Simvastatin Improves Retinal Ganglion Cell Survival and Spatial Vision after Acute Retinal Ischemia/Reperfusion in Mice

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Purpose. The major aims of this study were to evaluate the effect of retinal ischemia by behavioral testing and histologic analyses, to visualize ischemia-induced changes of cortical activity by optical imaging of intrinsic signals, and to test the therapeutic effectiveness of simvastatin.

Methods. Retinal ischemia was induced monocularly by elevating intraocular pressure. Visual function was tested behaviorally with a virtual reality optomotor system, physiologically with optical imaging of intrinsic signals, and histologically by counting the surviving retinal ganglion cells (RGCs) in the same animal.

Results. Visual acuity (−38%) and contrast sensitivity (−78%) were significantly reduced 6 days after ischemia compared with controls. The number of RGCs was reduced by 16%. In contrast, optical imaging revealed essentially unchanged cortical activity maps in spite of the lesion. Treatment of mice with simvastatin applied after the ischemic insult significantly improved both visual function as measured behaviorally (~95% visual acuity, ~165% contrast sensitivity) and RGC survival (~30%) compared with vehicle-treated animals (~42% visual acuity, ~85% contrast sensitivity).

Conclusions. This specific combination of behavioral measurements of visual function, cortical activity imaging, and histologic analyses is ideally suited to follow ischemia-induced changes and to monitor the effect of therapeutic approaches. Statin therapy may be a promising pharmacologic tool for the treatment of acute retinal ischemia in particular because, in our study, simvastatin was applied *after* ischemia, a treatment regimen with much greater clinical relevance than preventive administration, as in previous studies. (*Invest Ophthalmol Vis Sci.* 2011;52:2606–2618) DOI:10.1167/iovs.10-6005

Retinal ischemia, involving a reduction of retinal blood supply, is a serious and common clinical problem and is an important cause of visual impairment in retinal vascular occlusion, diabetic retinopathy, and glaucoma. Experimental studies have shown that retinal ganglion cells (RGCs) and their axonal fibers are lost after retinal ischemic reperfusion injury.[1] One of the models most frequently used to investigate molecular mechanisms and potential therapeutic strategies for retinal ischemia consists of acute elevation of intraocular pressure (IOP) above systolic levels followed by reperfusion.[2–4] This method produces global ischemia, with obstruction of both the retinal and the uveal circulation. It produces pathologic features almost identical with those observed after central retinal artery occlusion and represents a model of acute angle closure glaucoma.[5] Selles-Navarro et al.[6] found that both the duration of the initial transient period of ischemia and the duration of the survival period influence the proportion of RGC death. After 30 minutes of retinal ischemia, the RGC survival rate was 92%. After 45 minutes of retinal ischemia in rats, Adachi et al.[7] counted 29% less axons compared with control animals.

Quantitative analysis of cell damage after retinal ischemia/reperfusion has been achieved primarily by end-stage counting of the cells in the different retinal layers[4,8] or retrograde fluorescence labeling of RGCs.[6] Although morphologic studies provide important information about the number of surviving cells, they offer no information about the functional status of the retina and the dynamics of ischemic injury. Furthermore, the demonstration of functional drug efficacy constitutes an important additional measure of therapeutic outcome. To understand the pathologic mechanisms in retinal and optic nerve hypoxia and to evaluate potential therapeutic approaches, it is essential to develop strategies for the continuous and objective monitoring of visual function in easily accessible and reproducible animal models.

Previously, two different electrophysiological measures have been used to determine visual function after high IOP: electroretinography (ERG) and visual evoked potential (VEP) recordings. ERG recordings are a mass electrical response of the retina to photic stimulation, allowing partial differentiation of cell function within different retinal layers.[9,10] VEPs were recorded previously in rats from both the retina and the cortex after photoembolic stroke, allowing assessment of the function of the visual pathway up to the visual cortex.[10,11] In ERG recordings, the b-wave has been used as an index of retinal ischemia.[12] The b-wave results from an interaction of bipolar and Müller cells, with K^+ movement into the distal Müller cells after bipolar depolarization producing the current sink that is responsible for the b-wave.[13] In the high IOP model, an immediate attenuation of the scotopic b-wave is produced that flattens within minutes. Recent evidence shows, however, that modifications in ERG recordings do not directly predict visual capabilities. As we showed recently, Bassoon-mutant mice display a severely diminished and slowed b-wave[14] but have surprisingly robust vision when tested behaviorally.[15] It is, therefore, indispensable for a thorough understanding of both

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ischemia-induced vision loss and for the evaluation of potential therapeutic strategies to test vision behaviorally. We have used the recently developed virtual reality optomotor system[16] to follow spatial vision in mice after retinal ischemia and to test the efficacy of various therapeutic regimens. In addition, we have used optical imaging of intrinsic signals[17] as a fast and reliable method with which to visualize cortical activity in the same animals.[15,18]

Analysis of retinal function and morphology during and after retinal ischemia was previously investigated only in rats by means of electrophysiological and histologic techniques. Although retinal function has also been evaluated after IOP elevation in mice, there are no combined functional and morphologic analyses of the retinal response after well-controlled acute IOP elevation. Here, for the first time, we provide behavioral, optical imaging and histologic data from an experimental mouse model of IOP elevation that is increasingly important in studies of neurodegeneration and glaucoma and that might be applied to an increasing number of murine lines with well-defined genotypes and phenotypes in future studies.

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, generically termed statins, are potent and effective inhibitors of cholesterol synthesis and are widely prescribed to treat hypercholesterolemia. More recently, clinical trials have demonstrated that statins also exert beneficial effects when used as stroke prophylactic agents, reducing the sequelae of a stroke by 25% to 30%.[19,20] Prevention is believed to be achieved mainly through statin activity on blood vessel wall function. In addition to exerting antiatherosclerotic and antithrombotic effects, statins also have anti-inflammatory and neuroprotective actions that have been identified as cholesterol-independent or so-called pleiotropic effects.[21] Finally, accumulating evidence supports statin therapy for central nervous system diseases, including neurodegenerative pathologies affecting the visual system.[22] Systemic delivery of simvastatin significantly improves retinal ganglion cell survival after an optic nerve lesion[23] and after acute retinal ischemia/reperfusion in the rat retina in vivo.[4]

In this study, we examined the impact of retinal ischemia on retinal function and morphology in adult mice and evaluated the potential therapeutic use of statins as a pharmacologic tool to prevent both retinal neuronal injury and retinal dysfunction.

Materials and Methods

Animal Guidelines

Animals were kept in standard cages on a 12-hour light/12-hour dark cycle, with food and water available ad libitum. All experiments were performed in accordance with the European Convention for Animal Care and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Use of Laboratory Animals was approved by the local animal care committee.

Transient Retinal Ischemia

Male C57BL/6J mice, each weighing 25 to 30 g (12 weeks old), were anesthetized with intraperitoneal injection of chloral hydrate (500 mg/kg body weight; Fluka, Seelze, Germany). After application of topical anesthesia (oxybuprocaine-hydrochloride 4 mg/mL; Bausch & Lomb GmbH, Feldkirchen, Germany), transient ischemia was induced in the right eye according to Zheng et al.[3] Briefly, the anterior chamber of the right eye was cannulated with a 30-gauge needle connected to an elevated normal saline container. IOP was elevated above systolic pressure for 15, 30, or 60 minutes. For sham procedures, a needle attached to a saline reservoir was inserted into the anterior chamber of the contralateral untreated eye, but pressure was not increased and ischemia did not occur as verified by funduscopy. Blanching of the fundus because of interrupted blood supply confirmed retinal ischemia. IOP increase and maintenance were evaluated during the procedure using a hand-held tonometer (TonoLab; Tiolat Ltd., Helsinki, Finland). This method has been validated for the mouse eye.[23] After the corresponding period of ischemia, the needle was withdrawn from the anterior chamber, and the IOP was normalized. One drop of antibiotic solution (ofloxacin; Bausch & Lomb GmbH) was applied topically to the treated eye before and after cannulation of the anterior chamber. Eyes were inspected daily, and animals with signs of inflammation or cataract were excluded from further analyses.

Behavioral Evaluation of Visual Acuity

Visual acuity of the mice was assessed using a recently developed virtual reality optomotor system.[16] Briefly, freely moving mice were exposed to moving sine wave gratings of various spatial frequencies and contrasts and will reflexively track the gratings by head movements as long as they can see the gratings. Spatial frequency at full contrast and contrast at six different spatial frequencies were varied by the experimenter until the threshold of tracking was determined. Given that only motion in the temporal-to-nasal direction evokes tracking, it is possible to measure thresholds and contrast functions for both eyes separately by reversing the direction of the moving gratings.[24] The threshold for grating acuity and contrast thresholds at the following six spatial frequencies were measured: 0.031, 0.064, 0.092, 0.103, 0.192, 0.272 cyc/deg. Contrast sensitivity thresholds measured in percentages were converted to Michelson contrasts based on screen luminance (maximum − minimum)/(maximum + minimum) by taking the reciprocal of the threshold, which was multiplied by a factor of 0.985 for the proportion of minimal and maximal luminances. For example, 25% contrast is thus expressed as 4.06 in absolute contrast value. The animals were tested on days 1, 3, and 6 after retinal ischemia for the initial analyses of the functional effects of ischemia and again on days 9, 12, 15, and 18 after retinal ischemia in the 24-hour delay treatment/therapy group. Experimenters were always masked to each animal's previous treatment.

Surgical Preparations for Optical Imaging

After initial anesthesia with 2% halothane in a 1:1 mixture of O_2 and N_2O, each animal received an intraperitoneal injection of 50 mg/kg pentobarbital supplemented by chlorprothixene (0.2 mg/mouse, intramuscularly), atropine (0.3 mg, subcutaneously), and dexamethasone (0.2 mg/mouse, subcutaneously). Tracheotomy was performed, and the animal was placed in a stereotaxic apparatus. In addition, lidocaine (2% lidocaine jelly) was applied locally to all incisions. Body temperature was maintained at 37°C, and electrocardiographic leads were attached to continuously monitor heart rate. Anesthesia was maintained with 0.6% to 0.8% halothane in a mixture of 1:1 O_2/N_2O applied through the tracheal tube. Visual cortical activity was visualized through the intact skull, which was covered by low-melting point agarose (2.5% in saline) and a glass coverslip.

Optical Imaging of Intrinsic Signals and Visual Stimuli

All optical imaging experiments were performed in both normal mice and in mice that had undergone retinal ischemia for 30 minutes. Cortical activity was visualized on day 6, after completion of the behavioral experiments. Mouse visual cortical responses were recorded using the imaging method developed by Kalatsky and Stryker[17] and were optimized for the assessment of ocular dominance plasticity by Cang et al.[25] In this method, a temporally periodic stimulus is continuously presented to the animal, and the cortical responses at the stimulus frequency are extracted by Fourier analysis. Briefly, optical images of cortical intrinsic signals were obtained using a charge-coupled device (CCD) camera (1M30; Dalsa, Waterloo, Canada) controlled by custom software. A 50 × 50 mm tandem lens configuration was used (Nikon, Inc., Melville, NY). The surface vascular pattern and intrinsic signal images were visualized with illumination wavelengths

set by a green (550 ± 3 nm) or a red (610 ± 3 nm) interference filter, respectively. After acquisition of a surface image, the camera was focused 600 μm below the pial surface. An additional red filter was interposed between the brain and the CCD camera. Frames were acquired at a rate of 30 Hz, temporally binned to 7.5 Hz, and stored as 512 × 512 pixel images after spatial binning of the camera image. To enable simultaneous imaging of neuronal activity of the left and right visual cortexes, a high refresh rate monitor (D2128-TCO, 800 × 600 at 120 Hz; Dell, Round Rock, TX) was placed in front of the animal at a distance of 25 cm to display the visual stimuli. Visual stimuli consisted of drifting bars (2° wide) presented alternately to the left eye and the right eye. Horizontal bars elicited elevation maps, and vertical bars elicited azimuth maps.[15] The stimuli were shown across the full screen, generated by a video board (Matrox G450; Matrox Graphics, Inc., Quebec, Canada), and controlled by custom software. The distance between two bars was 80°, and they were presented at a temporal frequency of 0.125 Hz.

Analysis of the Optical Imaging Data

Maps were calculated from the acquired frames by Fourier analysis to extract the signal at the stimulation frequency using custom software.[17] Although the phase component of the signal was used for the calculation of retinotopy, the amplitude component represented the intensity of neuronal activation (i.e., response magnitude, expressed as a fractional change in reflectance × 10^{-4}). The neuronal activities for responses to left and right eyes were calculated from blocks of four runs. Runs with a response magnitude of $<1 \times 10^{-4}$ were excluded from further analyses. In each run, we obtained maps of response magnitude ($\times 10^{-4}$) for both control and ischemic eyes. We obtained at least four maps per eye; average activities were used for further statistical analyses. To assess the quality of retinotopic maps, we used the calculation introduced by Cang et al.[26] Both the elevation and the azimuth maps were used to select the most responsive 3000 pixels (1.60 mm^2 of cortical space) in the visual cortex. For each of these pixels, the difference between its position and the mean position of its surrounding 25 pixels was calculated. For maps of high quality, the position differences were small because of smooth progression. The SD of the position difference was then used as an index of the quality of retinotopic maps, with small values indicating high map quality and high values indicating low map quality.[26] To calculate map area, magnitude maps were thresholded at 30% of peak amplitude, and the area of all selected pixels (1 pixel = 5.76×10^{-4} mm^2) was summed up as described previously.[15]

In addition, we created an index to quantitatively compare cortical activities induced by visual stimulation through the control or ischemic eye in the two hemispheres of individual mice. This bihemispheric activity index was calculated as follows: (activity of the hemisphere contralateral to visual stimulation of the ischemic eye/activity of the hemisphere contralateral to visual stimulation of the control eye) + (activity of the hemisphere ipsilateral to visual stimulation of the ischemic eye/activity of the hemisphere ipsilateral to visual stimulation of the control eye) ÷ 2. The index equals 1 if activity elicited by both eyes is the same and <1 if activity after stimulation of the ischemic eye is smaller than after stimulation of the control eye, and vice versa.

Statin Administration

The commercially available statin simvastatin was used in the present study (Calbiochem, Darmstadt, Germany). Simvastatin was dissolved in ethanol (50 mg/mL) plus 1 N NaOH. Before use, simvastatin was activated by adding 1 N HCl (pH 7.2). The amount of statin to be injected was further dissolved in sterile phosphate-buffered saline (PBS). Simvastatin (10 or 20 mg/kg; 100 μL) was injected intraperitoneally for 4 days, starting 1 hour or 24 hours after retinal ischemia/reperfusion. The vehicle groups were injected with a solution consisting of the same mixture of ethanol and NaOH used to dissolve simvastatin in the "therapy" experiments.

Evaluation of Retinal Ganglion Cell Densities in the Retinas of Normal and Ischemic Animals

RGCs were identified and quantified by means of anti–β-III-tubulin immunostaining. To this end, mice were killed by overdoses of 30% chloral hydrate 6 or 18 days after ischemia/reperfusion. Eyecups were fixed in 4% paraformaldehyde/PBS (pH 7.4) for 30 minutes and cryoprotected by infiltration with 30% sucrose/PBS at 4°C. Tissue specimens were then embedded (Tissue-Tek; (Shandon, Pittsburgh, PA) and snap-frozen in liquid nitrogen. Cryostat transversal sections (16 μm) were permeabilized with 0.3% Triton X-100 in PBS and preincubated with 10% normal goat serum for 2 hours at room temperature, followed by reaction with antibodies raised against β-III-tubulin (1:500; Covance, Berkeley, CA) at 4°C overnight (each in 2% normal goat serum or normal donkey serum). Specific immunoreactions were detected using appropriate Alexa Fluor 488–conjugated secondary antibodies (1:500; Molecular Probes, Eugene OR). To improve cell permeability, reaction solutions contained 0.3% Triton X-100 (Sigma, Taufkirchen, Germany) in 3% BSA/PBS. Specificity of the signal was verified by omitting the primary antisera. To specifically locate immunolabeled cells, retinas were incubated in the presence of 4,6-diaminido-2 phenylindole (DAPI) for 2 minutes and then washed twice with PBS. After the staining procedure, retinal slices were embedded in mounting medium (Moviol; Calbiochem, Darmstadt, Germany). Immunostaining was evaluated by means of a laser scanning microscope (LSM510; Zeiss, Jena, Germany). To quantify the number of RGCs in normal and lesioned eyes, labeled cells were counted in every fifth slice, and the amount was averaged and corrected for the total amount of slices to obtain the average number of RGCs/retina. Quantitative analyses of RGC numbers were performed on day 6 (30 minutes of retinal ischemia without treatment and after vehicle, 10 mg/kg and 20 mg/kg simvastatin treatment 1 hour after ischemia) or day 18 (vehicle, 10 mg/kg and 20 mg/kg simvastatin treatment 24 hours after ischemia) after retinal ischemia.

Statistical Analysis

All intergroup comparisons were made by Student's two-tailed *t*-test. In analyses in which a within-subject factor was present (statin treatment or vehicle treatment), one-way ANOVA with repeated measures was performed. The levels of significance were set as $P < 0.05$, $P < 0.01$, and $P < 0.001$. Data are represented as mean ± SEM.

Results

Behaviorally Determined Visual Acuity and Contrast Sensitivity

Analyses in the virtual reality optomotor system[16] revealed that control eyes of our C57Bl/6 mice had an average visual acuity of 0.39 ± 0.01 cyc/deg. Pilot tests showed that 60 minutes of retinal ischemia caused a complete loss of visual function (data not shown). In contrast, after 15 and 30 minutes of ischemia, spatial vision (visual acuity and contrast sensitivity) was significantly reduced in lesioned compared with control eyes but was still measurable. Given that an ischemia time of 15 minutes did not render a significant reduction of the number of surviving RGCs 6 days after ischemia and that after 60 minutes of ischemia animals were irreversibly blind, we decided to perform all further imaging, morphologic, and therapeutic experiments with animals that underwent 30 minutes of retinal ischemia. Visual acuity and contrast sensitivity values of control animals and of the control eyes of the two vehicle groups (vehicle after 1 hour and after 24 hours) did not differ significantly (*t*-test, all comparisons; $P > 0.05$); therefore, values were pooled for further analyses and data display.

Visual Acuity. After 15 minutes of retinal ischemia (data not illustrated), control eyes had a visual acuity of 0.392 ± 0.0 cyc/deg. Visual acuities of lesioned eyes were significantly

reduced compared with those of control eyes. Visual acuity dropped to 0.175 ± 0.02 cyc/deg on the first day after ischemia (*t*-test, control vs. ischemic eyes; $P < 0.01$) but recovered significantly to 0.251 ± 0.03 cyc/deg on day 6 (*t*-test; $P < 0.05$; $n = 4$). Although the visual acuity of control eyes was constant throughout the observation period, the visual acuity of ischemic eyes increased significantly by 44.5% from baseline (ANOVA; $P < 0.05$). Visual acuity on day 6 after retinal ischemia, however, remained significantly different from that of control eyes (*t*-test, control vs. ischemic eyes; $P < 0.05$).

After 30 minutes of retinal ischemia (Fig. 1A), visual acuity of control eyes was stable at 0.39 ± 0.00 cyc/deg ($n = 35$). On day 1 after ischemia, the visual acuity of ischemic eyes was significantly reduced compared with that of control eyes. It dropped to 0.172 ± 0.01 cyc/deg on the first day after ischemia (*t*-test, control vs. ischemic eyes; $P < 0.001$) but recovered to 0.243 ± 0.01 cyc/deg on day 6 (*t*-test; $P < 0.001$; $n = 35$), corresponding to a significant increase of 46% from baseline (ANOVA; $P < 0.001$). Again, with −38%, the visual acuity on day 6 after retinal ischemia remained significantly different from control eyes (*t*-test, control vs. ischemic eyes; $P < 0.001$).

Taken together, retinal ischemia of 30 minutes led to a significant decrease in visual acuity compared with control eyes on day 1, but, over the 6-day observation window, a significant recovery of visual function occurred. Values after 6 days, however, remained significantly lower than in control eyes without ischemia. Importantly, visual acuity never reached control values and recovery in ischemic eyes was maximal after 6 days (*t*-test; $P > 0.05$) because even after longer recovery times (see Fig. 4), visual acuities did not increase further. Thus, retinal ischemia in mice causes a significant reduction of behaviorally determined visual acuity.

Contrast Sensitivity. We measured contrast sensitivity of control and ischemic eyes on days 1, 3, and 6 after retinal ischemia at six different spatial frequencies. In general, contrast sensitivities were maximal at spatial frequencies of 0.064 cyc/deg in both control and ischemic eyes (Fig. 1B). Because changes in contrast sensitivities at that spatial frequency were representative of changes at the other frequencies measured, we focused on values at this spatial frequency for presenting our results. Values at all other spatial frequencies can be taken from the graphs. Compared with control eyes, contrast sensitivity was dramatically reduced in ischemic eyes. Values recovered slightly during the 6 days after ischemia but never reached control values.

After 15 minutes of retinal ischemia (data not illustrated), control eyes had a contrast sensitivity of 18.4 ± 0.9 (corresponding to 5.7% contrast) at a spatial frequency of 0.064 cyc/deg. Contrast sensitivity of ischemic eyes was reduced at all six spatial frequencies tested compared with control eyes. At a spatial frequency of 0.064 cyc/deg, it dropped significantly to 3.9 ± 0.8 (corresponding to 29% contrast) on day 1 and recovered slightly to 6.1 ± 1.3 (corresponding to 19.6% contrast) on day 6, but this increase was not significant (*t*-test, ischemic eye, day 1 vs. day 6; $P > 0.05$; $n = 4$). The increase on baseline was 58.8% but was not significant (ANOVA with repeated measures over time; $P > 0.05$). The increase on baseline at 0.064 cyc/deg did not differ between control and ischemic eyes (ANOVA with repeated measures, control eyes vs. ischemic eyes; $P > 0.05$).

After 30 minutes of retinal ischemia (Fig. 1B), control eyes had a contrast sensitivity of 14.5 ± 0.1 (corresponding to 7.1% contrast) at a spatial frequency of 0.064 cyc/deg. Contrast sensitivity of ischemic eyes was reduced at all six spatial frequencies tested compared with control eyes. At a spatial frequency of 0.064 cyc/deg, it dropped significantly to 1.7 ± 0.2 (corresponding to 70.6% contrast) on day 1 (*t*-test, control eyes vs. ischemic eyes; $P < 0.001$) and recovered significantly to 3.2 ± 0.4 (corresponding to 48.3% contrast) on day 6 (*t*-test, ischemic day 1 vs. day 6; $P < 0.001$; $n = 35$). Contrast sensitivities were significantly different between control and ischemic eyes on days 1, 3, and 6 after ischemia (*t*-test, all comparisons; $P < 0.001$). The difference in contrast sensitivity in control versus ischemic eyes on day 6 was −78%. At spatial frequencies of 0.064 cyc/deg, contrast sensitivities of ischemic eyes significantly increased on baseline by 71.8% between days 1 and 6 (ANOVA with repeated measures, control vs. ischemic eyes; $P < 0.001$). The increase on baseline (days 1–6) for the other spatial frequencies was as follows: 0.031 cyc/deg = 27.6%; 0.092 cyc/deg = 79.1%; 0.103 cyc/deg = 76.6%; 0.192 cyc/deg = 84.8%.

In conclusion, retinal ischemia of 30 minutes led to a significant decrease of both visual acuity and contrast sensitivity in the ischemic compared with the control eyes of mice, even

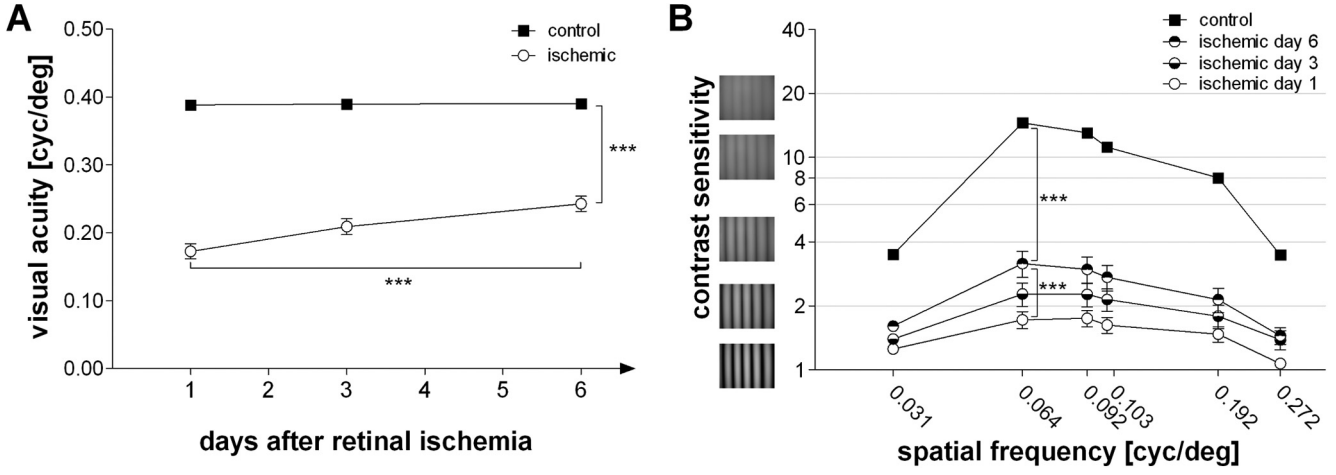

Figure 1. Retinal ischemia significantly impaired behaviorally determined visual capabilities of mice. Visual acuity (**A**) and contrast sensitivity (**B**) as measured by a virtual reality optomotor system on days 1, 3, and 6 after 30 minutes of retinal ischemia in both control and ischemic eyes. Control values were pooled values from control eyes of untreated or vehicle-treated ischemic mice. Note that visual acuity was significantly decreased on day 1 after ischemia and increased significantly between days 1 and 6, but values remained significantly lower than in controls. (**B**) Contrast sensitivity was measured at six different spatial frequencies. Contrast sensitivities of ischemic eyes were significantly reduced on day 1 after retinal ischemia compared with control eyes, recovered significantly during the 6-day recovery period, but remained significantly lower than in control eyes.

after 6 days of recovery. In ischemic eyes, visual acuity on day 6 was 0.306 ± 0.02 cyc/deg compared with 0.381 ± 0.00 cyc/deg in control eyes, corresponding to a reduction of 20%. Similarly, contrast sensitivities were significantly reduced in ischemic compared with control eyes. At a spatial frequency of 0.064 cyc/deg, contrast sensitivity of ischemic eyes was reduced to 3.2% ± 0.4% (48.3% contrast) compared with 14.6% ± 0.7% (7.0% contrast) in control eyes, corresponding to a reduction of 78%. Recovery was maximal after 6 days for visual acuity and for contrast sensitivities because even after longer recovery times, values did not increase further (*t*-tests; $P > 0.05$). Thus, retinal ischemia in mice lead to a significant reduction of both behaviorally determined visual acuity and contrast sensitivity in mice.

Optical Imaging

Mouse visual cortical responses were recorded in vivo using the imaging method developed by Kalatsky and Stryker[17] in both normal mice and in animals after monocular retinal ischemia. To address whether lesion-induced reduction of visual acuity was reflected in a similarly reduced visual cortical activity, we imaged this activity on day 6, immediately after the behavioral measurements in animals that underwent retinal ischemia for 30 minutes. Figure 2 shows two representative examples of the visual cortical activity maps after stimulation of either the ischemic or the control eye. Surprisingly, we recorded nearly identical activity maps after stimulation of normal and lesioned eyes, indistinguishable in both signal amplitude and quality of retinotopy. Visual cortical activity maps were recorded through the intact skull, and activities of both hemispheres were imaged simultaneously. As can be seen in the grayscale coded activity maps in Figures 2A and 2B, visual cortical activity in mice is dominated by input from the contralateral eye. After stimulation of the left (control) eye in the right hemisphere or the right (ischemic) eye in the left hemisphere, the activity patch is much darker, corresponding to higher cortical activation, than after stimulation of the ipsilateral eye. To thoroughly compare the optically recorded maps, we quantified both the magnitude of the visual cortical responses and the quality of the retinotopic maps according to published protocols.[26] In control animals, the magnitude of cortical activation after visual stimulation with a moving horizontal bar was 2.09 ± 0.09 ($n = 6$; Fig. 2C). In ischemic animals, cortical activation after visual stimulation of control eyes was similar at 2.26 ± 0.11 ($n = 5$; *t*-test; control animals vs. ischemic animals; $P > 0.05$). Visual cortical activation after stimulation of the ischemic eye was 2.19 ± 0.14 ($n = 5$; *t*-test; control eyes vs. ischemic eyes; $P > 0.05$) and thus indistinguishable from control values (Fig. 2C).

Similarly, after visual stimulation with a moving vertical bar (data not shown), the magnitude of cortical activation in control animals was 2.37 ± 0.15 ($n = 6$). In ischemic animals, cortical activation after visual stimulation of control eyes was similar at 2.47 ± 0.15 ($n = 3$; *t*-test; control animals vs. control eyes of ischemic animals; $P > 0.05$). Visual cortical activation after stimulation of the ischemic eye was 2.36 ± 0.35 ($n = 4$; *t*-test; control eyes vs. ischemic eyes; $P > 0.05$) and thus also indistinguishable from control values.

Because the magnitude of visual cortical activation was thus far always quantified in the hemisphere contralateral to the stimulated eye—that is, the hemisphere dominated by that eye (the left hemisphere after right [ischemic] eye stimulation and the right hemisphere after left eye stimulation)—activity values in different hemispheres were compared. To avoid any problems that might arise from lateralization of visual cortical activation or large interindividual variability, we designed an additional quantification (i.e., an index that takes activity in both hemispheres and in individual animals into account; Fig. 2D).

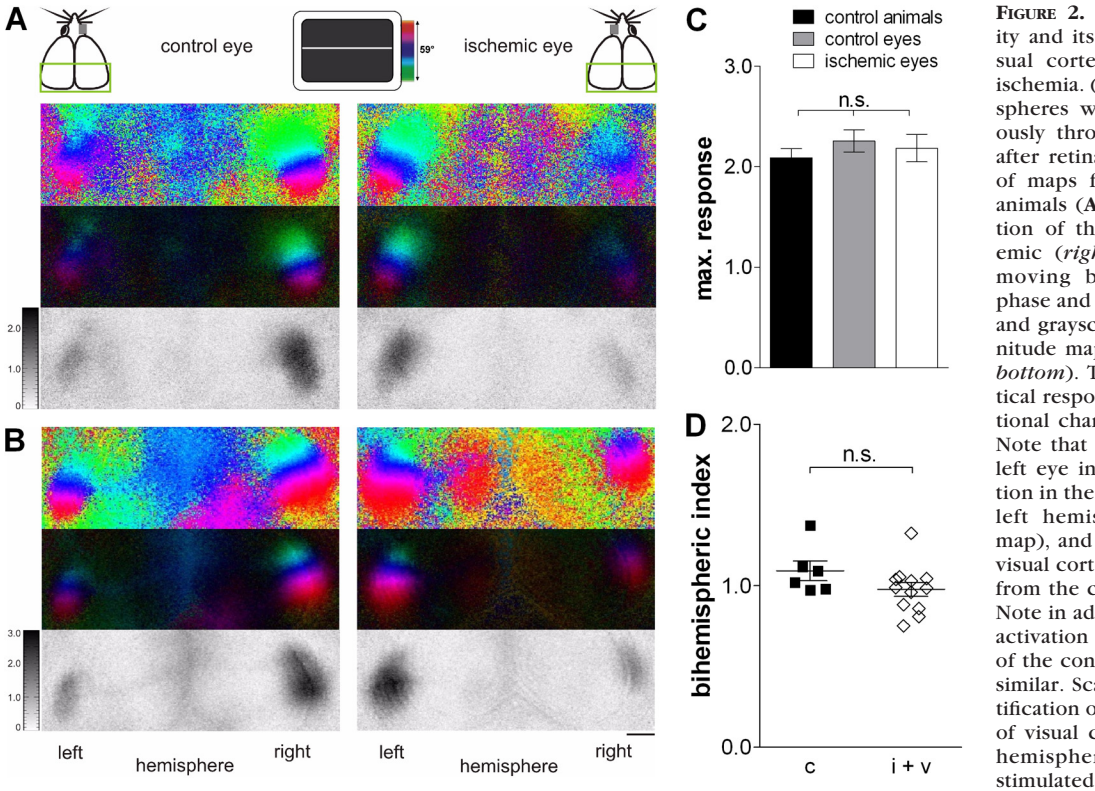

FIGURE 2. Optically imaged activity and its quantification in the visual cortex of mice after retinal ischemia. (**A**, **B**) Maps of both hemispheres were recorded simultaneously through the skull on day 6 after retinal ischemia. Comparison of maps from two representative animals (**A**, **B**) after visual stimulation of the control (*left*) or ischemic (*right*) eye with horizontal moving bars. Both color-coded phase and polar maps of retinotopy and grayscale-coded response magnitude maps are illustrated (*top* to *bottom*). The magnitude of the optical responses is illustrated as fractional change in reflection $\times 10^{-4}$. Note that visual stimulation of the left eye induced a stronger activation in the right compared with the left hemisphere (darker activity map), and vice versa, showing that visual cortex is dominated by input from the contralateral eye in mice. Note in addition that visual cortical activation induced by stimulation of the control or ischemic eye was similar. Scale bar, 1 mm. (**C**) Quantification of the magnitude ($\times 10^{-4}$) of visual cortical responses in the hemisphere contralateral to the stimulated eye in control and ischemic mice (after both ischemic and control eye stimulation). (**D**) Comparison of the bihemispheric activity index in control (c) and ischemic animals after vehicle treatment starting 1 hour after retinal ischemia (I + vehicle).

As detailed in Materials and Methods, our bihemispheric activity index equaled 1 if activity elicited by both eyes was the same and <1 if activity after stimulation of the ischemic eye was smaller than in control eye stimulation, and vice versa. In control animals (Fig. 2D, c), this bihemispheric activity index was 1.09 ± 0.06 (n = 6). In mice that received retinal ischemia and vehicle treatment after 1 hour (Fig. 2D, i + v), it was 0.98 ± 0.04 (n = 12). Thus, the indices did not differ significantly (t-test; $P > 0.05$), indicating that in individual animals, visual stimulation through the lesioned eye activated the visual cortex as strongly as after control eye stimulation (Fig. 2D).

Quantification of the quality of the retinotopic maps after visual stimulation of the control or ischemic eyes revealed no statistical differences for elevation or for azimuth maps (t-test; all comparisons; $P > 0.05$).

Given that reduced visual acuity could also have been reflected in a reduced cortical representation, we also quantified whether map area differed after stimulation of ischemic or control eyes. The cortical regions activated by moving horizontal bars (elevation map) were, on average, 2.53 ± 0.26 mm^2 (n = 3) for ischemic eyes, 2.26 ± 0.22 mm^2 (n = 2) for control eyes, and 2.31 ± 0.1 mm^2 (n = 6) for control animals. These sizes were not significantly different (t-test; $P > 0.05$). Thus, there were no differences in map area between control and ischemic eye stimulation or between ischemic and control animals.

Influence of Retinal Ischemia on Retinal Morphology and Retinal Ganglion Cell Survival

In anti–β-III-tubulin immunostained retinal sections, 15 minutes of retinal ischemia did not render a significant reduction of the number of surviving RGCs 6 days after ischemia. After 60 minutes of ischemia, animals were irreversibly blind as determined by optometry; therefore, we decided to perform all further experiments with animals that underwent 30 minutes of retinal ischemia.

As illustrated in Figure 3A, 30 minutes of retinal ischemia had a severe effect on gross retinal morphology in mice, primarily squeezing the inner plexiform layer, which showed an average of approximately 18% reduction in thickness.

Quantification of the histologic sections revealed a mean number of RGCs of 123,819 ± 3,951 (n = 6) in retinas of unoperated (control) animals (Fig. 3B). After 30 minutes of retinal ischemia, the number of RGCs/retina significantly decreased to 103,907 ± 5,090 (n = 6; t-test; $P < 0.05$), corresponding to a reduction of 16% compared with control retinas.

Visual Acuity and Contrast Sensitivity after Simvastatin Treatment

We analyzed a total of six groups of mice that received 30 minutes of retinal ischemia and underwent six different therapeutic regimens: ischemia only, ischemia + vehicle or 10 or 20 mg/kg simvastatin, applied either 1 or 24 hours after retinal ischemia (Figs. 4, 5). Animals that underwent treatment received statin or vehicle solution daily for 4 days after ischemia.

Visual Acuity

Groups Treated Starting 1 Hour after Lesion (Figs. 4A, 4B). Visual acuity of the control eyes in the vehicle-treated group was constant at 0.389 ± 0.00 cyc/deg throughout the observation period. Visual acuity of the ischemic eyes dropped to 0.179 ± 0.02 cyc/deg on day 1 and increased significantly to 0.246 ± 0.02 cyc/deg on day 6 (t-test; $P < 0.01$; n = 10; Fig. 4A). The increase on baseline was 41.9% and was significant (ANOVA with repeated measures; $P < 0.01$; Fig. 4B).

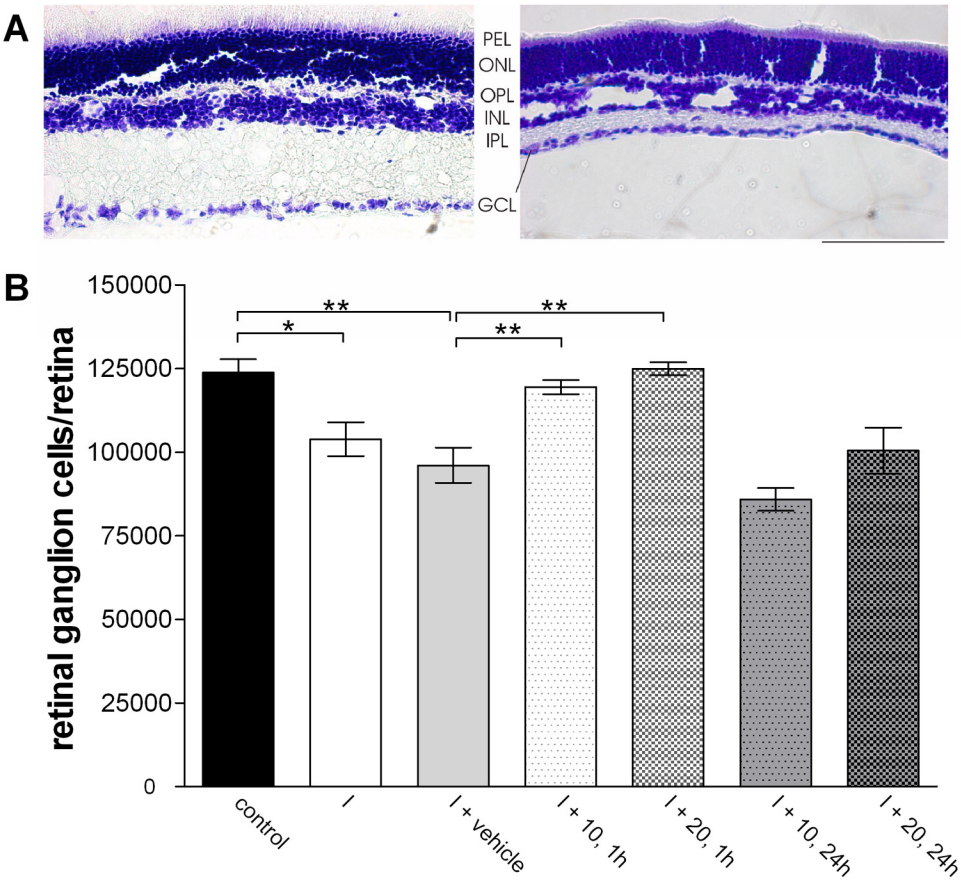

Figure 3. Effect of 30 minutes of retinal ischemia on (**A**) retinal morphology and (**B**) ganglion cell survival. (**A**) Representative section through both a control (*left*) and an ischemic (*right*) retina is illustrated. Note that after ischemia, retinal layers, particularly OPL and IPL, are severely shrunk. PEL, pigment epithelial layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 100 μm. (**B**) Number of retinal ganglion cells (RGCs) in the retinas of control and ischemic (I) mice and after various treatment strategies. Untreated, I; vehicle-treated, I + vehicle; administration of 10 mg/kg (I + 10, 1 hour) or 20 mg/kg simvastatin (I + 20, 1 hour) starting 1 hour after ischemia, administration of 10 mg/kg (I + 10, 24 hours) or 20 mg/kg simvastatin starting 24 hours after injury (I + 20, 24 hours).

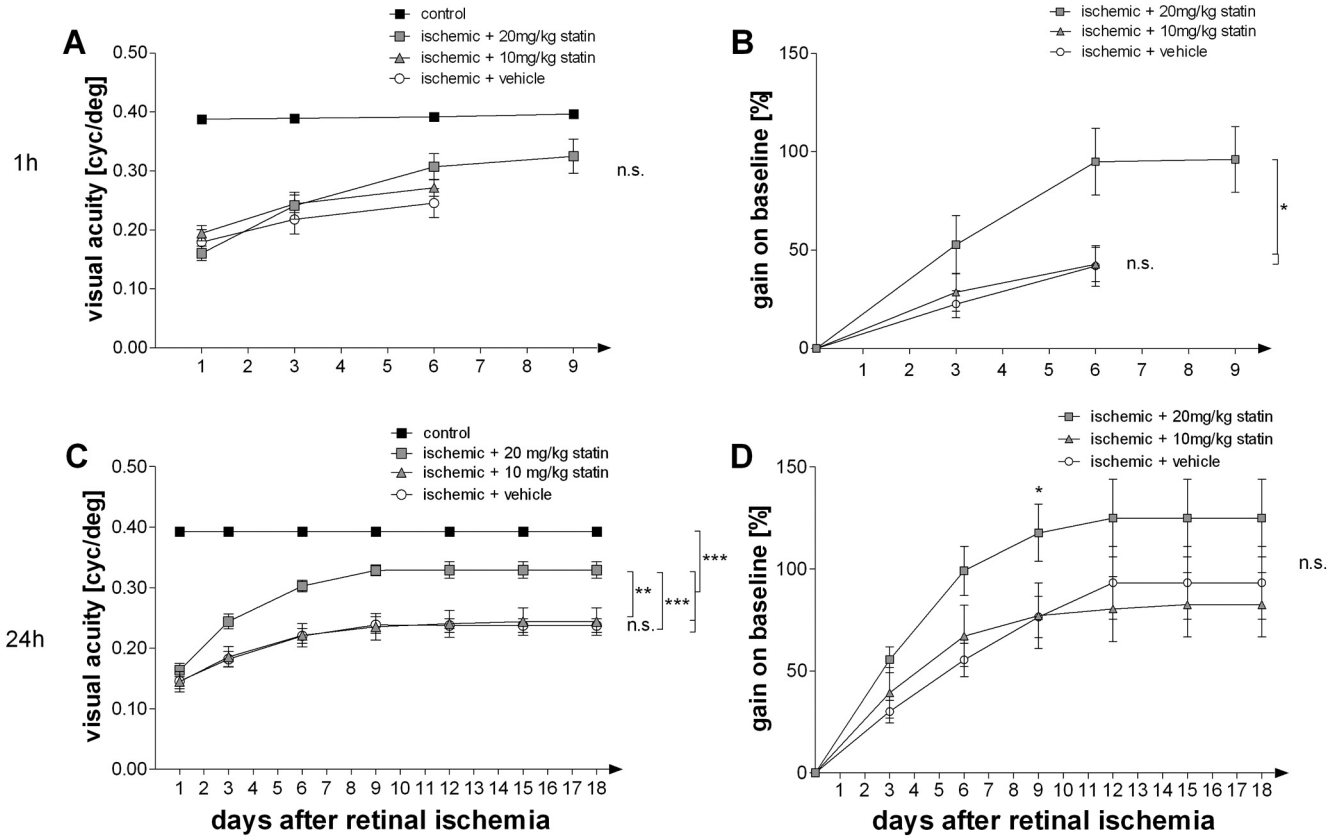

FIGURE 4. Effect of various regimens of simvastatin treatment on visual acuity in mice with retinal ischemia. Absolute changes (cyc/deg; **A**, **C**) and gain on baseline (%; **B**, **D**) are illustrated. Visual acuity was measured in a virtual reality optomotor system and plotted as a function of days after retinal ischemia. Statin treatment was started either 1 hour (**A**, **B**) or 24 hours (**C**, **D**) after retinal ischemia. When statin treatment was started 1 hour after retinal ischemia, there was no significant difference between visual acuities of the different treatment groups (**A**), but the gain on baseline was significantly higher after treatment with 20 mg/kg compared with 10 mg/kg or vehicle treatment (**B**). When statin treatment was started after 24 hours, there was a significant increase in visual acuity after treatment with 20 mg/kg compared with both 10 mg/kg or vehicle treatment (**C**) but not in gain on baseline (**D**).

After treatment with 10 mg/kg simvastatin, visual acuity of the control eyes was 0.39 ± 0.00 cyc/deg. Visual acuity of the ischemic eyes increased significantly from 0.195 ± 0.01 cyc/deg on day 1 to 0.271 ± 0.01 cyc/deg on day 6 (*t*-test; increased visual acuity; $P < 0.001$; $n = 11$; Fig. 4A). The increase on baseline was 42.8% and was significant (ANOVA with repeated measures; $P < 0.001$; Fig. 4B).

After treatment with 20 mg/kg simvastatin, visual acuity of the control eyes was 0.392 ± 0.00 cyc/deg. Visual acuity of the ischemic eyes significantly increased from 0.160 ± 0.01 cyc/deg on day 1 to 0.307 ± 0.02 cyc/deg on day 6 (*t*-test; increase visual acuity; $P < 0.01$) and to 0.325 ± 0.03 cyc/deg on day 9 (*t*-test; increased visual acuity; $P < 0.05$) (Fig. 4A). The increase on baseline was 95% until day 6 (ANOVA with repeated measures, increase on baseline over time; $P < 0.001$; $n = 5$) and 96.1% until day 9 (ANOVA with repeated measures; $P > 0.05$; $n = 3$) (Fig. 4B).

Because visual acuity values of the control eyes in the different treatment groups did not differ significantly (*t*-test; $P > 0.05$), acuity values were pooled for further analyses and data display. Taken together, recovery of visual acuity of the ischemic eye in the group that received 20 mg/kg simvastatin was significantly higher than in animals treated with vehicle. The gain on baseline in visual acuity for the 20-mg/kg simvastatin group was 95% compared with 42.8% for the 10-mg/kg group and 46% for the vehicle-treated group. This gain on baseline was significantly higher than in vehicle-treated animals and animals treated with only 10 mg/kg simvastatin (Fig. 4B; ANOVA, for both comparisons; $P < 0.05$). In addition, in all three groups, there was a significant increase in visual acuity on baseline over time, but values always remained significantly lower than in control eyes (see also Fig. 1A; ANOVA with repeated measures, over time; $P < 0.001$).

Groups Treated Starting 24 Hours after Lesion (Figs. 4C, 4D). Visual acuity of the control eyes in the vehicle-treated group was 0.393 ± 0.00 cyc/deg throughout the observation period. Visual acuity of the ischemic eyes in this group dropped to 0.145 ± 0.01 cyc/deg on day 1 and significantly increased to 0.237 ± 0.01 cyc/deg on day 18 (*t*-test, increased visual acuity; $P < 0.001$; Fig. 4C), corresponding to a significant increase on baseline of 93.1% (ANOVA with repeated measures, increase on baseline over time; $P < 0.001$; days 1–9: $n = 19$; days 12–18: $n = 10$; Fig. 4D).

After treatment with 10 mg/kg simvastatin, visual acuity of the control eyes was 0.392 ± 0.00 cyc/deg. Visual acuity of the ischemic eyes in this group also increased significantly from 0.145 ± 0.02 cyc/deg on day 1 to 0.244 ± 0.02 cyc/deg on day 18 (*t*-test, increased visual acuity; $P < 0.001$; Fig. 4C), corresponding to a significant increase on baseline of 82.4% (ANOVA with repeated measures, increase on baseline over time, $P < 0.001$; $n = 11$; Fig. 4D).

After treatment with 20 mg/kg simvastatin, visual acuity of the control eyes was 0.393 ± 0.00 cyc/deg. Visual acuity of the ischemic eyes in this group was 0.163 ± 0.01 cyc/deg on day 1 and increased significantly to 0.329 ± 0.01 cyc/deg on day 18 (*t*-test, increased visual acuity; $P < 0.001$; Fig. 4C), correspond-

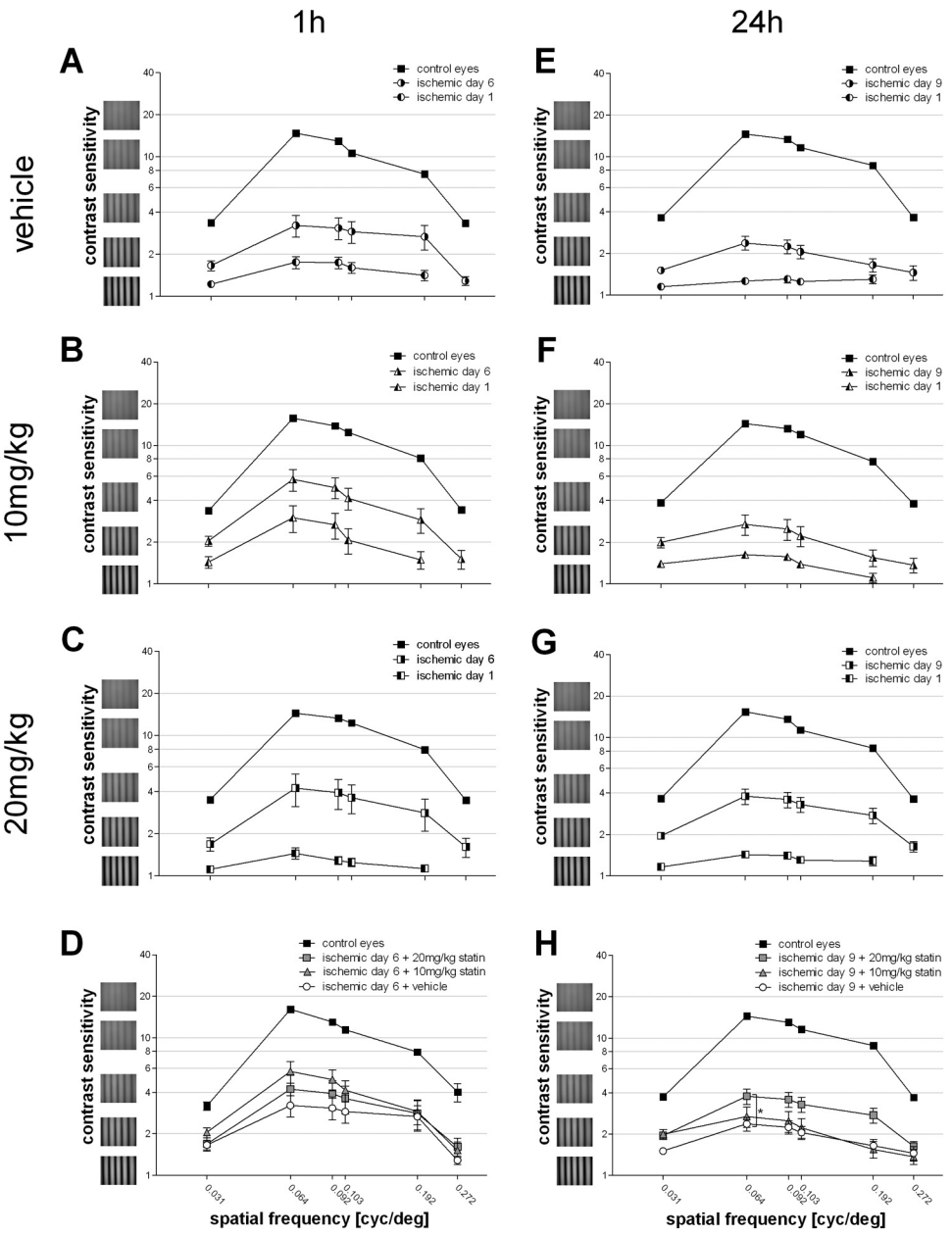

FIGURE 5. Effect of various regimens of simvastatin treatment on contrast sensitivity in mice with retinal ischemia. Contrast sensitivity, measured at six different spatial frequencies, after vehicle treatment (**A**, **E**) and treatment with either 10 mg/kg (**B**, **F**) or 20 mg/kg simvastatin (**C**, **G**), started 1 hour (**A–D**) or 24 hours (**E–H**) after retinal ischemia. (**D**) Comparison of contrast sensitivities on day 6 in the various treatment groups, when treatment started 1 our after retinal ischemia. (**H**) Comparison of contrast sensitivities on day 9 in the various treatment groups, when treatment started 24 hours after retinal ischemia.

ing to a significant increase on baseline of 124.9% (ANOVA with repeated measures, increase on baseline over time; $P < 0.001$; days 1–9, $n = 21$; days 12–18, $n = 11$; Fig. 4D).

Visual acuities after treatment with vehicle or 10 mg/kg simvastatin (24 hours) did not differ significantly from each other (ANOVA with repeated measures; $P > 0.05$). However, after treatment with 20 mg/kg simvastatin 24 hours after retinal ischemia, visual acuity of the ischemic eye was significantly higher than with both vehicle or 10 mg/kg treatment (ANOVA, 20 mg/kg vs. vehicle, $P < 0.001$; 20 mg/kg vs. 10 mg/kg, $P < 0.01$). The gain on baseline for visual acuity in the 24-hour delay treatment groups revealed, however, no significant increase after treatment with 20 mg/kg compared with treatment with vehicle or 10 mg/kg (ANOVA, for all comparisons; $P > 0.05$).

Thus, although acuity values generally increased with vehicle treatment (Figs. 1A, 1B, 4A, 4C), visual acuity values always remained lower than in simvastatin-treated animals. On the other hand, visual acuity of ischemic eyes never reached control values, even after simvastatin treatment.

Contrast Sensitivity

We measured contrast sensitivity of control and ischemic eyes at days 1, 3, and 6 (all groups; Fig. 5) and at days 9, 12, 15, and 18 (24-hour delay treatment groups; Figs. 5E-H) after retinal ischemia at six different spatial frequencies. Because contrast sensitivity values were highest at a spatial frequency of 0.064 cyc/deg, values at that frequency were exemplarily compared in the different treatment groups. As expected from our analyses with untreated and vehicle-treated mice, contrast sensitivity values of ischemic eyes decreased dramatically compared with control eyes on day 1 after ischemia. Values slightly recovered over the 6 or 18 days, respectively, after ischemia but never reached control values. Given that only some animals were tested until day 18 and there were no significant changes in contrast sensitivity after day 9 (test; $P > 0.05$), contrast sensitivity data are shown only until day 9.

Groups Treated Starting 1 Hour after Lesion (Figs. 5A–D). In the vehicle-treated group (Fig. 5A), contrast sensitivity of control eyes was 14.7 ± 0.2 (corresponding to 6.9%

contrast) at a spatial frequency of 0.064 cyc/deg. Thirty minutes of retinal ischemia reduced contrast sensitivity of the ischemic eyes at 0.064 cyc/deg nearly 10-fold to 1.7 ± 0.2 (corresponding to 63.9% contrast) on day 1, but sensitivity recovered significantly to 3.2 ± 0.6 (corresponding to 42.2% contrast) on day 6 (*t*-test; $P < 0.001$; $n = 10$). In ischemic eyes, there was a significant increase (81.1%) on baseline between days 1 and 6 (ANOVA with repeated measures; $P < 0.05$).

In the group treated with 10 mg/kg simvastatin (Fig. 5B), contrast sensitivity of control eyes was 15.7 ± 0.4 (corresponding to 6.6% contrast) at a spatial frequency of 0.064 cyc/deg. Contrast sensitivity of the ischemic eyes in this group was reduced approximately fivefold to 3.0 ± 0.6 (corresponding to 45.3% contrast) on day 1 and increased significantly to 5.7 ± 1.0 (corresponding to 26.6% contrast) on day 6 (*t*-test; $P < 0.001$; $n = 10$). The increase on baseline between days 1 and 6 was significant at 95.6% (ANOVA with repeated measures; $P < 0.01$).

After treatment with 20 mg/kg simvastatin (Fig. 5C), contrast sensitivity of the control eyes was 14.4 ± 0.2 (corresponding to 7.0% contrast) at a spatial frequency of 0.064 cyc/deg. Contrast sensitivity of the ischemic eyes in this group was reduced 10-fold to 1.4 ± 0.1 (corresponding to 72.3% contrast) on day 1 and increased significantly to 4.2 ± 1.1 (corresponding to 30.5% contrast) on day 6 (*t*-test; $P < 0.05$; $n = 5$). Although contrast sensitivity of treated ischemic eyes increased on average by 219.4% on baseline, this gain was not statistically significant (ANOVA with repeated measures; $P > 0.05$).

Groups Treated Starting 24 Hours after Lesion (Figs. 5E–H). After vehicle treatment (Fig. 5E) starting 24 hours after retinal ischemia, contrast sensitivity of the control eyes was 14.6 ± 0.2 (corresponding to 7.0% contrast) at a spatial frequency of 0.064 cyc/deg. Contrast sensitivity of the ischemic eyes in this group was decreased 11-fold to 1.3 ± 0.1 (corresponding to 83.9% contrast) on day 1 and increased significantly to 2.4 ± 0.3 (corresponding to 52.2% contrast) on day 9 after retinal ischemia (*t*-test; $P < 0.001$; $n = 19$). The increase on baseline of ischemic eyes was 84.7% and was significant (ANOVA with repeated measures; $P < 0.001$).

After treatment with 10 mg/kg simvastatin (Fig. 5F), contrast sensitivity of the control eyes was 14.4 ± 0.2 (corresponding to 7.1% contrast) at a spatial frequency of 0.064 cyc/deg. Contrast sensitivity of the ischemic eyes in this group was decreased 9-fold to 1.6 ± 0.1 (corresponding to 64.8% contrast) on day 1 after ischemia and increased significantly to 2.7 ± 0.5 (corresponding to 45.6% contrast) 9 days later (*t*-test; $P < 0.001$; $n = 11$). The increase on baseline was significant at 68.3% (ANOVA with repeated measures; $P < 0.01$).

After treatment with 20 mg/kg simvastatin (Fig. 5G), contrast sensitivity of the control eyes was 15.4 ± 0.2 (corresponding to 6.7% contrast) at a spatial frequency of 0.064 cyc/deg. Contrast sensitivity of the ischemic eyes in this group was reduced 11-fold to 1.4 ± 0.1 (corresponding to 74.5% contrast) on day 1 and increased significantly to 3.8 ± 0.5 (corresponding to 36% contrast) on day 9 (*t*-test; $P < 0.001$; $n = 21$). The increase on baseline was significant at 165.5% (ANOVA with repeated measures; $P < 0.001$).

Taken together, in all three "treatment" groups (vehicle, 10 mg/kg simvastatin, 20 mg/kg simvastatin), contrast sensitivities increased significantly between days 1 and 9 after retinal ischemia (*t*-test, all groups; $P < 0.001$) but never reached control eye values (test, $P < 0.001$). Intergroup comparison of the gain on baseline in percentage revealed a significant improvement after 20 mg/kg treatment (*t*-test, 20 mg/kg vs. 10 mg/kg and vehicle: $P < 0.05$; see also Fig. 5H).

Contrast sensitivities of control eyes did not differ among the six different treatment groups (*t*-test; $P > 0.05$ for all comparisons); therefore, these data were pooled for the summary plots in Figures 5D and 5H. After statin treatment, recovery of contrast sensitivities was slightly more pronounced than in vehicle-treated animals (Figs. 5B-D, 5F-H). Differences in contrast sensitivities were most pronounced at a spatial frequency of 0.064 cyc/deg. The summary plot in Figure 5D illustrates that treatment with 10 mg/kg simvastatin that started 1 hour after ischemia caused the largest recovery of contrast sensitivities compared with vehicle treatment, but differences were not significant at 0.064 cyc/deg (*t*-test; $P > 0.05$). At a spatial frequency of 0.064 cyc/deg, treatment with 20 mg/kg simvastatin after 24 hours improved contrast sensitivity by 166% compared with 85% in the vehicle-treated group. In addition, absolute contrast sensitivity values recovered to 2.4 (corresponding to 52% contrast) in the vehicle-treated group (Fig. 5E) and to 3.8 (corresponding to 36% contrast) in the 20 mg/kg simvastatin–treated group (after 24 hours; Fig. 5G). These differences between statin-treated and vehicle-treated animals were significant (*t*-test, improvement in contrast sensitivity, vehicle treatment [$n = 19$] vs. 20 mg/kg simvastatin treatment [$n = 21$]; $P < 0.05$). The intergroup comparison of the gain on baseline in percentage was significantly improved after treatment with 20 mg/kg statin after 24 hours (*t*-test, gain on baseline in percentage; 20-mg/kg treatment vs. 10-mg/kg treatment and vehicle treatment; $P < 0.05$).

In addition, contrast sensitivities of all treatment groups remained significantly lower than in control eyes (*t*-test, after 1 hour: control vs. ischemic eyes after vehicle [$n = 10$] or 10 mg/kg [$n = 10$], $P < 0.01$, control vs. ischemic eyes after 20 mg/kg [$n = 5$], $P < 0.05$; after 24 hours: control vs. ischemic eyes after vehicle [$n = 19$], 10 mg/kg [$n = 11$], or 20 mg/kg [$n = 21$]; $P < 0.001$).

Statin Treatment Rescues RGCs after Acute Retinal Ischemia Reperfusion

We tested four different simvastatin treatment regimens for their ability to improve the survival of RGCs after retinal ischemia/reperfusion, as follows: daily administration of 10 or 20 mg/kg simvastatin applied for 4 days, starting either 1 hour or 24 hours after ischemia (the quantification of ganglion cell numbers is illustrated in Fig. 3B); daily administration of 10 mg/kg simvastatin starting 1 hour after ischemia (I + 10, 1 hour) significantly improved RGC survival by 24% compared with vehicle-treated ischemic retinas (I + vehicle) after 9 days of reperfusion (119,465 ± 2164 [$n = 6$] vs. 96,049 ± 5282 [$n = 9$] RGCs/retina; *t*-test, $P < 0.01$). Similarly, the administration of 20 mg/kg simvastatin starting 1 hour after lesion significantly improved RGC survival by 30% compared with vehicle treatment (124,940 ± 1931 [$n = 4$] vs. 96,049 ± 5282 [$n = 6$] RGCs/retina; *t*-test; $P < 0.01$; Fig. 3B). RGC numbers of simvastatin-treated mice (10 or 20 mg/kg, delivered 1 hour after ischemia) were indistinguishable from controls (*t*-test, $P > 0.05$).

In contrast, the administration of 10 or 20 mg/kg simvastatin applied 24 hours after ischemia (I + 10, 24 hours; I + 20, 24 hours) had no significant effect on RGC survival compared with vehicle-treated mice (10 mg/24 hours: 85,871 ± 3419 RGCs/retina, $n = 4$, *t*-test, $P > 0.05$; 20 mg/24 hours: 100,488 ± 6858 RGCs/retina, $n = 9$, *t*-test, $P > 0.05$; Fig. 3B).

Intraperitoneal delivery of vehicle solution did not modify RGC numbers compared with untreated ischemic mice (96,049 ± 5282 [$n = 6$] vs. 103,907 ± 5090 [$n = 6$] RGCs/retina; *t*-test, $P > 0.05$).

To test whether the numbers of surviving RGCs influence visual acuity, we correlated RGC numbers with visual acuity in individual animals. Interestingly, however, there was no correlation between RGC numbers and visual acuity ($R = 0.318$; $P > 0.05$).

Taken together, treatment with 10 or 20 mg/kg simvastatin, delivered 1 hour after ischemia, significantly rescued RGC survival to values comparable to those of control animals, whereas statin treatment after 24 hours had no effect on cell survival.

Discussion

Summary of Results

Combining behavioral measurements of visual capabilities, intrinsic signal optical imaging, and anatomy, we here show that retinal ischemia significantly reduced visual acuity, contrast sensitivity, and RGC survival in mice. Although there was pronounced recovery of visual functions over 6 days without treatment or in vehicle-treated animals, visual acuity improvement was significantly higher in animals treated with 20 mg/kg simvastatin, applied in a therapeutic regimen either 1 hour or 24 hours *after* ischemia. The same dosage of simvastatin also significantly improved contrast sensitivity compared with vehicle treatment, but visual function never regained control values. Finally, 10 or 20 mg/kg simvastatin also significantly improved RGC survival when the drug was administered 1 hour after ischemia compared with administration after 24 hours or no treatment. Surprisingly, optical imaging of intrinsic signals in the same animals revealed that visual cortical activity was indistinguishable after stimulation of control or ischemic eyes.

Effect of Ischemia on Visual Capabilities

Effects of ischemia and possible neuroprotective actions are usually quantified by morphology.[8] However, functional analyses become especially important when pharmacologic principles are to be translated into clinical treatments.[27] Here we show for the first time that visual functional changes induced by a mild ischemic lesion can be repeatedly and accurately measured in awake mice after injury. Many diseases of the visual system are manifested as, and defined by, abnormalities in vision, and it is almost always the lack of normal vision that brings people to the clinic.[28] Therefore, an animal model of a visual disease must include a measure of visual function to ensure that it is relevant to the human condition. Prusky et al.[16] has developed a virtual reality optomotor system that provides a simple and precise method for rapidly and reliably quantifying mouse vision in awake animals, thus avoiding known effects elicited by narcotics on electrophysiological recordings.[29] Using this method, we could recently demonstrate that there is an age-dependent decline of sensory improvement after monocular deprivation in adult mice.[18] Since from the functional point of view the visual system is an integrated sensorimotor system, normal function is not possible without a variety of visuomotor reflexes to ensure that a relatively stable image of the visual field is maintained on the retina. One of these reflexes, the optokinetic response (OKR), compensates for the motion of the visual field, using the relative velocity of the image on the retina to induce head movements in the same direction and about the same velocity as in the external world in rodents.[28]

Standard ERG recordings have been used extensively for functional assessment of the retina before, during, and after ischemia. The b-wave of the ERG is taken as a particularly sensitive index of retinal ischemia because it has been shown both in humans[30] and in experimental models of retinal ischemia in vivo.[12] Although the origin of the normal b-wave is well established, the exact cause for its reduction during and after retinal ischemia is not.[5] Retinal ischemia produced by raised IOP lasting 90 minutes or more induced retinal damage that is most pronounced in the inner retinal layers— ganglion cells, inner plexiform layer, and inner nuclear layer—in rats.[31] An extinction of the b-wave can be observed immediately on raising the IOP.[32] The extent of recovery depends on the duration of the ischemia. In cats, elevated IOP for 90 or 120 minutes was followed by an incomplete recovery of the b-wave by as much as 50% or 10%, respectively, of the preischemic value.[33] In contrast, reperfusion after 30 or 50 minutes of ischemia resulted in a complete recovery of the b-wave.[33] More recently, Zhu et al.[34] found no recovery in the b-wave amplitude 1 week after 30 or 45 minutes of elevated IOP in mice. Since there are anatomic and biomechanical differences between eyes of different species, ERG responses, particularly the b-wave, might also be different after IOP elevation.

In mice it was shown that the inner retina–derived scotopic threshold response (STR), which has a significant ganglion cell contribution, is more sensitive to acute IOP elevation and recovers more slowly than other ERG components arising from photoreceptors (a-wave) and bipolar cells (b-wave). A reduction in positive STR (pSTR) and oscillatory potentials 1 week after IOP elevation suggested a persistent impairment of inner retinal function.[35] Available data suggest that functional assessment of ischemic damage by means of electrophysiological techniques, including ERG and VEP, may be a more sensitive parameter than conventional histopathologic quantification.[36] Several studies found a diminished b-wave despite near normal histology.[10]

Interestingly, here we show that although there was an increase in RGC survival and a significant recovery in spatial vision in mice after 30 minutes of ischemia and simvastatin treatment, visual acuity values never reached preischemic levels in the time evaluated. The persistent impairment in visual function might suggest that in spite of being protected, RGCs are still functionally impaired. In support of this, it was shown that not all RGCs that survive ischemia retain their capacity for retrograde axonal transport.[37] In addition, other retinal cell types, including amacrine cells, are affected by ischemic episodes, and this may contribute to visual impairment.[5] A further cause of persistent visual impairment might involve mechanical compression of retinal layers after elevated IOP, which affects light access to photoreceptors. The role of these additional aspects deserves further research.

McGill et al.[38] used full-field scotopic and photopic ERG amplitudes and spatial frequency thresholds of the OKR of adult rats to measure the effect of ciliary neurotrophic factor on normal visual function. The study demonstrated a clear segregation of the effect of ciliary neurotrophic factor on the ERG and OKR, with the OKR being more sensitive than the ERG. Results of our study indeed demonstrate that measurement of OKR allows for sensitive measurement of ischemia-induced changes in visual capabilities in mice and the effect elicited by pharmacologic treatments.

Lesion-Induced Changes in Cortical Activity

After the interruption of normal inputs to a region of somatosensory cortex, changes in the receptive fields of neurons can be observed even in the first hours after the lesion.[39,40] After the removal of visual input by focal binocular retinal lesions in monkeys and cats, electrophysiological recordings revealed changes in the sizes of receptive fields and the responsiveness of cortical cells near the edge of the retinal scotoma. Two months after the lesion, cortical areas that were initially silenced by the lesion recovered visual activity, and receptive field positions originally located at the center of lesion were shifted and enlarged.[41] In addition, short episodes of monocular deprivation cause a significant shift of the ocular dominance of neurons in the binocular part of the visual cortex toward the nondeprived eye in mice up to the age of 3 months,

as determined by intrinsic signal optical imaging.[18] We therefore hypothesized that a loss of visual input because of the lesion-induced death of RGCs might modify cortical activity in adult mice after acute retinal/reperfusion injury. Our data showed, however, that retinal ischemia had no measurable effect on visual cortical activity as recorded by intrinsic signal optical imaging. Size, magnitude and retinotopic patterns of activity were indistinguishable after stimulation of the control or lesioned eye. Presumably, a monocular loss of <20% of RGCs, as in our mouse model of mild ischemia, is insufficient to significantly decrease the activity of visual cortical cells. This conclusion is underscored by our recent observation in Bassoon-mutant mice[15] in which severely reduced visual acuity did not also result in altered visual cortical maps. The visual cortex of Bassoon-mutant mice was as strongly activated as in normal mice and sustained normal retinotopic maps, as observed in the present study. We take this as a strong indication of homeostatic mechanisms.[42] Although it is generally believed that Hebbian-type network modifications are crucial for shaping cortical circuits as a result of sensory experience, additional homeostatic plasticity may ensure that network compensation can be achieved in response to a wide range of sensory perturbations.[43,44] We therefore suggest that after retinal ischemia, homeostatic synaptic scaling has adjusted excitatory synaptic strengths of neurons in the afferent visual pathway or in the cortex to compensate for the reductions in activity in the retina. Overall our results demonstrate that the central visual system has an extraordinarily high potential to process altered inputs after an ischemic insult in the retina and, thus, to be instrumental in sustaining behaviorally relevant visual capabilities.

Treatment Strategies

The possible causes of retinal ischemia suggest a number of obvious targets for therapy; however, most approaches are associated with a temporal delay, during which the retina undergoes ischemic damage.[5] In many animal studies, potentially neuroprotective compounds were often administered *before* the induction of retinal ischemia. This is, however, unlikely to happen in the clinical management of ischemic retinopathies, during which administration of neuroprotective agents can be started only *after* onset of injury.[5]

Statin Therapy

To the best of our knowledge, only one study has actually documented the effects of statins on the function of the rat retina after ischemia/reperfusion injury (Bu P, et al. *IOVS*. 2007;ARVO E-Abstract 208). In this study, however, simvastatin (20 mg/kg, intraperitoneally) was administered prophylactically for 3 days. Statin treatment protected against lesion-dependent deficits in the retinal function of Lewis rats, as measured by scotopic ERGs. In marked contrast to untreated animals, ERG a- and b-wave responses from simvastatin-pretreated rats were not significantly affected by the ischemic/reperfusion insult (Bu P, et al. *IOVS*. 2007;ARVO E-Abstract 208).

In light of the clinical relevance a statin therapy might have after acute retinal ischemia/reperfusion, one of our aims was to investigate the therapeutic window of the postischemic neuroprotective action of simvastatin in adult mice undergoing ischemia. In this respect, our new observations are of direct clinical relevance because we show for the first time that therapeutic statin delivery started early *after* injury can still be effective and can significantly increase visual capabilities. In most other studies to date, statins were delivered *before* the induction of an ischemic lesion, a situation that cannot be transferred into a therapeutic regimen. Interestingly, the results are somehow different when RGC survival is taken into account. Here we show that simvastatin significantly increased RGC survival when delivered 1 hour after ischemia, whereas delaying the onset of treatment until 24 hours after ischemia revealed no morphologic protection. A similar result was obtained in a study involving brain ischemia as induced by middle cerebral artery occlusion (MCAO), showing that a strong beneficial effect of an acute dose of simvastatin was obtained when the drug was administered 1 hour or 3 hours after MCAO and lasted 48 hours; protection was, however, no longer present when the drug was administered 10 hours after MCAO.[45] Interestingly, in our paradigm, spatial vision was still improved when the statin was delivered 24 hours after lesioning, indicating that delayed onset of treatment only improves the functionality of surviving RGCs. Finally, simvastatin-mediated effects on visual acuity were dose dependent and were present only at a concentration of 20 mg/kg. The *dissociation* between behavioral visual acuity and RGC survival indicates that, in the case of a short-term injury, numbers of surviving RGCs do not adequately predict visual capabilities, a point that is also underscored by the absent correlation between visual acuities and RGC numbers in individual mice. In this regard, it has been proposed that mechanisms of injury, including excitotoxicity and free radical production, or recovery after short-term retinal ischemia may be different from those operating after long periods of ischemia.[46] Our data thus suggest that the effect of a retinal ischemia and possible therapeutic regimens should not be inferred from anatomic analyses of RGC numbers alone but be complemented by behavioral measurements of visual capabilities.

Therapeutic Time Window

Our morphologic data suggest that statin treatment might have a rather narrow therapeutic window, which may be a disadvantage for its application in the clinical setting because this would also imply a narrow time window for diagnosis and onset of treatment. However, our behavioral analyses clearly show that a therapeutic delivery of simvastatin after acute ischemia/reperfusion significantly improved spatial vision in mice, even when delivered 24 hours after lesioning. If similar time courses were to apply to human retinal ischemia, there would be at least 1 day for diagnosis and treatment onset, which is clinically feasible.

Possible Statin Mechanisms Involved in Functional Neuroprotection

It is unclear whether statins directly influence the central nervous system. Although lipophilic statins (e.g., lovastatin and simvastatin) readily cross the blood brain barrier and lipophobic statins (e.g., pravastatin) do not, no clinical differences in the neuroprotective properties have been reported thus far. Indeed, both types of statins were effective in preventing RGC death in a model of retinal ischemia/reperfusion in rats[4] and may also slow the progression of retinopathy in diabetic patients.[47] Experiments in mice and guinea pigs have demonstrated that high doses of statins affect brain cholesterol production but not brain cholesterol content.[48] Furthermore, it was shown that some adult neurons no longer require endogenous cholesterol synthesis and can fully meet their cholesterol needs by uptake from their surroundings.[49] Therefore, the neuroprotective effects demonstrated by the present work are probably cholesterol independent.

Systemic administration of simvastatin induced an increase in blood velocity and blood flow in retinal arteries and veins in the healthy human retina.[50] In addition, it was shown that simvastatin treatment increased the plasma nitrite/nitrate levels and decreased the IOP, probably through the increase in

nitric oxide. Furthermore, simvastatin-mediated increases in nitric oxide elicited the dilation of isolated porcine retinal arterioles, which also may account for an increase in blood velocity.[51] Interestingly, the enhancement of enzyme expression, detected by immunostaining, was also observed in animals receiving simvastatin after MCAO.[52]

Bartoli et al.[53] have shown that systemic statin delivery (fluvastatin, intraperitoneally) prevents neovascularization in a model of oxygen-induced retinopathy in young mice. The beneficial effects appear to result from antioxidant and anti-inflammatory properties of statins. In the same model, a similar effect was described for simvastatin at low doses (0.2 mg/kg). Here it increased retinal microvascular repair by promoting intraretinal revascularization. By contrast, high-dose simvastatin (20 mg/kg) significantly prevented revascularization and concomitantly increased pathologic neovascularization.[54] Molecular mechanisms underlying statin-mediated neuroprotective effects remain far from being completely understood and may differ according to the type of insult (global, focal, transient, or permanent) and the stage of brain development.[45]

Taken together, our study shows for the first time that optometry is a very sensitive method, allowing the measurement of subtle changes in visual function after a mild retinal ischemic injury. In addition, this method represents a new and powerful tool with which to evaluate the effectiveness of putative pharmacologic therapeutic approaches to overcome damage after retinal injury. Our new observations show that, although statins can be considered promising therapeutical candidates for the treatment of acute retinal ischemia/reperfusion and can further demonstrate neuroprotective effects of this class of drugs, there is a narrow therapeutic window for its application after lesion. In addition, although there was a significant increase in cell survival and visual function, the latter never regained preischemic levels, suggesting a partial and possibly temporary neuroprotective effect that might limit its clinical application for acute processes.

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