for the combined evaluation of the ERG responses and the PLR and may be a very useful strategy to precisely locate cell populations affected by ischemic damage in acute and/or chronic hypertensive rat eyes.

Tonometry

Elevated intraocular pressure is the most frequently recognized and medically (or surgically) targeted risk factor which can influence glaucoma. Due to the significance of the rat model in studying glaucoma, we have been interested to characterize physiological properties of the IOP in healthy rat eyes under experimental conditions. Other studies have used various devices for the non-invasive monitoring of the IOP in rats (5,6,7,9). In our study we used the Tonopen which is the most frequently used device for measuring IOP in rats. Our results showed relatively high levels of IOP in healthy rat eyes (24 mmHg). However these results are reasonably similar to data from previous study (23) where IOP levels in non-anesthetized Brown Norway rats were in the range from 19.3 to 31.1 mmHg. Our study did not reveal significant difference when IOP values were compared between different eyes or rats of different sexes. These results suggest that monitoring of the IOP in rat models of glaucoma might be achieved by comparing values between operated and non-operated (control) eyes. We confirmed a suppressive effect of the combined isoflurane and NO anesthesia on the IOP levels as been published before (24), but not with the combination of halothane and NO. A potential explanation for these results might be due to a very light level of anesthesia which was produced by 1% halothane and 30% NO (pinching of the tail would awaken the animal which was not the case when same percent of the isoflurane anesthesia was used). Multiple reports described variations in the IOP rates due to the diurnal cycle (4,8). While we detected some trend of elevation of the IOP in animals during the night cycle, statistical analysis did not reveal significant difference between IOP values during night and day periods under our experimental conditions. Potential explanation might be small size of our experimental group (n=10) which could not provide sufficient statistical power.

We have evaluated several physiological parameters which are likely to provide important information about the development of functional deficits in chronic hypertensive eyes. While numerous pharmacological studies have examined the end-product of ischemic damage to the retina and optic nerve - histopathological examination and cell count, monitoring of the PLR and ERG are objective and non-invasive techniques which might provide novel information about dynamics of retina and optic nerve pathology *in vivo* in experimental rat models of the ocular hypertension.

Acknowledgments

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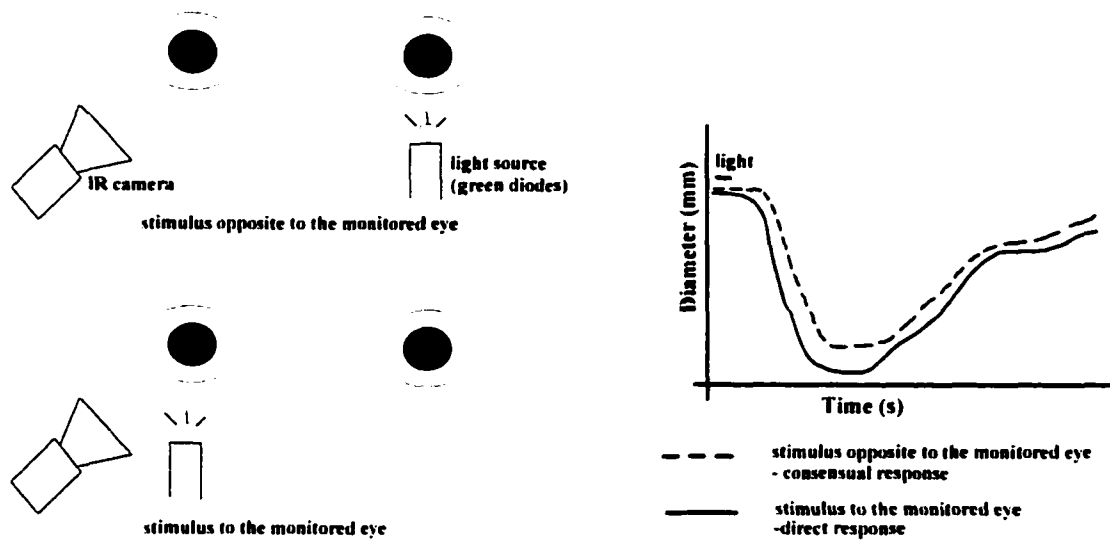
Figure 1. Schematic presentation of the PLR monitoring technique. One chanel computerized pupillometer was used to record the movement of one pupil while the stimulus light was alternated between the right and left eye.

Direct response – light stimulus delivered to monitored eye

Consensual response – light stimulus delivered to the opposite (non-monitored) eye
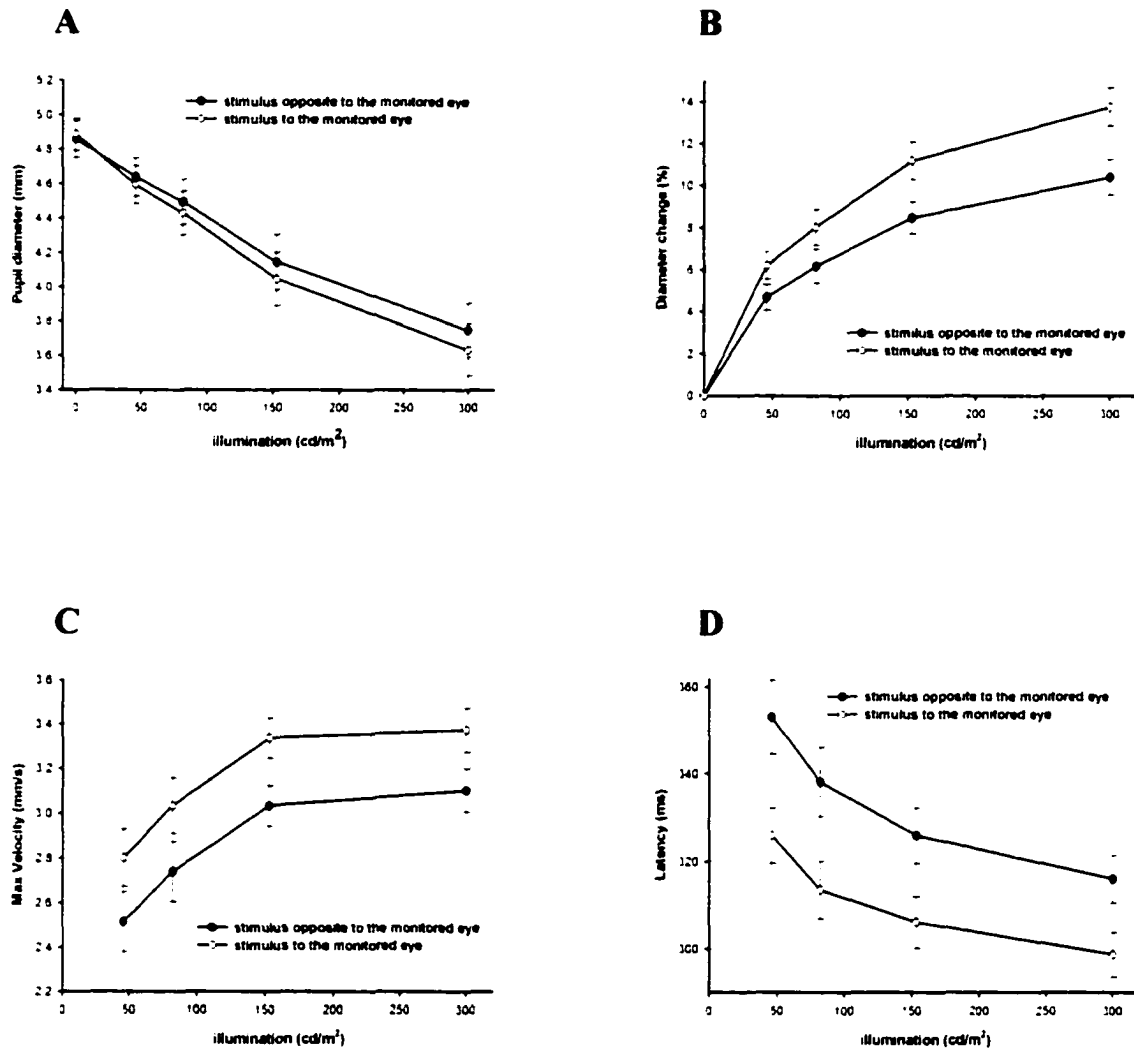
Figure 2. Characterization of the PLR. The absolute amplitude (A) and the percent of the pupil diameter change (B) of the pupillary response when light stimulus was delivered opposite to the monitored eye (consensual response) were significantly smaller compared to the direct response (p=0.03). The velocity (p=0.0003) of the pupil constriction was significantly slower during the consensual pupillary response (C). The constriction of the pupil is significantly delayed (p=0.001) during consensual light stimulation (D).
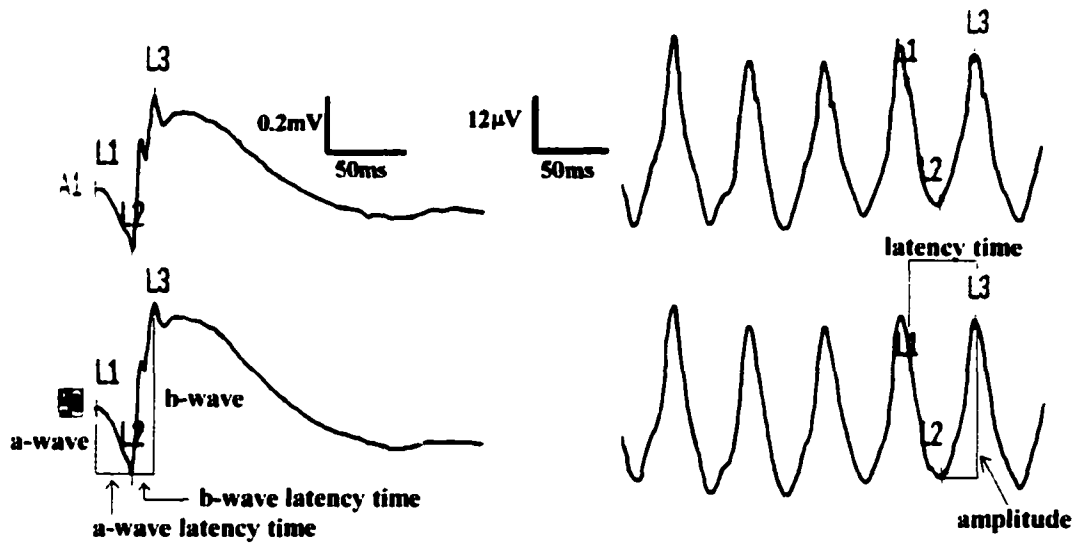
Figure 3. Original tracings for the flash and flicker ERG. Amplitudes and latency times for the a-wave (L1-L2) and b-wave (L2-L3) of the flash ERG and amplitudes (L2-L3) and latency times (L1-L3) of the flicker ERG do not differ between eyes. Channel A1 represents tracings from the left eye, while channel A2 represents simultaneous tracings from the right eye.
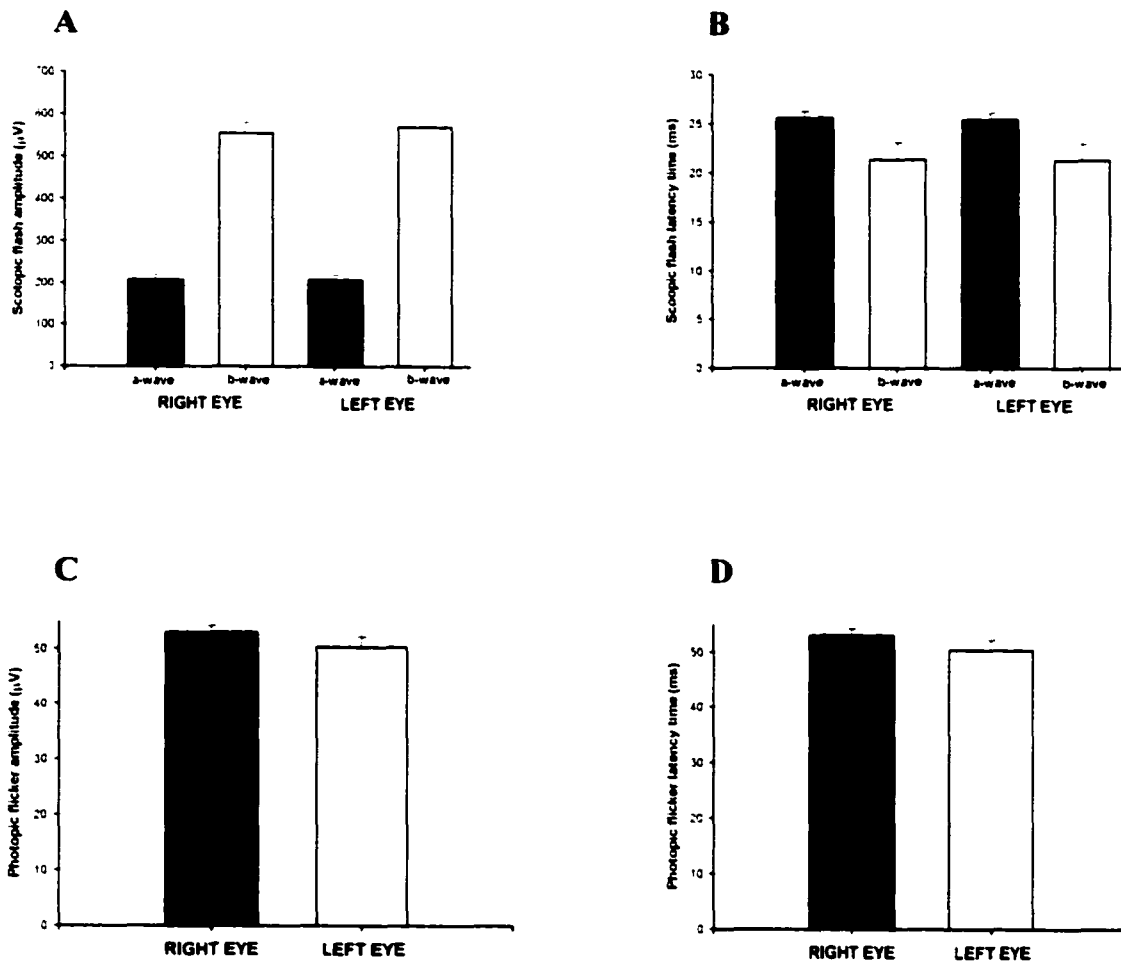
Figure 4. Characterization of the electroretinographic responses. There is no significant difference between tested eyes in amplitudes and latency time for the scotopic flash (A,B) and photopic flicker (C,D) ERG (p>0.05).
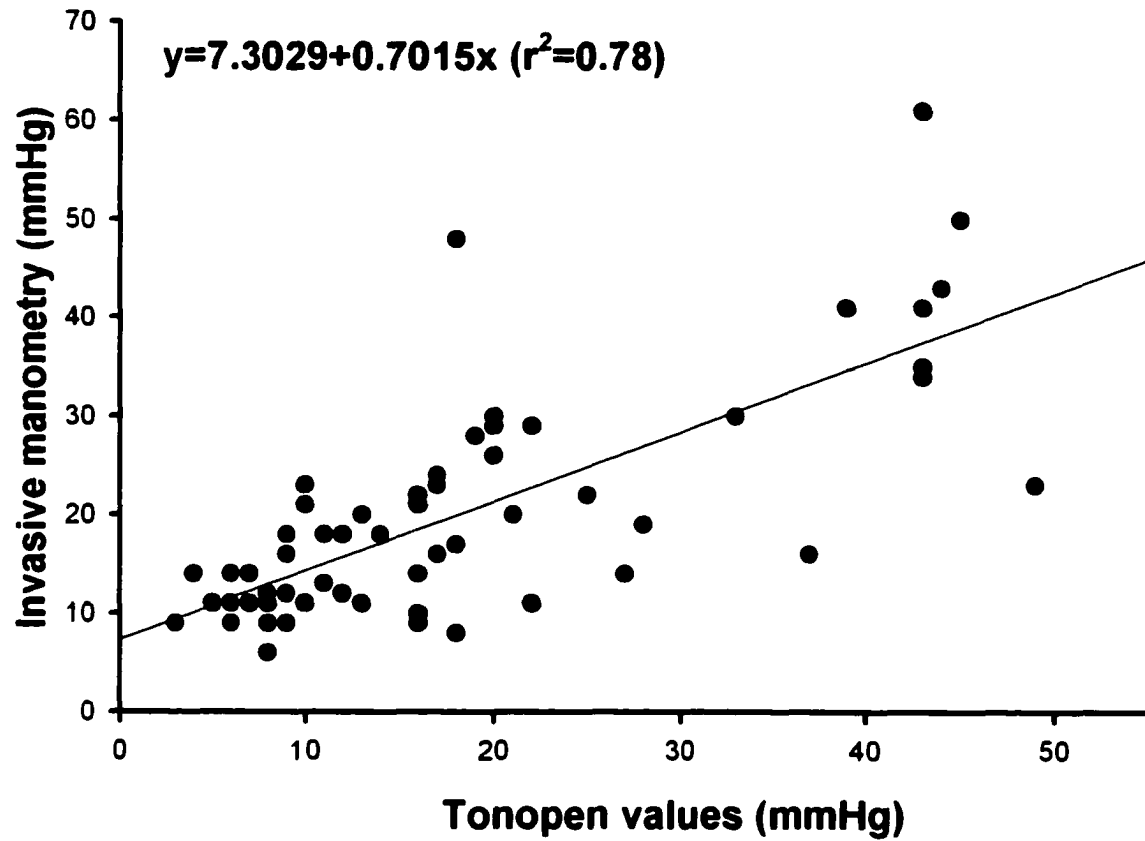
$$y=7.3029+0.7015x \ (r^2=0.78)$$

Figure 5. Tonopen values reasonably well correlate with invasive manometry recordings of IOP (n=5)
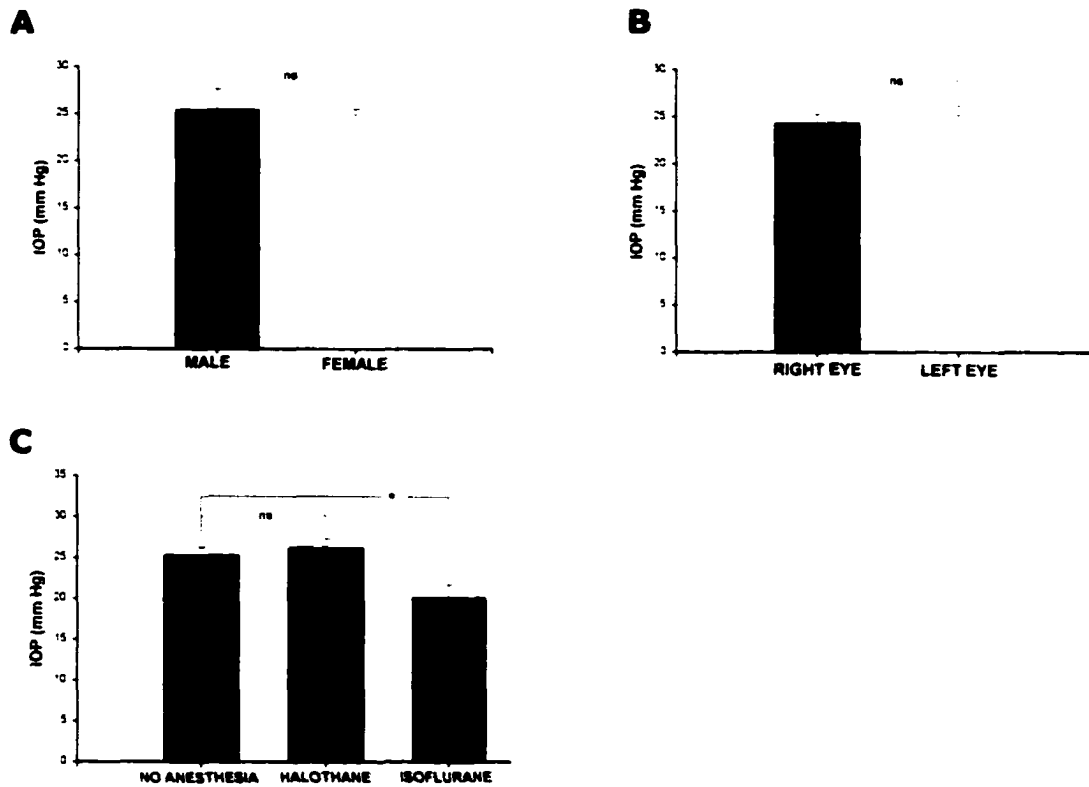
Figure 6. Characterization of the IOP. Tonopen measurement of the IOP revealed no significant difference between male and female animals (A) or right and left eyes (B). While isoflurane anesthesia suppressed IOP values (p<0.05), halothane anesthesia did not have significant effect (C).

CHAPTER 3 – FUNCTIONAL CHARACTERIZATION OF RETINA AND OPTIC NERVE AFTER ACUTE OCULAR ISCHEMIA IN RATS

Sinisa D. Grozdanic, Donald S. Sakaguchi, Young H. Kwon, Randy H. Kardon and Ioana M. Sonea

Abstract

Purpose: To functionally characterize the status of the rat retina and optic nerve after acute elevation of the intraocular pressure (IOP) and determine the dynamics of the pathological changes in the ischemic retina and optic nerve.

Methods: Retinal ischemia was induced in rats by acutely increasing the IOP (110 mmHg/60 minutes). Direct and consensual pupil light reflexes (PLR) were recorded from the unoperated eye, and electroretinograms (flash and flicker ERG) were recorded from the operated and control eyes preoperatively and postoperatively. Amplitudes and latency times were calculated for each recording session.

Results: Preoperative values for the PLR_{ratio} (ratio=consensual/direct PLR) were 76.7$\pm$2.6 (mean$\pm$SEM; %). 24h postoperatively the PLR_{ratio} was 15.2$\pm$12.8, 10 days postoperatively 11.6$\pm$9.8, 20 days postoperatively 26.5$\pm$8.0 and 28 days postoperatively PLR_{ratio} was 33.27$\pm$9.3. However, at day 35 the PLR was significantly recovered when compared to the 24h postoperative values (PLR_{ratio}=41.1$\pm$7.3%, p<0.01, Repeated measures ANOVA). 42 days after surgery the PLR started to decrease once again in the operated eyes (PLR_{ratio}=28.7$\pm$5.9). Electroretinographic amplitudes (full field flash ERG) followed a similar pattern. Cone responses (flicker ERG) were measured 42 days postoperatively and revealed defects in operated eyes (control eyes: 46.6$\pm$2.9μV, operated eyes: 3.4$\pm$1.7μV). Histological analysis revealed ischemic damage to all retinal layers with the primary defects localized to central retina.

Conclusions: Acute ocular ischemia causes significant decrease in retinal function as measured by pupillary light reflex and electroretinogram although over time the rat retina and optic nerve show partial regain of function.

INTRODUCTION

Ischemic insults to the retina and optic nerve are frequently observed in glaucoma, acute ocular hypertension, diabetic retinopathy, hypertension and vascular occlusion and giant cell arteritis and can lead to serious perturbation of neuronal and glial retinal elements and can ultimately lead to blindness. Traditionally, retinal damage due to ischemia has been considered as a potentially incurable condition in humans and animals due to the lack of the regenerative capacity of the mammalian central nervous system. Few reports described the recovery of visual function in human patients who suffered severe retinal ischemia after central retinal artery occlusion[1,2], while multiple reports confirmed that severe ischemic events were followed by almost complete and irreversible loss of visual function[3,4,5,6]. To understand pathological mechanisms occurring in retinal and optic nerve hypoxia it is essential to develop strategies for the continuous and objective monitoring of visual function in easily accessible and reproducible animal models. One of the most frequently used models for the investigation of molecular mechanisms, and potential therapeutic strategies for retina and optic nerve ischemia has been a rat model of acute elevation of the intraocular pressure, characterized by an ischemia-reperfusion injury. Numerous studies have identified molecular mediators with neurotoxic properties during ischemia-reperfusion injury of the rat retina and optic nerve, such as excitatory amino acids[7], free oxidative radicals[8,9] and cytokines[10,11]. However, quantitative analysis of the damage has been achieved only by end stage counting of the cells in different retinal layers[12] or retrograde fluorescent labeling of retinal ganglion cells[13]. Relatively few studies have carried out a functional analysis of the retina after ischemia-reperfusion injury using electroretinography for a prolonged period of time[14,15] and to our knowledge, there is only one published study, which actually documented function of the retina and optic nerve in rats after ischemia-reperfusion injury[16]. While morphological studies provide important information about the numbers of surviving cells, only electrophysiological studies offer precise information about the functional status of the retina and the dynamics of ischemic injury. The goal of this study was to functionally characterize the electrophysiological

status of the retina and optic nerve after ischemia-reperfusion injury in rats over time (6 weeks), by using electroretinography and computerized pupillometry. Also we were interested to observe the dynamics of the functional deficit *in vivo* and correlate electrophysiological changes with histological and morphometric analysis of the retina from the operated eyes.

MATERIALS AND METHODS

Induction of retinal ischemia by elevated intraocular pressure (IOP)

All animal studies were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and procedures were approved by the Iowa State University Committee on Animal Care. A previously published procedure to generate an ischemia-reperfusion insult in rats, with slight modifications, was used[17]. Briefly, adult Brown Norway rats (n=11) were initially anesthetized with 4% halothane, 30% nitrous oxide (NO) and 70% oxygen (O_2). Anesthesia was maintained with 2.5% halothane, 30% NO and 70% O_2 and body temperature was maintained using a heating pad. The pupils were dilated with topical 2.5% phenylephrine hydrochloride and 1% tropicamide. After topical instillation of 0.5% propracaine hydrochloride, the anterior chamber was cannulated with a 25-gauge needle connected to a reservoir containing 0.9% NaCl. The intraocular pressure in experimental eyes was controlled by the height of the reservoir to maintain a pressure of 110 mmHg for 60 minutes. Retinal ischemia was confirmed by the blanching of the iris and retinal circulation. At the end of the period of elevated IOP, the needle was removed and reperfusion of the retinal vasculature was confirmed by ophthalmoscopic examination. To prevent potential infection, antibiotic ointment (neomycin + polymyxin B + bacitracin; Bausch & Lomb Pharmaceuticals Inc; Tampa, FL 33637) was applied topically after the procedure.

Computerized pupillometry

The pupil light reflex was evaluated with a custom-made computerized pupillometer (University of Iowa, Iowa City, IA) preoperatively and on days 1, 10, 20, 28, 35 and 42 postoperatively. Animals were anesthetized initially with 4% halothane, 30% NO and 70% O_2. A light plane of anesthesia was maintained with 1% halothane, 30% NO and 70% O_2 to avoid suppression of the pupil light reflex response as detected with the use of higher doses of anesthetic. The computerized pupillometer was attached to 2 infrared sensitive CCTV video cameras for simultaneous visual monitoring of both pupils. However, one channel computerized pupillometer was used to record the movement of the pupil from the control (non-operated) eye, while the stimulus light was alternated between the control and operated eye. The stimuli were delivered through a mask equipped with 4 green (light stimulus delivering) and 3 infrared (iris illuminating) diodes per eye. Optimal positioning of the light stimulus with aspect to the eye was obtained by inserting calibrated photosensitive diodes into the orbits of a preserved rat head. The surface of each diode corresponded approximately to the surface of the dilated rat pupil ($50mm^2$); alternating light stimuli were delivered to each diode and the system adjusted until the light stimuli measured by both diodes were identical. This ensured that the alternated light stimulus used in animals was equal in luminance for the right eye and left eye stimulation. Each camera was equipped with cutoff filters, to avoid detection of the corneal reflection of the stimulus light produced by green diodes. Custom made software routines (Winnana software, University of Iowa) were used to analyze the recorded tracings of the pupil movements in response to light stimuli and to objectively determine the timing and amplitude of the pupil reflex responses. Since the opposite, non-operated eye was used as a control at the same testing time, any defect, which was caused by ischemia-reperfusion injury, was monitored longitudinally over time (Figure 1)

CONTROL   OPERATED

Operated eye stimulated but control eye
monitored - consensual response

CONTROL   OPERATED

Control eye stimulated and monitored
- direct response

lat-oper
lat-cont

amplitude-oper

velocity-oper

velocity-cont

amplitude-cont
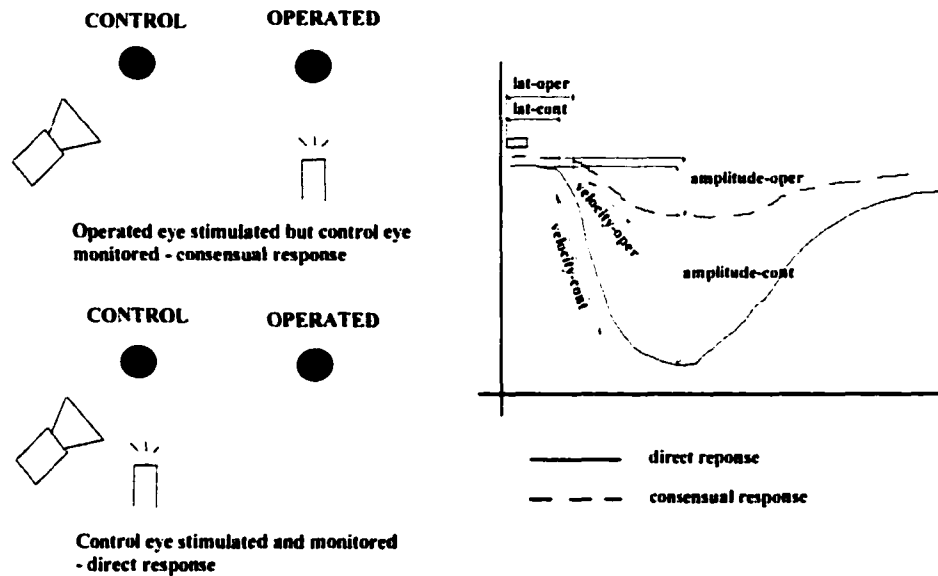
——————— direct response
— — — consensual response

FIGURE 1 Schematic presentation of the PLR monitoring technique. One channel computerized pupillometer was used to record the movement of the pupil from the control (non-operated) eye while the stimulus light was alternated between operated and non-operated eye. Software routines were used to analyze the recorded tracings of the pupil movements in response to light stimuli and to objectively determine the latency time (lat-cont and lat-oper), maximal velocity (velocity-cont and velocity-oper) and amplitudes of responses for control (cont) and operated (oper) eyes.

Electroretinography

To quantitate post-ischemic recovery after acute elevation of the IOP, electroretinography was performed 48h, 12d, 22d, 32d and 42d post-operatively. Animals were dark adapted for at least 6h, anesthetized as described previously and the pupils dilated with 1% tropicamide and 2.5% phenylephrine. The animals were

placed in a specially designed dome whose interior was completely covered with aluminum foil to obtain a Ganzfeld effect. Body temperature was maintained using a microwave-heated thermal pad (R.G. Barry Corporation, Pickerington, OH). A light stimulus was delivered through the ceiling of the dome using a PS-22 stimulator (Grass-Telefactor, West Warwick, RI). To avoid any possibility of direct illumination of the eyes from the light source, the ceiling port was protected by a foil-wrapped baffle, which prevented direct dispersion of the light to the animal eyes which would result in the unequal illumination of the whole retinal field. The homogeneity of the light field at the level of the rat eyes was examined by using a cadmium-sulphide photo-sensitive diode with a 50 mm^2 surface area (approximate surface area of the rat dilated pupil). The luminance of the Ganzfeld dome illuminated surface was measured with a J17LumaColorTM photometer equipped with a J1803 luminance head (Tektronix, Willsonville, OR) in 5 different quadrants of the dome positioned in the front, dorsally, ventrally and laterally (right and left) from the rat head. Measured luminance in all quadrants was 1600$\pm$ 200 cd/m^2. Two cotton-wick electrodes, containing Ag-AgCl cells immersed in saline, were used to simultaneously obtain signals from both eyes. The reference electrode was positioned in the ear, while the ground electrode was on the back (subcutaneous). A Neuropack-MEB 7102 Evoked Potential Measuring System (Nihon-Kohden America, Foothill Ranch, CA) was used to deliver a triggered output to the flash stimulator and collect signals from both eyes. A flash ERG routine was delivered at a 0.2 Hz frequency (20 averaged signals per recording session). Isolated cone responses were recorded from previously light adapted eyes by delivering stimuli at 20 Hz (100 averaged signals per recording session). To avoid potential bias due to electrode differences, recordings were repeated with electrodes switched to opposite eyes. The difference between right and left eyes preoperatively, was never above 20% of recorded amplitudes.

Histological examination

Forty nine days postoperatively, rats were deeply anesthetized with a high dose of phenobarbitol (100mg/kg) and perfused intracardially with ice-cold heparinized

saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Optic nerves (dissected 1mm posterior to the sclera) and eye globes were postfixed, paraffin embedded, and 7□m thick sections of the retina were collected onto poly-l-lysine coated glass slides, and stained with hematoxylin and eosin while transverse sections of the optic nerves were stained with 1% methylene blue. Tissue sections were examined with a Nikon Microphot FXA photomicroscope (Nikon Corporation, New York, NY). Images were captured using a Kodak Megaplus Camera (Model 1.4; Kodak Corp, San Diego, CA) connected to a MegaGrabber Framegrabber in a Macintosh 8100/80 AV computer (Apple Computer, Cupartino, CA) using NIH Image 1.58 VDM software (W.Rasband, NIH, Bethesda, MD). The morphometric analysis of the retina was performed with slight modifications of a previously described procedure[12]. Briefly, sections of the retina at the level of the optic nerve head were prepared and each section was divided into 12 optic fields (6 fields of the central retina and 6 fields of the peripheral retina). Within each field we measured the thickness of the inner plexiform (IPL), inner nuclear (INL) and outer nuclear layer (ONL) by measuring 4 values per each layer and calculating averaged values per section. Measurements of retinal layer thickness were performed using a calibrated scale of the objective of the light microscope.

Statistical analysis

Statistical analysis was performed by using Student's t-test, Paired t-test, Repeated measurement ANOVA and Kruskal-Wallis nonparametric test (as indicated in the text) with the GraphPad (GraphPad, San Diego, CA) software.

RESULTS

Assessment of optic nerve function using the pupil light reflex (PLR)

The measurement of the PLR was used as an assay to investigate possible changes in retina and optic nerve function following acute elevation of the IOP. The reflex contraction of the pupil to a light stimulus provides an objective measure of the

afferent conduction of the visual system. Damage to the retina or optic nerve reduces the amplitude of the pupil contraction to light. Since the motor output of the neuronal reflex of pupil contraction to light is distributed to both pupils, monitoring the pupil from just the non-operated eye is sufficient to assess any asymmetry of light input between the operated and non-operated eye. Because the fellow eye was used as a control at the same testing time, any defect, which was caused by acute elevation of the IOP, was monitored longitudinally over time. All pupil parameters were calculated by comparing values from the operated and non-operated (control) eyes in the same animal. Evaluation of the PLR revealed defects of all components (amplitude, latency time and maximal velocity) in all operated animals (Fig. 2 and 3).

Preoperative values for the PLR_{ratio} (ratio=consensual/direct PLR, Fig. 1a) were 76.7$\pm$2.6 (mean$\pm$SEM; %). 24h postoperatively the PLR_{ratio} was 15.2$\pm$12.8, 10 days postoperatively 11.6+9.8, 20 days postoperatively 26.5$\pm$8.0 and 28 days postoperatively the PLR_{ratio} was 33.27$\pm$9.3. However, at day 35 the PLR displayed significant recovery when compared to the 24h postoperative values (PLR_{ratio}=41.1$\pm$7.3%, $p<0.01$, Repeated measures ANOVA with Bonferroni posttest). Forty two days after surgery the PLR started to decrease once again in the operated eyes (PLR_{ratio}=28.7$\pm$5.9) and was not significantly different compared to the condition 24h postoperatively ($p>0.05$, Repeated measures ANOVA with Bonferroni posttest). The PLR_{ratio} never recovered to the preoperative values ($p<0.0001$, Repeated measures ANOVA) (Fig. 2).
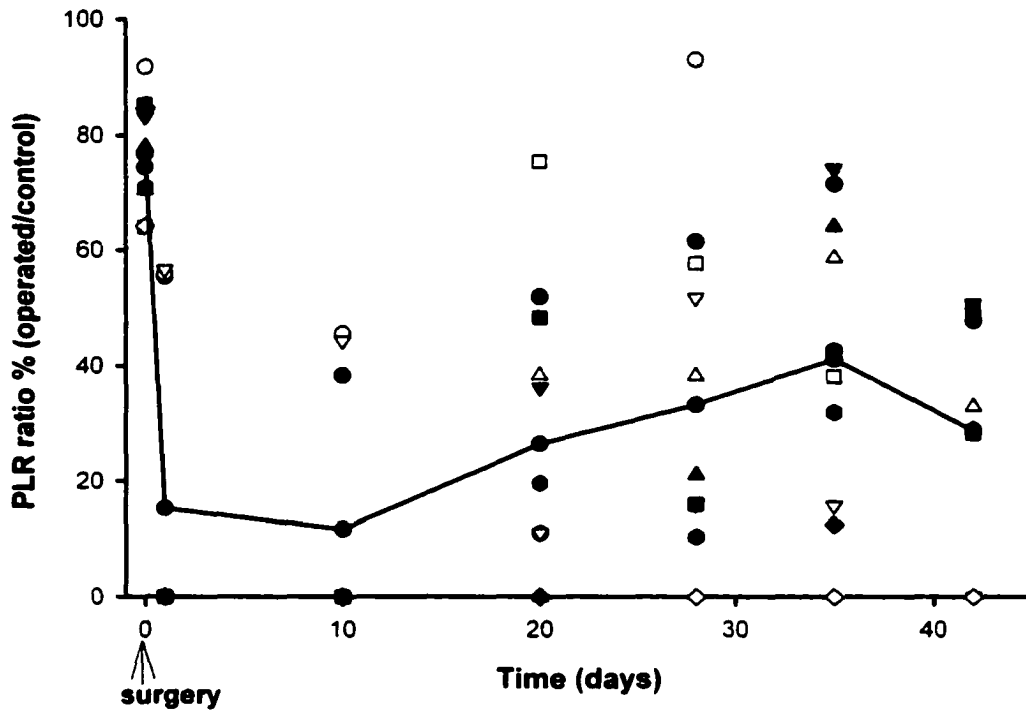
FIGURE 2 The dynamics of the PLR recovery. The PLR was detectable in 3 of 11 operated animals 24 hours after surgery. However, at day 35 the PLR displayed significant recovery when compared to the 24h postoperative values ($p<0.01$, Repeated measures ANOVA with Bonferroni posttest). Forty two days after surgery the PLR started to decrease once again in the operated eyes and was not significantly different compared to the condition 24h postoperatively ($p>0.05$, Repeated measures ANOVA with Bonferroni posttest). Black line connects mean values for each group.

We analyzed latency time and velocity of the PLR (latency time - time from onset of stimuli to the start of the pupil constriction, velocity – maximal speed of the pupil constriction) as parameters of the retina and optic nerve function after ischemic

insult and detected significant decrease of the PLR velocity (p<0.0004, Kruskal-Wallis nonparametric test) and increase in the latency time (p<0.004, Kruskal-Wallis nonparametric test) after stimulation of the operated eye compared to the stimulation of the non-operated (control) eye (Fig. 3).
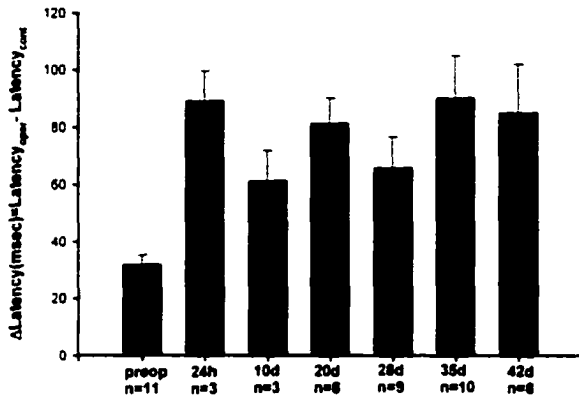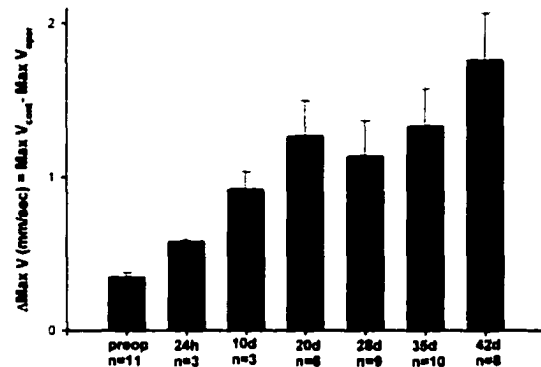

Figure 3A

Figure 3B

FIGURE 3. Bar histograms representing combined data for operated eyes which had detectable pupil responses when operated eye was stimulated and control (non-operated) eye monitored. A) While mean levels of the ratio between consensual and direct pupillary response showed recovery of function with a peak at 35 days, latency time defects (statistical comparison to preoperative values revealed significant difference p<0.05, Kruskal-Wallis nonparametric test with Dunn's posttest) were detected at 20 and 35 days postoperatively but not at 28 days postoperatively (p>0.05). B) The maximal velocity deficit followed a similar pattern.

The consensual latency time was greatly prolonged following surgery. By subtracting latency time values (latency time$_{oper}$ - latency time$_{cont}$) we determined next values: preoperative = 31.8$\pm$3.4 (mean$\pm$SEM; msec, n=11), 24h postoperatively 88.9$\pm$10.7 (n=3), at 10 days 61.1$\pm$10.7 (n=3), at 20 days 81.3$\pm$8.8 (statistical comparison to preoperative values revealed significant difference p<0.05, Kruskal-Wallis nonparametric test with Dunn's posttest, n=8), at 28 days 65.7$\pm$10.9 (difference

was not statistically significant, p>0.05, n=9), at 35 days 90 ± 15 (p<0.05, n=10) and 42 days after surgery 85 ± 17 (p<0.05, n=8). Calculation of the velocity parameters (velocity$_{cont}$ – velocity$_{oper}$) revealed next values: preoperative 0.34 ± 0.03 (mean$\pm$SEM; mm/sec), 24h postoperative 0.58 ± 0.02 (n=3), at 10 days 0.92 ± 0.11 (n=3), at 20 days 1.26 ± 0.23 (statistical analysis revealed significant difference comparing to preoperative values: p<0.01, Kruskal-Wallis nonparametric test with Dunn's posttest, n=8), at 28 days 1.13 ± 0.22 (p<0.05, n=9), at 35 days 1.33 ± 0.24 (p<0.01, n=10) and 42 days after surgery 1.76 ± 0.31 (p<0.001, n=8).

Analysis of amplitude parameters (Fig. 4) revealed loss of function in eight of eleven operated rats (PLR was not detectable when operated eye stimulated) 24h and 10 days after surgery. However, 20 days after the ischemic event the PLR function was detected in eight of eleven operated rats, although latency time and maximal velocity did not show signs of recovery. The peak in the PLR recovery was detected 35 days after surgery when 10 of 11 operated eyes had detectable activity, however 7 days later pupil parameters declined leaving 8 of 11 operated eyes with detectable responses. One animal never recovered pupil or electroretinographic responses in the operated eye after acute elevation of the IOP. Since we are monitoring function of the healthy non-operated eye while stimulating the operated eye, we excluded the potential effect of ischemia on the iris sphincter muscle as a source of the deficit in analyzed pupil responses.
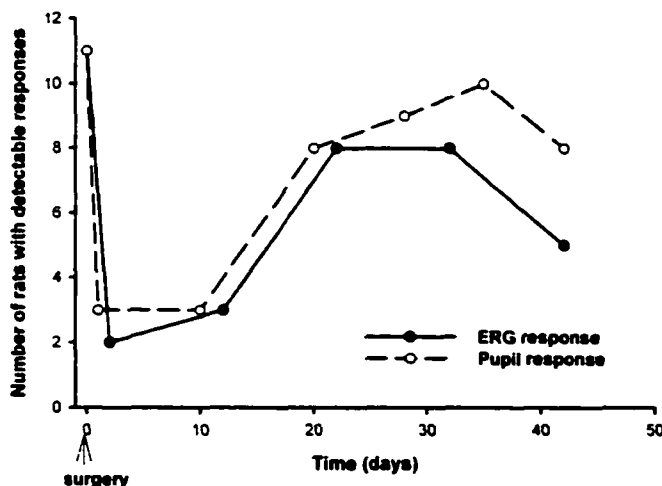

FIGURE 4 Recovery dynamics –ERG and PLR. Operated eyes showed the peak of recovery at day 35 post-operatively for PLR responses (10 of 11 operated eyes) and day 22 and 28 for ERG responses (8 of 11 operated eyes).

Electroretinography

Electroretinography was used as an objective method to evaluate the functional status of the inner and outer retina with the exception of the retinal ganglion cells. To evaluate the effect of the acute elevation of the IOP on different populations of photoreceptors we used full field flash and flicker ERG. Acute elevation of the IOP caused a dramatic reduction of electroretinographic activity (Fig. 4,5,6).

The dynamics of the recovery (number of animals with detectable signals) revealed the peak of recovery of the ERG and pupil responses at 22 and 32 days post-operatively (Fig 4). Rats with a- and/or b-wave amplitudes smaller than $3\mu V$ (noise level) were considered as animals without detectable signal. Electroretinographic data expressed as a ratio between control and operated eyes revealed higher values of a-wave amplitudes at all tested time points compared to b-wave values, but without statistical significance ($p>0.05$, Paired t-test) (Fig. 5a): 48h postoperative: a-wave=2.9 ± 1.4 (mean$\pm$SEM, %), b-wave=2.6 ± 1.1; 12 days postoperative: a-wave=4.5 ± 1.6, b-wave=$0.7\pm0.$; 22 days postoperative: a-wave=5.4 ± 1.2, b-wave=4.1 ± 1.1, 32 days postoperative: a-wave=8.1 ± 2.2, b-wave 4.8 ± 1.1 and 42 days postoperative: a-wave=1.89 ± 0.6, b-wave=1.35 ± 0.42.

Complete absence of activity was detected in 9 of 11 operated eyes after 48h of reperfusion period, while amplitudes of the a-wave (45 ± 24; mean$\pm$SEM;μV) and b-wave (95 ± 10) in the remaining 2 operated eyes were dramatically reduced compared to control (non-operated) eyes (a-wave: 236.46 ± 14; b-wave: 595.117 ± 37.3). Ten days after acute elevation of the IOP, amplitudes further decreased (a-wave: 10 ± 7; b-wave: 3.3 ± 2.3), but an ERG was detectable in 3 operated eyes. Twenty two and 32 days post-operatively, ERG responses were detected in 8 operated eyes (22d: a-wave=39.6 ± 13.1, b-wave=44.1 ± 22.8; 32d: a-wave=37.8 ± 5.7, b-wave=45.1 ± 16). Forty two days after surgery ERG amplitudes were detectable in 5 operated eyes (a-wave=12.5 ± 2, b-wave=31.3 ± 1).

The latency time (Fig 5b) was significantly increased compared to control, non-operated eyes at 22 and 32 days postoperatively (22d: a-wave latency=39.6 ± 13.1 ($p=0.02$, Paired t-test), b-wave latency=44.1 ± 22.8 ($p=0.04$); 32d: a-wave

latency=37.8±5.7 (p=0.007), b-wave latency=45.1±16 (p=0.03). The latency time for a- (12.5±2) and b-wave (31.3±1) were not significantly decreased (p>0.05) compared to control eyes at 42 days postoperatively (the last recorded time point) but the number of operated eyes which had detectable ERG signals decreased to five.
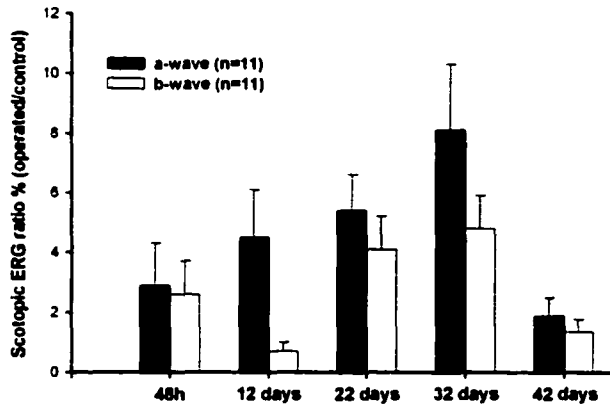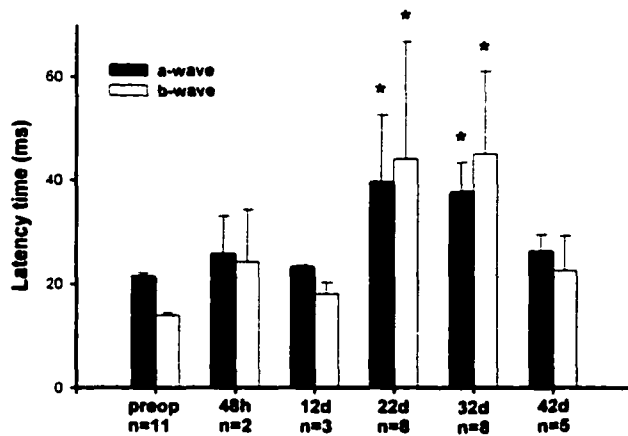

Figure 5A

FIGURE 5 The dynamics of the full field flash ERG amplitude recovery (A) followed dynamics of the PLR recovery (ratio for rats w/o detectable response was calculated as zero), however latency time (B) at the peak recovery time points were significantly longer comparing to the control, non-operated eyes (32d: a-wave latency p=0.02, b-wave latency p=0.03; 42d: a-wave latency p=0.007, b-wave latency p=0.024, Paired t-test).

Figure 5B

Isolated cone response (flicker ERG) revealed existence of recorded activity in 3 of 11 operated eyes 42 days after surgery with average amplitude of 34.3±5.3 μV and latency time of 52.7±0.7 ms for control eyes and 3.9±1 μV and latency time of 61.3±0.1 ms for operated eyes. Statistical analysis revealed significant amplitude (p=0.0069, Paired t-test) and latency time (p=0.0012, Paired t-test) defects for the operated eyes compared to control eyes.
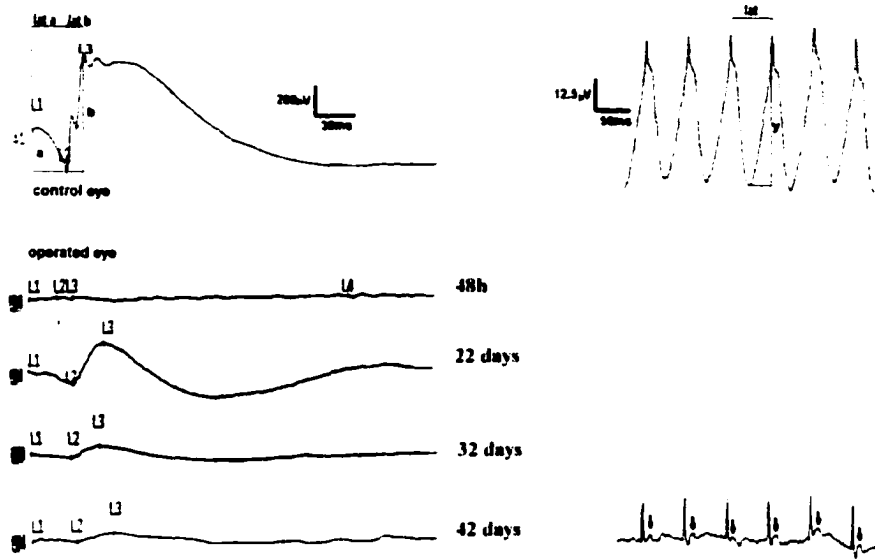
FIGURE 6 Electroretinographic characteristics from one animal with acute elevation of the IOP which displayed detectable ERG responses 42 days postoperatively. The acute elevation of the IOP caused dramatic reduction in the amplitudes of a-wave and b-wave in operated eyes (lat a – latency time for a-wave, a-amplitude of a-wave; lat b –latency time for b-wave, b-amplitude of b-wave). The recording of the isolated cone response (flicker ERG) revealed barely detectable amplitudes in the operated eyes 42 days after surgery (lat-latency time, y–amplitude of the response).

Histological analysis

Our electrophysiological studies revealed a dramatic reduction of the function in all retinal layers and thus, we performed a histological analysis to determine if functional defects were in agreement with morphological appearance of the operated eyes.

Light microscopy analysis revealed vacuoalization of the optic nerve axons (7a,b), complete loss of inner retinal layers at the level of central retina (7e,f) and significant thinning of inner retina structures at the periphery (7i,j). Dramatic reduction

of the cellular organization of the central retina and reduced thickness compared to control, non-operated eyes suggests that ischemic effect damaged both the inner and outer retina structure (7e,f).
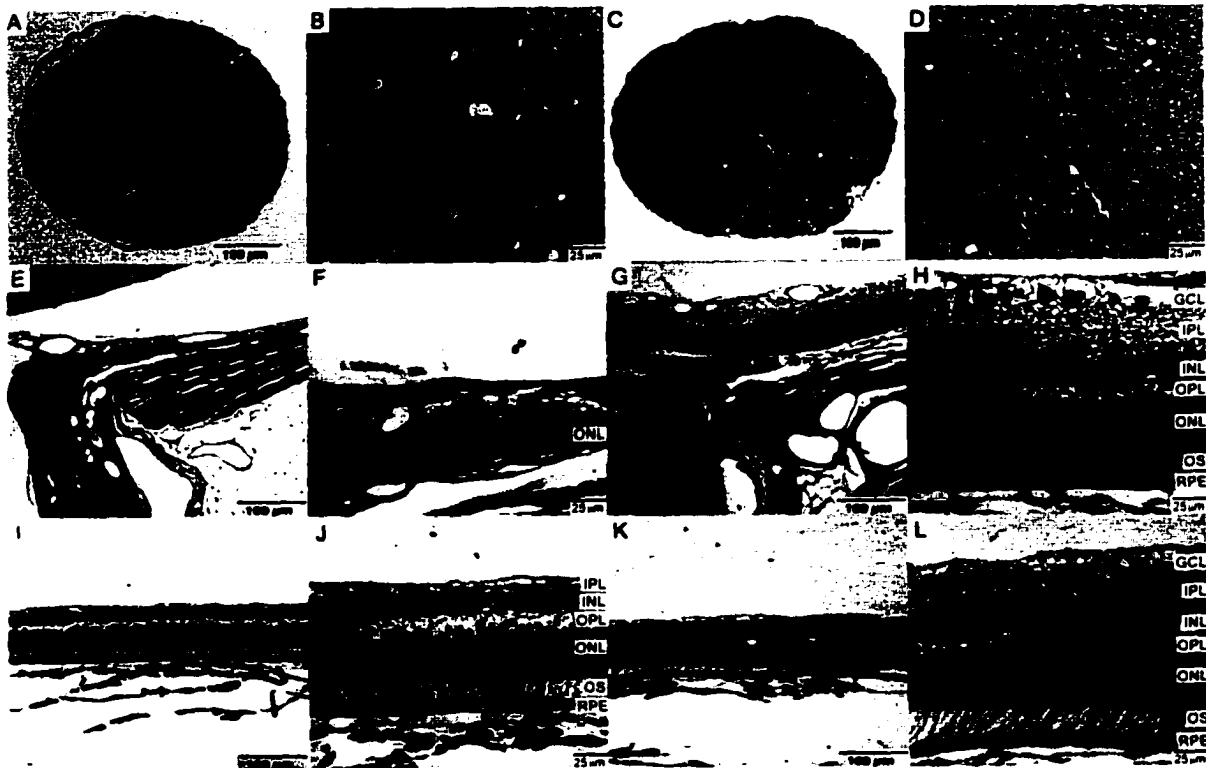

FIGURE 7 Histological examination of the operated eyes revealed optic nerve degeneration (**A** – 100X magnification, **B** – 400X, **C** and **D** – non-operated eye), complete loss of central retina architecture (**E** – 100X, **F** – 200X, **G** and **H** – non-operated eye) and dramatic reduction of the inner plexiform and inner nuclear layers of the peripheral retina (**I** -100X, **J** - 400X, **K** and **L** – non-operated eye).

For the analysis we compared measurement of the thickness of the outer nuclear layer (ONL), inner nuclear layer (INL) and inner plexiform layer (IPL) between the control and operated eyes at central and peripheral retinal locations (Table 1). Retinal thickness, expressed as a ratio (operated/control), for peripheral retina was:

ONL=97.3±8.5 (mean±SEM;%), INL=67.6±5.6, IPL=36.4±3.6 and for central retina: ONL=23.9±6.2, INL=5.5±1.8, IPL=16.5±5.1. The ONL, INL and IPL absolute values were measured and showed significant reduction in thickness (Table 1): ONLcontrol=50.2±5.2 (Ave±SEM; μm), ONLoperated=12.8±4.6 (p=0.0001, Paired t-test); INLcontrol=50.2±5.2, INLoperated=16.6±1.1 (p<0.0001, Paired t-test); IPLcontrol=34.2±1.1, IPLoperated=1.8±0.6 (p<0.0001, Paired t-test). The photoreceptor layer was completely devoid of outer segments. Organization of the retinal layers was preserved at the retinal periphery with no reduction in the thickness of the ONL (ONLcontrol=38.8±4.4, ONLoperated=38.1±5.2 (p=0.47, Paired t-test)). However the thickness of the INL and IPL were dramatically reduced in the peripheral retina compared to control, non-operated eyes: INLcontrol=12.1±0.96, INLoperated=7.9± 0.6 (p=0.0006, Paired t-test); IPLcontrol=25.3±1.5, IPLoperated=8.9±0.9 (p<0.0001, Paired t-test).

RETINAL LAYER	CENTRAL RETINA		PERIPHERAL RETINA	
	CONTROL	OPERATED	CONTROL	OPERATED
ONL(μm)	50.2 ± 5.2	12.8 ± 4.6*	38.8 ± 4.4	38.0 ± 5.2 ns
INL (μm)	16.5 ± 1.1	2.6 ± 0.9*	12.1 ± 1.0	7.9 ± 0.6*
IPL (μm)	34.2 ± 1.1	1.8 ± 0.6*	25.3 ± 1.5	9.0 ± 1.0*

TABLE 1 Morphometric analysis of the retina revealed significant reduction of the thickness of ONL, INL and IPL layers in operated eyes with the exception of the ONL in the peripheral retina.
(* p<0.001; ns = not significant (p>0.05); Paired t-test).

Functional monitoring and histological analysis revealed dramatic effects of the ischemia-reperfusion injury on retinas and optic nerves of the operated eyes. Histological analysis revealed the major impact of injury on all retinal layers in the central retina and the inner layers of the peripheral retina.

DISCUSSION

The experimental approach used in this study allowed us to precisely monitor dynamics of ischemic retinal damage for prolonged periods of time (up to 6 weeks) after acute elevation of the IOP. Retinal ischemia-reperfusion injury was induced by increasing intraocular pressure to 110 mmHg for a period of 60 minutes by inserting a cannula connected with a 0.9% NaCl reservoir to the anterior chamber. Functional characterization of the status of the retina and optic nerve were evaluated by electroretinography and computerized pupillometry. Since ischemia itself can damage iris muscles we recorded pupil responses from the untreated (non-operated) eye while stimulating the operated eye to obtain data regarding the direct effect of ischemia on the retina and optic nerve. To quantify morphological damage we performed morphometric analysis comparing sections from operated and control retinas.

Use of computerized pupillometry to identify deficits of the retina and optic nerve

Analysis of the PLR parameters (amplitude of pupil constriction, latency time and velocity) proved to be an effective strategy for monitoring of the retina and optic nerve status after acute ocular hypertension (ischemia-reperfusion) injury. We used the PLR parameters more as an objective method to evaluate function of the optic nerve rather than photoreceptors of the retina since previous reports showed that PLR activity does not correlate with the number of photoreceptors in rodents[18,19]. Furthermore, recent evidence suggests that the PLR might be driven by light-sensitive, melanopsin-containing retinal ganglion cells (RGCs)[20]. Our results indicate dramatic suppression of the PLR in the first 20 days of the reperfusion period. Surprisingly, the PLR amplitude partially recovered in almost all (10/11) operated rats 35 days after injury, but showed a decrease at 42 days after injury. The latency time did not improve, however maximal velocity recovered 28 days after injury but afterwards continuously decreased till the end of the experiment. The recent study using retrograde labeling of RGCs after temporary retinal ischemia has confirmed that death of RGCs is an

ongoing process which lasts up to 3 months after ischemic injury[21] which might offer potential explanation for the late loss of the pupil function.

Electroretinography as a method for the evaluation of retinal integrity

To obtain information about the status of the photoreceptors and inner nuclear layer neurons we used full field flash and flicker ERG examination. Complete ocular ischemia for 1 hour almost completely abolished ERG amplitudes up to 22 days after surgery. Contrary to previous reports[14,15], which did not observe significant alteration of photoreceptor function, we observed a dramatic reduction of a- and b-wave amplitudes 24 hours after pressure induced ischemia-reperfusion insult. However, our data are consistent with a previous ultrastructural study where significant damage of the outer nuclear layer with combined features of apoptosis and secondary necrosis were noticed after acute elevation of the IOP[22]. The ERG examination revealed temporary recovery of function at 22 and 32 days of the reperfusion period, but latency times for a- and b-waves were significantly increased which might implicate changes in physiological activity of the recovered cells. The kinetics of the ERG response revealed minimal recovery of the function 22 and 32 days after injury. However, 42 days after ischemic insult function started to decrease again. This data suggests a potential susceptibility of photoreceptors to delayed neurodegenerative damage due to the ischemic insult as previously been suggested[23].

Histological and morphometric analysis of the retina and optic nerve

The morphometric analysis of the retinal layer thickness implicated location of the insult to ONL, INL and IPL layers in the central retina. These types of changes revealed the existence of neurotoxic damage not only at the inner retina but also at the outer retina (photoreceptors) of central retinal regions. However, the peripheral retina was more spared from the damage. We do not know particular mechanism responsible for the better survival of the peripheral retina but previous studies suggested existence of different mechanisms which might protect retinal function and structure after ischemic insult[24,25,26,27].

CONCLUSION

The continuous monitoring of the retina and optic nerve function revealed the interplay between regenerative and pathological events in ischemic rat retinas and optic nerves. The exact characterization of the timing and identification of the mechanisms responsible for the transient recovery of function and late neurodegeneration might open new avenues for the treatment of glaucoma and ischemic retinal and optic nerve diseases.

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CHAPTER 4 – FUNCTIONAL CHARACTERIZATION OF RETINA AND OPTIC NERVE AFTER CHRONIC ELEVATION OF THE INTRAOCULAR PRESSURE IN RATS

A paper to be submitted to Experimental Eye Research

Sinisa D. Grozdanic, Daniel M. Betts, Donald S. Sakaguchi, Young H. Kwon, Randy H. Kardon and Ioana M. Sonea

Abstract

PURPOSE: To evaluate visual function in rats with chronic elevation of intraocular pressure (IOP).

METHODS: Chronic ocular hypertension was induced in the left eye of 14 adult Brown Norway rats by cauterizing 3 vortex veins and 2 major episcleral veins; the right eye served as a non-operated control. A control group (n=5) was sham operated on the left eye. Prior to surgery, the IOP was measured with a Tonopen, the pupil light reflex (PLR) evaluated with a custom-made computerized pupillometer and electroretinograms (ERGs) were recorded simultaneously from both eyes post surgically: IOP was measured on weeks 1, 3, 5 and 8 post-operatively, pupil light reflexes on weeks 1, 4 and 8 post-operatively, and ERGs on weeks 4 and 8 post-operatively. Sixty five days postoperatively, rats were euthanized and optic nerves and eye globes were prepared for histological analysis.

RESULTS: Seven days after surgery 5/14 rats developed significant elevation of the IOP in operated eyes (control eyes: 25.1±0.5 mmHg; operated eyes: 34.1±0.6 mmHg; mean±SEM; p=0.0004; Paired t-test). Elevation of the IOP was sustained at 3 (p=0.002) and 5 (p=0.007) weeks postoperatively. However, IOP values did not significantly differ between control and operated eyes 8 weeks postoperatively (p=0.192, Paired t-test). Sham operated animals showed no elevation of the IOP 7 days postoperatively. Rats with elevated IOP had significant pupil defects at 7 (7.6±0.4% (mean±SEM); p<0.05, Repeated measures ANOVA, n=5) and 28 days postoperatively (6.7±0.4; p<0.05), but not at 62 days postoperatively (3.7±1.3%; p>0.05) compared to preoperative values (direct - consensual pupil response was 1.4±0.8%). Rats whose IOP values did not rise after surgery and sham operated rats did not develop pupil deficits 4 weeks postoperatively.

Rats with elevated IOP displayed a significant decrease in ERG amplitudes in operated eyes at 4 (a-wave$_{operated}$/a-wave$_{control}$ (a-wave ratio) = 42±14% (mean±SEM); b-wave$_{operated}$/b-wave$_{control}$ (b-wave ratio) = 43±16%) but not at 8 weeks postoperatively (a-wave ratio = 88±8.4%; b-wave ratio = 82.9±9%). Sham operated and rats whose IOP values remained non-elevated after surgery did not develop ERG deficits 4 weeks after surgery. Histological analysis did not reveal any damage in the eyes of animals with elevated intraocular ocular pressure with the exception of one rat, which still had ERG and pupil deficits at the end of experiment.

CONCLUSIONS: Development of ERG and PLR deficits are proportional to the elevation of the IOP in the rat model of chronic ocular hypertension. Functional monitoring of the ERG and PLR are sensitive techniques for the detection of retina and optic nerve deficits.

INTRODUCTION

The permanent loss of retinal ganglion cells (RGCs) is a hallmark of glaucoma (Quigley, 1999). Glaucoma is thought to affect over 67 million people worldwide (Quigley, 1996). Glaucoma is characterized as a progressive optic neuropathy with characteristic optic disc changes and progressive visual field defects (Quigley, 1999). The exact mechanism for the initiation of ganglion cell death in glaucoma is unknown, but different hypotheses have been reviewed recently (Quigley, 1999; Osborne et al, 2000; Farkas and Grosskreutz 2001). Evidence from the experimental animal models implicate that retinal ganglion cells die in a slow manner via apoptosis (Quigley et al, 1995). In order to achieve a better understanding of the glaucomatous pathology, and eventually develop new strategies for the protection of the optic nerve from glaucomatous neuropathy, it is essential to develop animal models and adequate tools for the *in vivo* monitoring of function during progression of the disease. The laboratory rat has become an easily induced and reproducible animal model system for studying glaucoma (Garcia-Valenzuela et al, 1995; Morrison et al, 1997; Ueda et al, 1998). While elevated intraocular pressure is still the major parameter for the evaluation of the rat glaucoma models, data describing retina and optic nerve function deficits during chronic elevation of the IOP are lacking. Currently, a damage estimation in the experimental rat models of chronic ocular hypertension is usually evaluated by histological or immunocytochemical analysis after euthanasia. Unfortunately, this approach does not provide any information about dynamics of the disease in different time points. Two recent studies described some of the electroretinographic properties of chronically hypertensive rat eyes (Mittag et al, 2000; Bayer[a] et al, 2001); there are no studies evaluating the function of the optic nerve in rat models of chronic ocular hypertension. The goal of this study was to investigate if it is possible to non-invasively monitor the function of the optic nerve and retina in this rat model of chronic ocular hypertension and to correlate electrophysiological deficits with the histological analysis.

MATERIALS AND METHODS:

Rat model of chronic ocular hypertension

All animal studies were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and procedures were approved by the Iowa State University Committee on Animal Care. A previously published procedure to generate chronic ocular hypertension in rats was used (Shareef et al, 1995). Briefly, Brown Norway rats (4 months of age, n=19) were initially anesthetized with 4% halothane, 30% nitrous oxide (NO) and 70% oxygen (O_2). Anesthesia was maintained with 2.5% halothane, 30% NO and 70% O_2 and body temperature was maintained using a heating pad. Two millimeter-long incisions through the conjunctiva and Tenon's capsule were made on the limbal periphery of the dorsal, ventral and temporal quadrants of the left eye. The right eye was not operated and it served as a control. Two radial incisions were made at the edges of the initial incision and tissue was recessed posteriorly, exposing the episcleral and vortex veins. A hand-held ophthalmic cautery was applied to 3 vortex and 2 major episcleral veins (or resected conjuctiva – sham operated rats), thereby blocking their corresponding area of venous drainage, and elevating intraocular pressure. Sham surgery was performed in the same manner without cauterizing vortex and episcleral veins. To prevent potential infection, antibiotic ointment (neomycin-polymyxin B-bacitracin; Bausch & Lomb Pharmaceuticals Inc; Tampa, FL) was applied topically after each procedure.

Intraocular pressure monitoring

Intraocular pressure was measured with a hand-held tonometer (Tonopen XL, Mentor, Norwell, MA). A calibration of the Tonopen was performed by comparing IOP results measured using invasive manometry as previously been described (Moore et al, 1993; Grozdanic et al, submitted). Readings from the manometer and Tonopen were obtained simultaneously and a regression line was calculated ($y=7.3029\pm0.7015x$, $r^2=0.78$). Six hours after start of the dark cycle animals were anesthetized with 3% halothane and 30% NO with 70% O_2. After rats were

immobilized, the concentration of the anesthetic was decreased to 1% of halothane and corneas were anesthetized with 0.5% proparacaine hydrochloride. The Tonopen was held perpendicular to, and applied toward the center of the cornea. All readings which were obtained after contact with the cornea were used to calculate the mean values for each eye.

Computerized pupillometry

The pupil light reflex was evaluated with a custom-made computerized pupillometer (University of Iowa, Iowa City, IA) preoperatively and on weeks 1, 4 and 8 postoperatively as previously been described (Grozdanic et al, submitted). Briefly, animals were anesthetized initially with 4% halothane, 30% NO and 70% O_2. A light plane of anesthesia was maintained with 1% halothane, 30% NO and 70% O_2 to avoid suppression of the pupil light reflex response detected with the use of higher doses of anesthetic. One channel computerized pupillometer was used to record the movement of the pupil from the control (non-operated) eye, while the stimulus light was alternated between the control and operated eye. Custom made software routine (Winnana Software, University of Iowa, Iowa City, IA) was used to analyze the recorded tracings of the pupil movements in response to light stimuli and to objectively determine the timing and amplitude of the pupil reflex responses. Since the opposite, non-operated eye was used as a control each defect caused by chronic ocular hypertension, was monitored longitudinally over time (Figure 1)

Electroretinography

To quantitate potential damage to the retina due to chronic elevation of the IOP, electroretinography was performed 4 and 8 weeks post-operatively as previously described (Grozdanic et al, submitted). Briefly, animals were dark adapted for at least 6h and placed in a specially designed dome whose interior was completely covered with aluminum foil to obtain a Ganzfeld effect. A Neuropack-MEB 7102 Evoked Potential Measuring System (Nihon-Kohden America, Foothill Ranch, CA) was used to deliver a triggered output to the flash stimulator and collect signals from both eyes. A flash ERG routine was delivered at a 0.2 Hz frequency (20 averaged signals per

recording session). To avoid potential bias due to electrode differences, recordings were repeated with electrodes switched to opposite eyes. The difference between right and left eyes preoperatively was never above 20% of recorded amplitudes.

Histological examination

Sixty five days postoperatively, rats were deeply anesthetized with a high dose of phenobarbitol (100 mg/kg) and perfused intracardially with ice-cold heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Optic nerves (dissected 1 mm posterior to the sclera) and eye globes were postfixed, paraffin embedded, and 7 μm thick sections of the retina were collected onto poly-l-lysine coated glass slides, and stained with hematoxylin and eosin; transverse sections of the optic nerves were stained with 1% methylene blue. Tissue sections were examined with a Nikon Microphot FXA photomicroscope (Nikon Corporation, New York, NY). Images were captured using a Kodak Megaplus Camera (Model 1.4; Kodak Corp, San Diego, CA) connected to a MegaGrabber Framegrabber in a Macintosh 8100/80 AV computer (Apple Computer, Cupertino, CA) using NIH Image 1.58 VDM software (W.Rasband, NIH, Bethesda, MD).

Statistical analysis

Statistical analysis was performed by using Paired t-test and Repeated Measures ANOVA with Dunnett's Multiple Comparison test (as indicated in the text) with the GraphPad (GraphPad, San Diego, CA) software. A p value of <0.05 was considered significant.

RESULTS

Tonometry

Seven days after surgery 5 of 14 operated rats developed significant elevation of the IOP in operated eyes (Figure 2). However, IOP values returned to preoperative

values and did not significantly differ between control and operated eyes 8 weeks postoperatively.

Pupillometry

Evaluation of the PLR revealed functional defects in the 5 operated animals which developed chronic elevation of the IOP (Figure 3a and b). Sham operated and rats which received cauterization of the vortex and episcleral veins but did not develop elevation of the IOP in operated eyes did not develop any significant PLR defects.

Preoperative values for the direct-consensual pupil response for rats with chronically elevated IOP were 1.4±0.8% (n=5). Rats with elevated IOP developed significant pupil defects at 1 week postoperatively (7.6±0.4% (mean±SEM); $p<0.05$, Repeated Measures ANOVA with Dunnett's Multiple Comparison test, n=5). Four weeks postoperatively the PLR defect was still significant compared to preoperative values (6.7±0.4; $p<0.05$, Repeated Measures ANOVA with Dunnett's Multiple Comparison test). However, at 8 weeks postoperatively the PLR response returned to preoperative values in 4 rats with previously elevated IOP, and there was no statistically significant difference compared to the preoperative levels (3.7±1.3%; $p>0.05$, Repeated Measures ANOVA with Dunnett's Multiple Comparison test, n=5). One rat had sustained pupil and ERG deficits throughout the entire post-operative period.

When the ratio between consensual and direct PLR (PLR_{ratio}=consensual/direct PLR, Fig. 3b) was examined, preoperative values were 92.2±4% (mean±SEM), 1 week postoperatively 65+4% (significantly different from preoperative values, $p<0.05$ Repeated Measures ANOVA with Dunnett's Multiple Comparison test, n=5), 4 weeks postoperatively 60.6+3.2% ($p<0.01$, n=5). By 8 weeks postoperatively, pupil responses had essentially recovered 75.4+6.9% ($p>0.05$, n=5). Rats whose IOP values remained normal after surgery and sham operated rats did not develop pupil deficits 4 weeks postoperatively (4.4±0.8%) compared to the corresponding preoperative values: 2.6±0.3% ($p>0.05$, Paired t-test, n=9). Sham operated rats had

preoperative values of 2.9±0.7% and 4 weeks after surgery 3.3±0.9% (p>0.05, Paired t-test, n=5).

Since recordings were obtained from the non-operated (control) eye, the latency time and velocity of the PLR (latency time - time from onset of stimuli to the start of the pupil constriction, velocity – maximal speed of the pupil constriction) were observed as parameters of retinal and optic nerve function after chronic elevation of the IOP in the operated eye. We detected a significant decrease of PLR velocity (p=0.02, Repeated Measures ANOVA with Dunnett's Multiple Comparison test) but not increase in the latency time (p>0.05, Repeated Measures ANOVA with Dunnett's Multiple Comparison test) in operated eyes compared to non-operated (control) eyes of rats which developed chronic ocular hypertension. By subtracting latency time values (latency time$_{oper}$ - latency time$_{cont}$) we determined next values for the rats with chronic ocular hypertension: preoperative = 47.3±3.6 (mean±SEM; ms, n=5), 1 week postoperatively 48.7±8.3 (p>0.05 (n=5), Repeated Measures ANOVA with Dunett's Multiple Comparison test), at 4 weeks 40.4±4.1 (p>0.05 (n=5)), and 8 weeks after surgery 36.7±8.2 (p>0.05 (n=5), Repeated Measures ANOVA with Dunnett's Multiple Comparison test).

Velocity parameters (velocity$_{cont}$ – velocity$_{oper}$) revealed next values: preoperative 0.28±0.06 (mean±SEM; mm/s), 1 week postoperative 0.71±0.22 (p>0.05 (n=5), Repeated Measures ANOVA with Dunnett's Multiple Comparison test) at 4 weeks 0.68±0.09 (statistical comparison to preoperative values revealed significantly slower pupil contraction when operated eye stimulated p<0.05, n=5), and 8 weeks after surgery 0.39±0.18 (p>0.05, n=5).

Electroretinography

Full field scotopic flash electroretinography was used as an objective method to evaluate the functional status of the inner and outer retina with the exception of the retinal ganglion cells.

Chronic elevation of the IOP caused significant reduction of ERG activity (Figure 4). Electroretinographic data were presented as a difference in amplitudes

between control (non-operated) eyes and operated eyes (Δamplitude = amplitude$_{control}$ − amplitude$_{operated}$). Rats which developed chronic elevation of the IOP had significant deficits in both a-wave and b-wave amplitudes 4 weeks after surgery (Δamplitude$_{a\text{-wave}}$ = 253$\pm$42 μV (mean$\pm$SEM; p<0.05, Repeated measures ANOVA), Δamplitude$_{b\text{-wave}}$ = 616$\pm$107 μV (p<0.05, Repeated measures ANOVA)) but not 8 weeks after surgery (Δamplitude$_{a\text{-wave}}$ = 47$\pm$21 μV (p>0.05, Repeated measures ANOVA), Δamplitude$_{b\text{-wave}}$ = 139$\pm$65 μV (p>0.05, Repeated measures ANOVA)) when compared to preoperative values (Δamplitude$_{a\text{-wave}}$ = 10$\pm$37 μV, Δamplitude$_{b\text{-wave}}$ = -32$\pm$26 μV).

Operated animals which did not develop elevation of the IOP and sham operated animals did not develop significant ERG deficits when recorded 4 weeks after surgery (operated w/o elevated IOP: Δamplitude$_{a\text{-wave}}$ = 16$\pm$19 μV (p>0.05, Paired t-test), Δamplitude$_{b\text{-wave}}$ = 18$\pm$51 μV (p>0.05, Paired t-test); sham operated: Δamplitude$_{a\text{-wave}}$ = 253$\pm$42 μV (p>0.05, Paired t-test), Δamplitude$_{b\text{-wave}}$ = 253$\pm$42 μV (p>0.05, Paired t-test)).

Electroretinographic data of rats with elevated IOP, expressed as a ratio between control and operated eyes again revealed a significant decrease in ERG amplitudes in operated eyes at 4 (a-wave$_{operated}$/a-wave$_{control}$ (a-wave ratio) = 42$\pm$14% (mean$\pm$SEM); b-wave$_{operated}$/b-wave$_{control}$ (b-wave ratio) = 43$\pm$16%, p<0.05, Repeated measures ANOVA) but not at 8 weeks postoperatively (a-wave ratio = 88$\pm$8.4%; b-wave ratio = 82.9$\pm$9%, p>0.05, Repeated measures ANOVA).

The latency time was not significantly increased in rats which developed chronic elevation of the IOP compared to control, non-operated eyes at 4 weeks postoperatively when amplitude deficits were detected: a-wave latency$_{control}$=21.4$\pm$2.6 ms (mean$\pm$SEM, n=5), a-wave latency$_{operated}$=18.5$\pm$1 ms (p>0.05, Paired t-test), b-wave latency$_{control}$=11.3$\pm$1.3 ms, b-wave latency$_{operated}$=11.6$\pm$0.5 ms (p>0.05).

Histological analysis

Histological analysis did not reveal any damage in the eyes of animals with elevated intraocular pressure with the exception of a single rat which sustained ERG

and pupil deficits throughout the experiment. The retina of the rat with sustained ERG and PLR deficits was characterized by thinning of the nerve fiber layer (Fig 5 - arrows, fig5B – central retina) and vacuolarization of the optic nerve axons (fig 5C), while other retinal layers did not have visible changes examined by light microscopy (Fig 5A, B and C). Rats whose IOP, PLR and ERG values returned to preoperative values by 8 weeks postoperatively (Figure 5D, E and F), sham operated rats, and rats which did not develop elevation of the IOP after surgery did not have detectable damage when examined by light microscopy.

DISCUSSION

Cauterization of 3 vortex and 2 episcleral veins induced temporary elevation of IOP in 5 of 14 operated rats (35.7%). While histological analysis revealed optic nerve damage in only one of fourteen operated rats (7%), functional monitoring of the PLR and ERG revealed significant deficits which correlated with IOP levels: out of 5 rats with elevated IOP, all displayed ERG deficits (100%) and PLR deficits (100%) 1 and 4 weeks postoperatively. Previous report (McKinnon et al, 1999) revealed that f49% of operated animals developed chronic elevation of the IOP, but only 20% of animals had detectable morphological damage of the optic nerve) after vortex and episcleral vein cauterization. Mittag et al (2000) also described that IOP was only temporarily elevated for 15 days in the absence of 5-fluorouracil injections in this vortex/episcleral vein cauterization model. Previous ERG studies of the same model (Mittag et al, 2000; Bayer[a] et al, 2001) and an electroretinographic study (Bayer[b] et al, 2001) of DBA/2NNia mice (mice with spontaneous occurrence of changes which mimic secondary glaucoma) also revealed a decrease of a- and b-wave amplitudes and oscillatory potentials. Our results also imply damage to all retinal layers since we detected a decrease of both a- and b-wave amplitudes. Since the major disadvantage of this model is that venous circulation might be seriously disrupted and cause the disturbance of choroidal circulation (Goldblum and Mittag, 2002), we were concerned that observed ERG deficits might be caused by the altered blood supply to photoreceptors. Since the ERG and PLR analysis did not reveal deficits in the eyes

which received cauterization of the veins but did not elevate IOP we hypothesized that the observed ERG and PLR deficits were probably caused by the chronic elevation of the IOP. However, we cannot exclude the possibility that rats with elevated IOP developed some type of choroidal circulation problems which affected photoreceptor function.

Simultaneous PLR and ERG monitoring proved to be a useful and sensitive, non-invasive method for the evaluation of the retina and optic nerve function. Since strategies for the induction of damage due to chronically elevated intraocular pressure usually involve surgery of one eye, monitoring of the pupil parameters of the control, non-operated eye, provided very precise information about retinal and optic nerve functional integrity.

We were not able to discriminate if PLR deficits were due to retinal ganglion cell damage or to damage of the outer retina since the PLR response depends on the status of the outer retina. However, since the ERG records function in the outer retina and Muller cell function, but not ganglion cell function per se, the combination of the PLR and ERG data might provide some localizing information. We were capable to demonstrate deficits of the outer retina (a-wave deficits) and Muller cells (b-wave deficits).

We can conclude that functional monitoring (ERG and PLR) of the retina and optic nerve are sensitive techniques, since we were capable to detect temporary loss of function.

In order to better understand the pathological mechanisms which occur during chronic ocular hypertension and evaluate potential success of neuroprotective strategies, it is essential to use techniques which would be sensitive enough to detect disruption of the retina and optic nerve function before occurrence of the cellular death.

ACKNOWLEDGEMENTS

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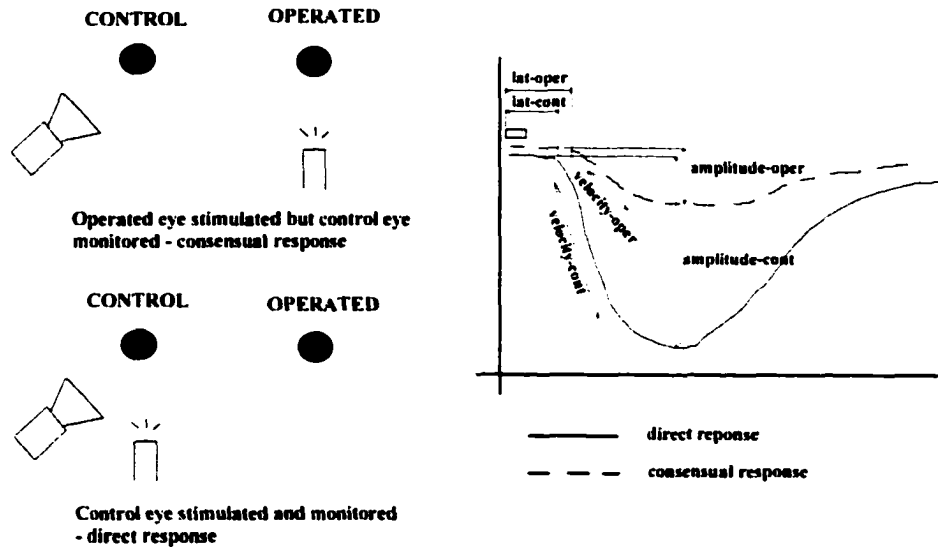
FIGURE 1. Schematic presentation of the PLR monitoring technique. One channel computerized pupillometer was used to record the movement of the pupil from the control (non-operated) eye while the stimulus light was alternated between operated and non-operated eye (Abbreviations: lat-cont = latency time for control eye; lat-oper = latency time for operated eye; velocity-cont = maximal velocity for control eye; velocity-oper = maximal velocity for operated eye; amplitude-cont = amplitudes of responses for control eye; amplitude-oper = amplitudes of responses for operated eye).
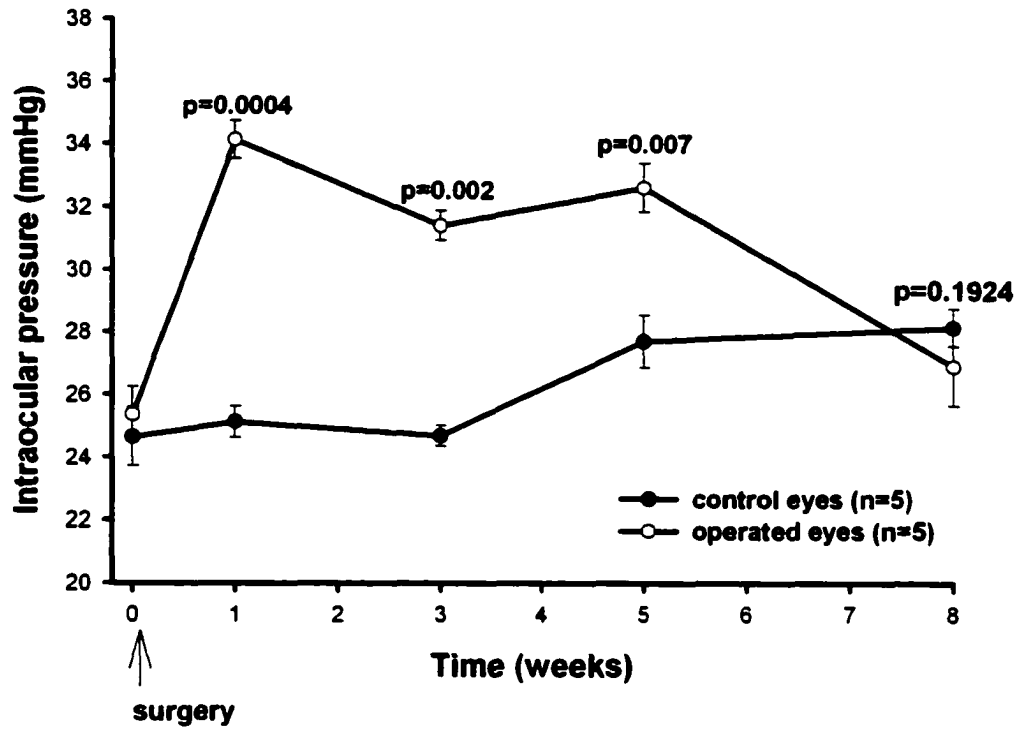
FIGURE 2 The cauterization of vortex and episcleral veins caused significant elevation of the IOP at 1, 3 and 5 weeks postoperatively in 5 of the 14 operated rats. However, IOP values returned to preoperative values in all 5 rats 8 weeks after surgery.
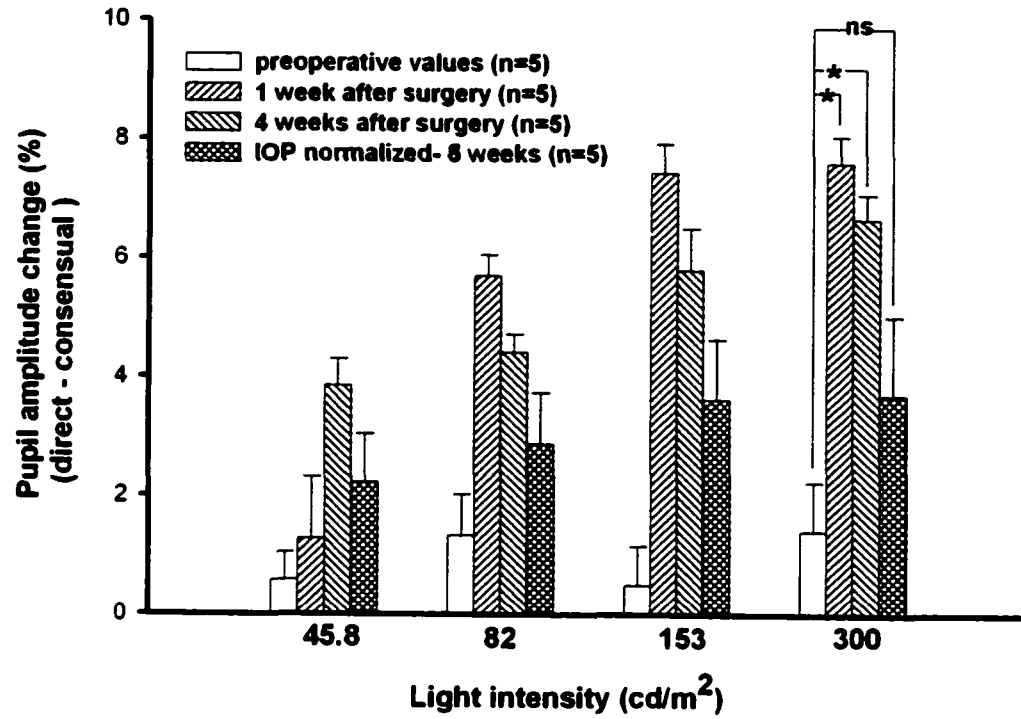
FIGURE 3a. Rats which developed elevation of the IOP had significant PLR deficits at 1 and 4 weeks postoperatively, which were proportional with the increase of light intensity. However, the PLR function returned towards normal values after 8 weeks ("*" for $p<0.05$; ns for $p>0.05$).
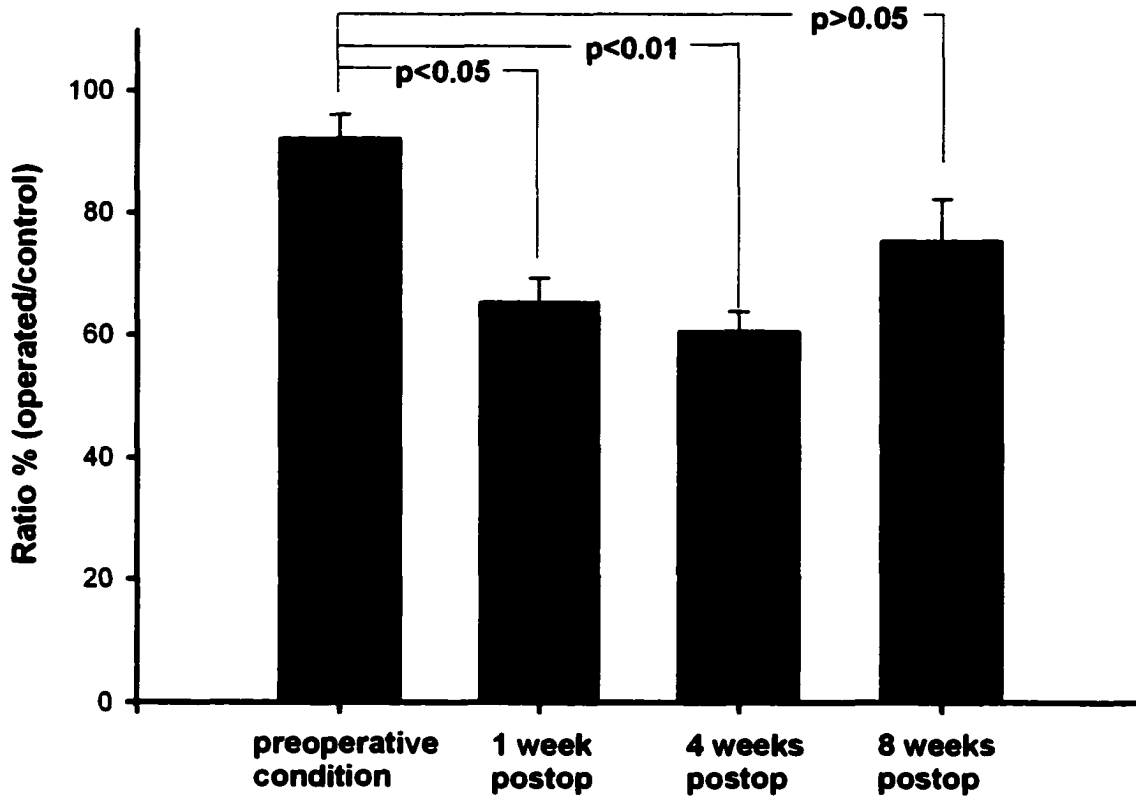
FIGURE 3b. The PLR values expressed as a ratio between operated and control eyes. Rats which developed elevation of the IOP had significant PLR deficits at 1 ($p<0.05$) and 4 weeks postoperatively ($p<0.01$), which were proportional with the increase of light intensity. However, the PLR function returned towards normal values after 8 weeks ($p>0.05$).
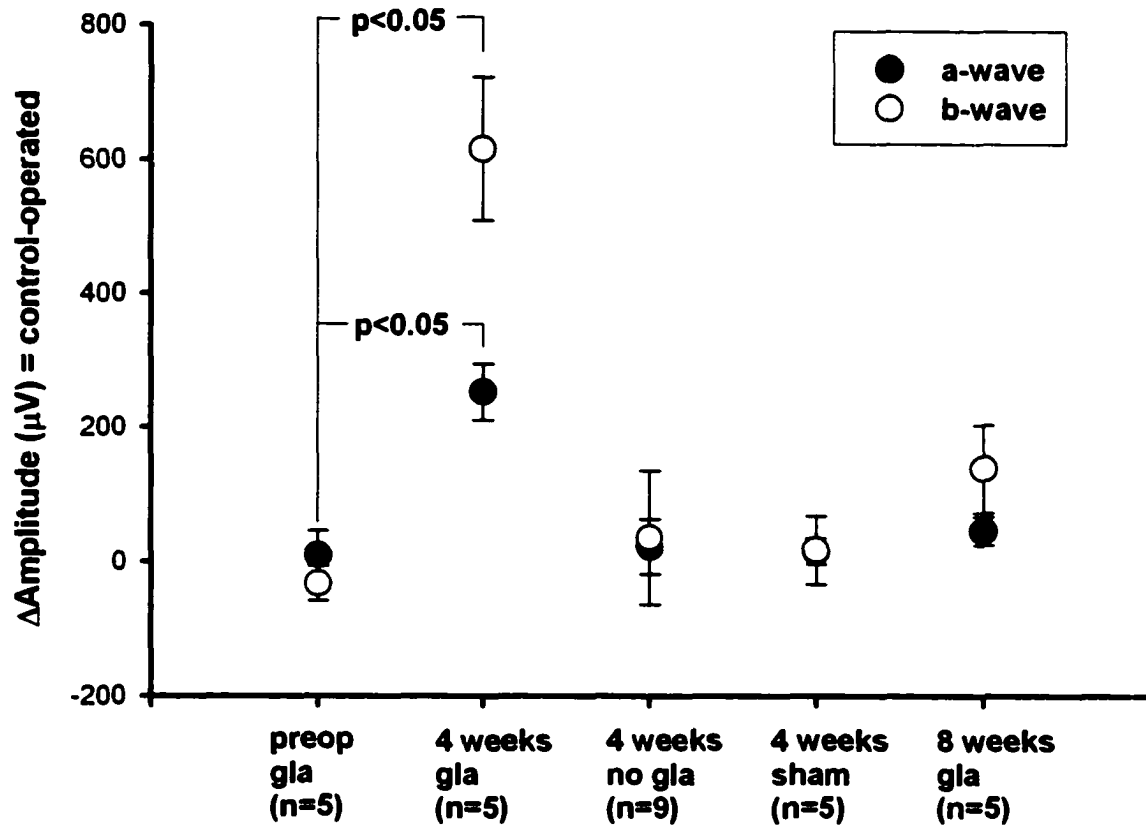
FIGURE 4. Rats which developed chronic elevation of the IOP (n=5) had significant deficits in both a-wave and b-wave amplitudes 4 weeks after surgery but not 8 weeks after surgery, when compared to preoperative values. Operated animals which did not develop elevation of the IOP (n=9) and sham operated animals (n=5) did not develop significant ERG deficits when recorded 4 weeks after surgery (operated w/o elevated IOP: a-wave (p>0.05, Paired t-test), b-wave (p>0.05, Paired t-test); sham operated: a-wave (p>0.05, Paired t-test), b-wave (p>0.05, Paired t-test)).
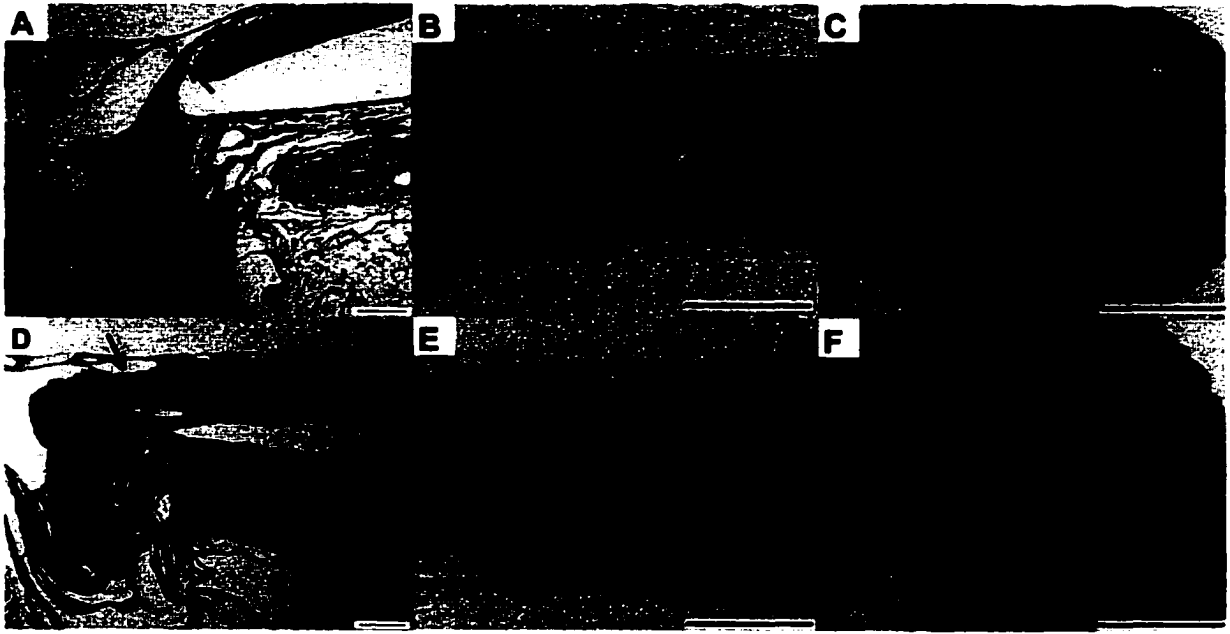
FIGURE 5. Histological analysis revealed damage in the operated eye of one rat which had sustained ERG and pupil deficits by the end of experiment (A,B,C). Damage was characterized by the thinning of the nerve fiber layer (Fig 5 - arrows, fig5B – central retina) and vacuolarization of the optic nerve axons (fig 5C). Figures 5D, E, and F represent optic nerve head, central retina and transverse section of the optic nerve in one of the rats which showed PLR and ERG deficits 1 and 4 weeks postoperatively, but not at 8 weeks. There was no visible damage to any of structures observed by light microscopy (Abbreviations: RGC = retinal ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; OS = outer segments; bar = 100μm).

CHAPTER 5 – GENERAL CONCLUSIONS

General discussion

The principal goal of this study was to develop non-invasive monitoring techniques for the characterization of rat eyes after acute and chronic elevation of the IOP and correlate functional with morphological changes.

The experimental approach used in these studies allowed us to precisely monitor the dynamics of pressure induced optic nerve and retina ischemic damage for prolonged periods of time (up to 8 weeks) after acute and chronic elevation of the IOP. Since ischemia itself can damage iris muscles we recorded pupil responses from untreated (non-operated) eyes while stimulating the operated eye to obtain data regarding the direct effect of ischemia on the retina and optic nerve. To quantify morphological damage we performed morphometric analysis comparing sections from operated and control retinas and optic nerves

Use of computerized pupillometry to identify deficits of the retina and optic nerve

Analysis of the PLR parameters (amplitude of pupil constriction, latency time and velocity) proved to be an effective strategy for monitoring of the retina and optic nerve status after acute and chronic ocular hypertension. The PLR are more useful as an objective method to evaluate function of the optic nerve rather than of photoreceptors, since previous reports showed that PLR activity does not correlate with the number of photoreceptors in rodents (Kovalevski et al, 1995; Lucas et al, 2001). Furthermore, recent evidence suggests that the PLR might be driven by light-sensitive, melanopsin-containing retinal ganglion cells (Hattar et al, 2002).

Our results showed a dramatic suppression of the PLR in the first 20 (acute ischemia model) and 28 (chronic ocular hypertension model) days after surgical procedure. Our findings implicate development of the ischemic damage in all retinal layers in both models. The PLR amplitude partially recovered in almost all (10/11) rats with acute ocular ischemia 35 days after injury, while rats which developed chronic

elevation of the IOP almost completely recovered ERG and PLR parameters 8 weeks after surgery. The partial recovery of function in acute model and almost complete recovery in the chronic model imply the existence of neuroprotective mechanisms or retina and optic nerve ischemia tolerance, which might be preserved even in dramatically damaged retinas and optic nerves. Hayreh and Weingeist (1980) showed that the monkey retina suffered irreparable damage after ischaemia of 105 minutes but recovered well after ischaemia of 97-98 minutes. They hypothesized that retinal neurons are more resistant to ischemia as compared to the brain. Iijima et al (2000) showed that the glutamate release in the retina does not proceed as rapidly as that in the cerebral cortex during 20 min of ischemia. They hypothesized that the difference shown by the two organs may be due to the slow depletion rate of ATP in the retina. These data (Hayreh and Wingeist, 1980; Iijima et al, 2000) may partially explain the neuronal tolerance to ischemia in the retina.

At this point we have no exact molecular evidence for an explanation for the functional recovery. However, previous study from Yu et al. (1999) described a reactive glial upregulation of and neuroprotective effect the ciliary neurotrophic factor in the ischemic retina in the very same model of acute ocular ischemia that we used. Other groups reported different mechanisms which could potentially play role in the neuroprotection after ischemic insult to the retina and optic nerve such as regulation of different growth factors (Vecino et al, 1998) and redistribution of anti-oxidative substances from glial to neuronal cells (Schutte and Verner, 1998)

Electroretinography as a method for the evaluation of retinal integrity

To obtain information about the status of the photoreceptors and inner nuclear layer neurons we used full field flash and flicker ERG examination. Acute elevation of the IOP almost completely abolished ERG amplitudes up to 22 days after surgery. Rats with chronic elevation of the IOP also developed significant ERG deficits. Previous results from the vortex vein-cauterization model (Mittag et al, 2000; Bayer^ et al, 2001) and electroretinographic study (Bayer et al, 2001) from the DBA/2NNia mice (mice with spontaneous occurrence of changes which mimics secondary glaucoma) revealed a decrease of a- and b-wave amplitudes and oscillatory potentials. Our

results also imply ischemic damage to all retinal layers since we detected a decrease of both a- and b-wave amplitudes. While glaucoma in humans is not characterized by flash ERG deficits, electroretinographic findings from our group and others (Mittag et al, 2000; Bayer et al, 2001) support the idea that the rodent outer retina might be more sensitive to the ischemic damage than human retina. However, there is possibility that detected ERG defects in rodent models develop due to a difference in the chronic ocular hypertension induction. This is a reminder that the extrapolation of results from animal models to the human disease has to be extremely careful.

Since a major disadvantage of the vortex and episcleral vein cauterization model is that venous circulation might be seriously disrupted and cause the disturbance of choroidal circulation (Goldblum and Mittag, 2002), we were concerned that observed ERG deficits in our model are caused by the inadequate blood supply to the photoreceptor layer. While ERG function recovered by 8 weeks postoperatively in the chronic model, ERG amplitudes dramatically decreased in eyes of the ocular ischemia model. We do not know exactly what particular mechanism is responsible for the delayed decrease in the ERG function.

Histological analysis

Our electrophysiological studies revealed a reduction of the function in all retinal layers of the acute and chronic hypertensive rat eyes. We performed a histological analysis to determine if functional defects were in agreement with morphological appearance of the operated eyes.

Light microscopy analysis of eyes, of the ocular ischemia model, revealed vacuolarization of the optic nerve axons, complete loss of inner retinal layers at the level of central retina and significant thinning of inner retina structures at the periphery. However, in the eyes with chronic elevation of the IOP, histological analysis did not reveal any damage in the eyes of animals with normalized intraocular pressure 8 weeks after surgery. The exception was one rat, which had sustained ERG and pupil deficits by the end of experiment. Retina of the rat with sustained ERG and PLR deficits had detectable loss of retinal ganglion cells while other retinal layers did not

have visible changes examined by light microscopy. Optic nerve head had signs of the gliosis and transverse sections of the optic nerve revealed axonal vacuolarization and swelling.

Recommendations for future research

The principal goal of this study was to develop non-invasive monitoring techniques for the characterization of rat eyes after acute and chronic elevation of the IOP and correlate functional with morphological changes. The continuous monitoring of the retina and optic nerve function revealed the interplay between the loss and recovery of the function in ischemic rat retinas and optic nerves after acute and chronic elevation of the IOP. We successfully demonstrated capability to continuously monitor function of the ischemic rat retina and optic nerve. Since recent report (Liang et al, 2001) implicated the discordance of functional and structural results (cilliary neurotrophic factor provided long-term protection of retinal structure but not function in animal models of retinitis pigmentosa), it become essential to couple the exact functional characterization of retina and optic nerve changes with the histological picture. That approach might open new avenues for the treatment of the glaucoma and ischemic retinal and optic nerve diseases.

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