Quantification of Ischemic Damage in the Rat Retina: A Comparative Study Using Evoked Potentials, Electroretinography, and Histology

Thomas Jehle, Karin Wingert, Cornelia Dimitriu, Wolfram Meschede, Julia Lasseck, Michael Bach, and Wolf A. Lagrèze

PURPOSE. To identify objective criteria to quantify visual function in the rat for developing therapeutic strategies to protect neuronal cells after ischemia. The impact of ocular ischemia on luminance and frequency-modulated contrast vision was compared with the function of outer retinal cells and the number of intact retinal ganglion cells (RGCs).

METHOD. Ischemia was induced in Brown-Norway rats by elevating the intraocular pressure to 120 mm Hg for 30, 45, 60, and 90 minutes. Visual function was evaluated by visual evoked potentials (VEPs) in awake, freely moving rats. Retinal function was analyzed with scotopic and photopic electroretinography (ERG). RGCs were quantified in retinal flatmounts after postischemic injection of tracer into the superior colliculus.

RESULTS. The response to flicker stimulation in VEP recordings decreased as the ischemic episodes increased. The susceptibility to ischemic damage was more pronounced when potentials were evoked with stimuli at higher frequencies. In ERG recordings, ischemia reduced oscillatory potentials and photopic flicker responses more intensely than scotopic a- and b-waves. In counting the RGCs, the reduced cell density correlated significantly with all electrophysiological parameters. The duration of ischemia with half-maximal inhibitory effect was between 36 and 58 minutes for VEPs and between 36 and 41 minutes for ERG, and it was 51 minutes for RGCs.

CONCLUSIONS. The amounts of reduction in VEPs, ERG, and RGCs differed as the duration of ischemia increased. The electrophysiological parameters presented in this study may serve as a useful addition to morphologic evaluations in future neuroprotection studies in vivo. (Invest Ophthalmol Vis Sci. 2008;49:1056–1064) DOI:10.1167/iovs.07-1050

Ocular ischemia plays an important role in the pathophysiology of various ocular diseases such as diabetic retinopathy,1,2 retinal vascular occlusion,3 anterior optic neuropathy,4 and possibly glaucoma.5–7 It may ultimately lead to neuronal death by inducing apoptosis8 or necrosis.9 Of the different retinal neurons, the retinal ganglion cells (RGCs) are thought to be most vulnerable to ischemia.10,11 Neuroprotection is a paradigm that aims to reduce or even prevent neuronal damage by pharmaceutical intervention or molecular genetic techniques. Neuroprotective effects are usually quantified by morphology.12,13 However, such methods provide no information about the functional state of single neurons or the entire retina. The growing body of research on the structure-function relationship reflects the potential dissociation between the functional and morphologic state.14–17 Functional investigations become especially important when pharmacologic principles are to be translated into clinical treatments.

Visual function in animals can be assessed by recording evoked potentials of the retina and cortex.18,19 Electroretinography (ERG) displays retinal activity, allowing partial differentiation of cells within different layers.18,20,21 Visual evoked potentials (VEPs) reflect the function of the visual pathway19,22–24 up to the visual cortex. VEPs can be recorded by stimulating of the entire visual field (Ganzfeld) or by pattern stimulation. The latter became standard in clinical testing25 but requires adequate refraction and the ability to fixate. However, in rats, this would require anesthesia, altering the VEPs.26,27 Ganzfeld stimulation overcomes this restriction because it allows examination of awake and freely moving animals.

Among the different dimensions of vision, contrast perception depends on the ability to discriminate intensities of light varying in space or time. Spatial28,29 and temporal modulation transfer function30,31 were found to be sensitive in detecting anterior visual pathway damage in glaucoma. For detecting impaired RGC function in animals, the recording of VEPs with Ganzfeld flicker stimulation allows the flicker response function (more correctly, modulation transfer function) to be evaluated across contrast at various temporal frequencies.

The intention of this study was to apply such Ganzfeld stimuli in rats, a technique not reported so far. With this paradigm, we aimed to test whether low modulation depths high stimulation frequencies, or both, can detect ischemia insults more sensitively than other stimulation parameters. To address this question, we induced ocular ischemia of different durations in rats. We then recorded steady state VEP with stimuli modulated in luminance and frequency, transient VEP with various stimuli, and scotopic and photopic ERGs. To enable comparison with morphology, we counted RGCs, which were labeled after the ischemic insult.

METHODS

Animals

Adult male Brown Norwegian rats (180–200 g) were obtained from Charles River Laboratories (Sulzfeld, Germany). Rats had free access to food and water and were kept in temperature-controlled rooms with a 12-hour light/12-hour dark cycle. 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 Committee of Animal Care of the University of Freiburg. All types of surgery, manipulations, and ERG recordings were performed under general anesthesia with isoflurane/O₂. Body temper-
ature was maintained at 37°C ± 0.5°C with a heating pad and a rectal thermometer probe. While recovering from anesthesia, the animals were placed in separate cages, and gentamicin ointment (Reofacin; Merck, Darmstadt, Germany) was applied on ocular surfaces and skin wounds.

**Ocular Ischemia**

Rats were anesthetized, and the anterior chamber of the left eye was cannulated with a 30-gauge needle connected to a reservoir containing 0.9% NaCl. Intraocular pressure was increased to 120 mm Hg, and ocular ischemia was confirmed by interruption of the ocular circulation. Rats without recovery of retinal perfusion 3 minutes after the end of the ischemic period, or those with lens injuries, were excluded. Forty rats underwent ocular ischemia and were divided into four groups, each receiving a different duration of ischemia, as follows: 30 minutes (n = 14), 45 minutes (n = 11), 60 minutes (n = 8), and 90 minutes (n = 7) ischemia. To exclude effects of the manipulation of the rat eye on VEP, ERG, or RGC survival, each left eye of six rats was cannulated without increasing the intraocular pressure.

**VEP**

To permit VEP recording, rats were anesthetized, and stainless steel screws were implanted 3 mm lateral to the lambda and 5 mm behind the bregma. Reference electrodes were placed 2 mm lateral to the lambda and 2 mm in front of the bregma. The electrode assembly was encased in dental acrylic, and the wounds were sutured. At least 5 days were allowed for recovery before electrophysiological testing. For recording, the unanesthetized rats were placed in a cage surrounded by a Ganzfeld bowl (Fig. 1). The bowl had a diameter of 45 cm, and the cage was placed in the middle of it. The position of the animal and the eye opening was controlled using a small infrared camera connected to an additional monitor. Monitoring the EEG allowed the detection of sleeping phases and the interference or interruption of measurements. A patch of aluminum foil in addition to an ointment containing coal particles reversibly blinded the right, nontreated eye. The electrodes were connected to an amplifier, and the potentials were recorded in real time. Visual stimuli (maximum stimulus luminance was 38 cd/m²) were generated with a computer-based system (EP2000 [http://www.michaelbach.de/ep2000/index.html]) and was displayed on a video monitor (FlexScanP56; EIZO, Ishikawa, Japan). The aperture (24 × 34 cm) of the Ganzfeld bowl was set in contact with the monitor. The recorded signals were amplified and band-pass filtered (5–300 Hz). First, we evoked steady state VEP by flicker stimulation at five contrast levels (modulation depth: 5%, 10%, 20%, 40%, 80%). Because we were initially uncertain which frequency would be most appropriate, we applied 2 frequencies, 7.5 Hz and 19 Hz. The full contrast (modulation depth, 100%) was applied at six frequencies of 2.9, 4.7, 7.5, 12.5, 19, and 38 Hz. Second, transient VEPs were evoked by single-flash stimulation (1.9 Hz, 12 ms) and by a low-frequency (1.9 Hz) on- and off-flicker stimulus. The number of sweeps per average generally ranged between 40 and 80. Recordings were performed on day 4 after ischemia.

**ERG**

Animals were dark adapted overnight and prepared for recording under dim red light using LED illumination (>600 nm). After anesthesia, the pupils were fully dilated with tropicamide (0.5%; Pharma Stulln, Stulln, Germany) and phenylephrine (5%; Uropharm, Bonn, Germany). The cornea was also anesthetized with proparacaine hydrochloride (Dr. Winzer Pharma GmbH, Berlin, Germany). Retinal signals were recorded from the cornea using an DTI electrode. The reference silver needle electrode was placed subcutaneously in the nose, and a grounding silver needle electrode was placed in the tail. Methylcellulose sodium (0.5%) and a custom-made contact lens (Hecht, Freiburg, Germany) ensured sufficient electrical contact. Ten additional minutes of dark adaptation followed. In the scotopic stimulation paradigm, series of flashes (76 cd/m², 10-ms duration, 0.1-Hz frequency) were delivered from the back of the animal to avoid any direct illumination of the eyes. Neutral density filters ranging from 5 to 0 log units in 0.33-unit steps were used to attenuate the flashes. Signals were amplified, recorded (500-ms analysis time), and band-pass filtered (0.5–300 Hz). In the photopic stimulation, paradigm rats were adapted to a background luminance of 22 cd/m² for 10 minutes. Series of flashes with frequencies of 2.9 to 38 Hz were used to evoke photopic ERG. The recordings were performed on day 4 after ischemia.

**Quantification of RGCs**

Four days after ischemia, RGCs were labeled retrogradely by injection of 4 μL fluorescent tracer (FluoroGold; Fluorochrome, Denver, CO) into both superior colliculi using a stereotactic device (Stoelting, Kiel, WI). Rats were killed 6 days after labeling by an overdose of chloral hydrate. The eyes were removed and fixed for 30 minutes in paraformaldehyde. Retinas were then dissected, flattened mounted on gelatin-coated glass slides, and embedded in mounting media (Vectashield; AXXORA Deutschland, Loerrach, Germany). Tracer (FluoroGold; Fluorochrome)-positive RGCs were counted in a blinded fashion under a fluorescence microscope (AxioImager; Carl Zeiss, Jena, Germany) in 12 distinct areas measuring 0.04 mm² each.

**Data and Statistical Analyses**

Steady state VEP and photopic flicker ERG responses were extracted by Fourier analysis with image processing and programming software.

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**Figure 1.** Experimental setup. (A) Rat connected to the amplifier with one eye occluded. (B) Cage in the Ganzfeld bowl in front of the monitor.
In VEP, the recordings' amplitudes were added up to the third harmonic. The amplitudes of the transient VEPs were calculated from the main negative trough to the main positive peak. For scotopic ERG, the a-wave was calculated as the difference between the baseline and the trough of the first negative deflection. The b-wave amplitude was the difference between the trough of the a-wave and the peak of the b-wave. Oscillatory potentials were extracted as a bandpass of 75 Hz to 300 Hz from the unattenuated scotopic flash ERG and were quantified as the difference between the highest peak and the lowest trough.

All averaged data are presented as means with their corresponding SEM or with SD in case of estimated parameters in sigmoidal curve fitting. Statistical significance was assessed using ANOVA or by t-testing, followed by Tukey-Kramer post hoc testing for multiple comparison procedures. Differences were considered significant at \( P < 0.05 \). Sigmoidal curves were fitted (Igor Pro; WaveMetrics) to the mean amplitudes using the formula: 

\[
F(x) = I_{\text{max}} \left( \frac{1}{1 + e^{\left( \frac{T_{50} - x}{s} \right)}} \right),
\]

with \( I_{\text{max}} \) maximum inhibition, \( T_{50} \) time of ischemia with 50% of the inhibitory effect of the response, and \( s \) slope of the point of inflection. The Pearson coefficient of correlation was calculated (Prism 4.0b; GraphPad Software, San Diego, CA).

To describe the modulation of luminance paradigm with only one parameter, we calculated the slope (\( s \)) of the amplitude's regression line plotted over the logarithm of the modulation depth. To describe the modulation of frequencies with one single parameter, we calculated the product of all amplitudes with their stimulation frequency \( (TR \times A_i \times F_i) \), with \( TR \) temporal response, \( A_i \) amplitude, \( F_i \) frequency. This product respects the fact that high-frequency responses were more vulnerable to pharmacologic or physical manipulation.
RESULTS

VEP

Estimation of Noise. To estimate the level of background noise, we first recorded potentials without visual stimulation \((n = 8)\). Amplitudes were \(1.1 \pm 0.2 \mu V\) (mean amplitude \(\pm SEM\)) in steady state and \(12.3 \pm 2.1 \mu V\) in transient stimulation. To confirm that the eyes of awake rats were sufficiently blinded, both eyes were occluded. These recordings did not differ from background noise.

Steady State Stimulation. Averaged steady state VEP traces showed a quasi-sinusoidal waveform and were dominated by the first or second harmonic of the input signal. Figure 2 shows potentials evoked by different visual stimuli after various durations of ischemia. With regard to luminance modulation, we observed a monotonic rise in amplitudes with an increase in modulation depth. Ischemia reduced the amplitudes to all modulation depths proportionally. The slope of the corresponding regression line was \(9.67 \pm 0.59\) at \(19\) Hz and \(6.00 \pm 0.32\) at \(7.5\) Hz in untreated rats. The slope parameter of

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933445/) Effect of ocular ischemia on VEP recordings stimulated by (A) modulation of luminance, (B) modulation of frequencies, and (C) transient stimuli. Ischemia markedly affected all the responses with varying susceptibility. \(T_{50}\) values are compared in Figure 8. All values are means with corresponding SEMs.
the sham-operated rats was 10.3 ± 1.1 at 19 Hz and 6.7 ± 1.0 at 7.5 Hz. Ischemia reduced the slope in the 19-Hz group sooner and to a more pronounced degree than in the 7.5-Hz group (Figs. 3, 4A). Regarding frequency modulation, the amplitudes of controls decreased with increase of stimulation frequency. An intermediate minimum appeared at 7.5 Hz. All amplitudes decreased as the ischemia rose. Responses at high frequencies were more vulnerable to ischemia than were responses at lower frequencies (Fig. 3). The TR of untreated rats was 1697 ± 98, and that of sham-operated rats was 1777 ± 182. The effect of the ischemic durations on TR is shown in Figure 4B.

**Transient Stimulation.** As shown in Figure 2, the transient VEP waveform evoked by a single flash or a low-frequency flicker consists a major positive (P1) and a major negative (N1) component. Mean amplitudes of control animals were 65.7 ± 3.0 μV for single flashes, 70.8 ± 3.6 μV for ON stimuli, and 38.3 ± 2.6 μV for OFF stimuli. The effect of ocular ischemia on VEP responses is shown in Figure 2 for representative recordings and in Figure 4C. The amplitudes of sham-operated rats 4 days after the procedure were 70.0 ± 13.2 μV for single flashes, 69.1 ± 16.9 μV for ON stimuli, and 45.7 ± 12.6 μV for OFF stimuli.

**ERG**

Examples are shown in Figure 5. Amplitudes decreased with the duration of ischemia. As shown in Figure 5A, oscillatory potentials decreased because of ocular ischemia and disappeared after 60 minutes. In the photopic ERG, amplitudes

![Figure 5. Effect of ocular ischemia on ERG. (A) Responses to single flashes (0.02 Hz) with an increase in flash intensity. (B) Photopic flicker stimulation at different frequencies. Responses revealed a typically sinusoidal waveform, with a period corresponding to the rate of stimulation. Ischemia led to a marked decrease in amplitudes. It is evident that ocular ischemia reduced amplitudes with an increase in its duration, with an emphasis on oscillatory potentials and photopic responses.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933445/)
depended on the frequency of the flashes. Oscillatory potentials and photopic responses were more sensitive to ischemia than the scotopic a- and b-wave. The sham operation did not significantly alter the a-wave (maximal amplitude, $221 \pm 35 \mu A$), the b-wave (maximal amplitude, $460 \pm 47 \mu A$), oscillatory potentials ($115 \pm 23 \mu A$), or photopic flicker ERG (19 Hz, $23 \pm 3 \mu A$) compared with preischemic controls (Fig. 6).

**FIGURE 6.** (A, B) Effect of ocular ischemia on scotopic a- and b-waves. Mean amplitudes with SEM were plotted as a function of flash intensity. Ischemia led to a marked, but not complete, inhibition of responses. (C) Mean amplitudes with SEM of photopic ERGs. Responses were plotted as a function of stimulus frequencies. For all responses, ischemia led to a proportional inhibition of amplitudes. (D) Normalized responses plotted as a function of the duration of ocular ischemia. Sigmoidal curves were fitted to mean amplitudes in control percentages. Ischemia affects oscillatory potentials and responses of flicker ERG almost identically with complete inhibition; a- and b-waves remained detectable even after 90 minutes of ocular ischemia.
RGCs

The mean density of RGCs was 2307 ± 38 cells/mm² in control eyes (n = 18); a sham operation did not significantly alter the number of countable RGC (2256 ± 76 cells/mm²; n = 6). Their number dropped with the duration of ischemia (Fig. 7). If ischemia lasted longer than 45 minutes, more than 50% of RGCs were lost.

Comparison and Correlation

Linear regression analysis revealed significant correlations between the number of RGCs and all electrophysiological parameters measured here. With regard to the ERG, the Pearson correlation quotient was 0.78 (P < 0.0001) for the a-wave, 0.65 (P < 0.0001) for the b-wave, 0.84 (P < 0.0001) for the oscillatory potentials, and 0.72 (P < 0.0001) for the photopic 19-Hz flicker. Concerning VEPs, the Pearson correlation quotient was 0.65 (P < 0.0001) for the single flash, 0.62 (P < 0.0001) for the ON stimulus, 0.56 (P < 0.0001) for the OFF stimulus, 0.68 (P < 0.0001) for the slope 19 Hz, and 0.57 (P < 0.0001) for the slope 7.5 Hz. After Bonferroni-Holm correction for multiple testing, all results remained significant. The highest correlation was seen between RGCs and the amplitudes of oscillatory potentials. Ischemia reduced all tested parameters in direct proportion to the duration of ischemia. This susceptibility can be expressed as the half-maximum inhibition time (T₁/₂); it ranged, as shown in Figure 8, from 36 to 58 minutes in VEP amplitude and from 36 to 41 minutes in ERG amplitude, and it was 51 minutes in RGC density.

DISCUSSION

We designed this study to evaluate new electrophysiological paradigms at different amounts of RGC loss induced by ocular ischemia in rats. To ensure they were independent of anesthesia, which alters the VEP,26,27 animals were not restrained and were recorded while moving freely. Because awake animals do not fixate reliably, we chose homogenous visual stimuli without contours, illuminating the entire visual field (Ganzfeld stimulation). Another advantage of this stimulation mode is its independence of refraction. To encompass different aspects of contrast vision, the stimuli were modulated in depth and frequency. In normal rats, VEP amplitude increased with modu-

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**Figure 7.** (A) Sections of RGCs in wholemount retinas retrogradely labeled with tracer applied to the superior colliculi 4 days after ocular ischemia. Only cells with active retrograde transport were stained. Animals were processed 6 days later. Left to right: 0, 30, 45, 60, and 90 minutes of ischemia. RGCs decreased markedly with an increase in ischemic episodes. Scale bar, 100 μm. (B) Mean numbers with SEM of RGCs. Cells per square millimeter were plotted as a function of ischemic episodes. Ischemia over 90 minutes led to a loss of retrograde transport capacity of almost 100%. Differences between all groups were significant (P < 0.05).
Quantification of Ischemic Damage

...have been reported by others, but the dependence of ERG parameter change on ischemic duration has not been previously presented. Oscillatory potentials and amplitudes of flicker ERG were more sensitive to ischemic damage than a- and b-waves. These results confirm earlier studies showing a high susceptibility to ischemic and glaucomatous damage.

Quantification of RGCs is used to evaluate the effectiveness of neuroprotective drugs, but few studies provide information on whether the remaining RGCs are dysfunctional or healthy. Therefore, we labeled RGCs after the ischemic insult. With this method, only cells with intact retrograde transport were stained and counted as "vital" RGCs. We demonstrated that even 30 minutes of ischemia led to a significant decrease in RGCs. Selles-Navarro et al. labeled RGCs before the insult and found that ischemia up to 45 minutes does not induce RGC loss. Because not all RGCs that survive ischemia retain their capacity for retrograde axonal transport, we assumed that the reductions in RGCs after 30 and 45 minutes of ischemia in our study resulted from functional deficits rather than apoptosis. We propose that the approach labeling RGCs after insult may supplement information about potential effects in neuroprotective studies.

All electrophysiological parameters correlated significantly with the number of intact RGCs. The highest correlation was observed in oscillatory potentials with ERG and in TR with VEPs. Larger variance of individual VEPs may account for the fact that correlation is lower in VEPs than in the ERG recordings.

To compare the susceptibility of functional parameters to ischemia, we estimated the half-maximal inhibition time ($T_{50}$). To our knowledge, these data have not been reported for either VEP or ERG recordings. The $T_{50}$ ranged from 36 to 58 minutes for VEPs and 36 to 41 minutes for ERG, and it lasted 51 minutes in RGCs. It is conceivable that sigmoid fitting is not always appropriate for reflecting recordings, depending on the duration of ischemia. A more complex curve form may be likely under some conditions. However, for better comparability, all curves were fitted identically. This may explain the large standard derivations for $T_{50}$ values in complex stimulation modalities such as the modulation of luminance.

The $T_{50}$ of TR was most similar to that of RGCs, and we conclude that TR could apply to neurodegenerative animal models to reflect RGC loss over time. VEPs after 7.5-Hz stimulation were most resistant to ischemic damage. Low-frequency stimulation parameters, therefore, may be used in models in which greater damage is expected, such as with the optic nerve crush, longer ischemia, or severe glaucoma. Responses to 19-Hz modulation depth and ON stimuli are most susceptible to ischemia.

In conclusion, the amounts of reduction in VEPs, ERG, and RGCs differed as the duration of ischemia increased. The electrophysiological parameters presented in this study may serve as useful additions to morphologic evaluations in future neuroprotection studies in vivo.

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