Hypothermia Promotes Survival of Ischemic Retinal Ganglion Cells

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Purpose. Ischemic stroke in retinal arteries leads to death of neural tissue and ultimately to blindness. The retina is known to die within 4 hours after onset of ischemia. It is debated whether hypothermia might increase the time window for medical treatment and thereby the chance of recovering sight. In order to characterize the time course of cell death during ischemia and potential beneficial effects of hypothermia in more detail, we investigated the survival of ganglion cells in ischemic pig and human retina as a function of time and temperature.

Methods. Eyes were obtained from minipigs and from human donors post mortem. Enucleated minipig eyes were stored for defined durations at three different temperatures (37°C, 21°C, and 4°C). In order to assess the viability of the tissue, we measured ganglion cell activity (spiking) with multielectrode arrays.

Results. Minipig retinal ganglion cell function was severely compromised after 2 hours of ischemia at body temperature. After 4 hours, ganglion cells did not fire action potentials anymore. However, at 21°C, ganglion cell activity was maintained under ischemic conditions for up to 12 hours, and for at least 50 hours at 4°C. In postmortem human retina, we recorded ganglion cell activity in retinas received up to 27 hours after death.

Conclusions. Our results demonstrate that hypothermia greatly increases survival of retinal ganglion cells exposed to ischemia. These results might be relevant for the future treatment of retinal ischemia.

Keywords: electrophysiology, function, human retina, hypothermia, ischemia, pig retina

Circulatory disorders may cause a deficiency of oxygenation and nutrient supply to neural structures, ultimately leading to serious damage or even death of the nerve tissue.[1] Such ischemia also is indicated as a critical factor in a number of ophthalmic diseases, like diabetic retinopathy, glaucoma, and central retinal artery occlusion.[2–4] Numerous studies examining ischemia in the brain have established that only a few minutes of ischemia can result in irreversible nerve damage.[5] The retina, however, appears to be considerably more resistant,[6] as ganglion cells in rhesus monkeys survive up to 4 hours of ischemia.[7,8]

Currently, there is no effective treatment to prevent ischemic damage.[4] However, temperature is known to have an important influence. Hypothermia is regarded as one of the oldest, yet most effective treatments for limiting cellular injury during ischemia.[4] Even a reduction of 3 to 5°C led to a clear improvement in survival rate of cortical neurons,[9,10] and hypothermia is, therefore, rated as one of "the most potent therapeutic approach for reducing experimental ischemic brain injury identified to date."[11,12] In addition, the efficacy of hypothermia in stroke patients has been successfully investigated in early clinical trials.[13] Hypothermia might be particularly effective at extending retinal survival, because in the retina, the time window for a promising treatment is considerably longer than that in the rest of the brain. Positive effects of hypothermia on the survival of ischemic retina have been demonstrated by cooling the eyes of rats to 33°C.[4]

These results prompted us to investigate the influence of hypothermia systematically. This question has been addressed by several studies in rodents,[14–16] but investigations with an animal model system closer to the human are still missing. Here we used retinas of minipigs to investigate the time course of retinal damage and the protective potential of hypothermia, and we compared these results with data from postmortem

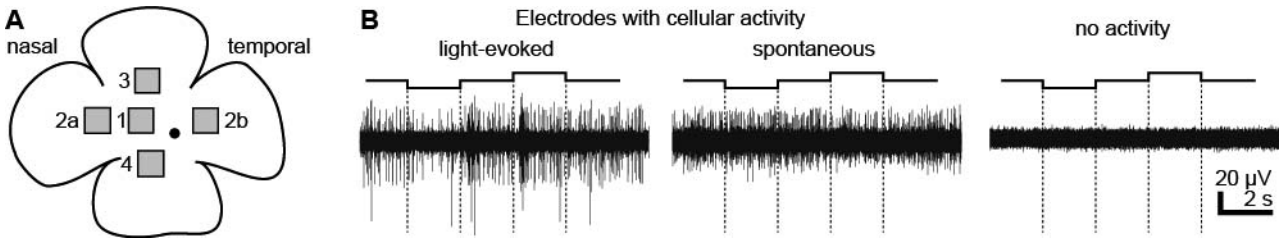

FIGURE 1. Retinal recordings and outcome measures. (**A**) Retinal regions used for recordings. Up to four regions were tested in each retina to probe for ganglion cell activity after the retina had been exposed to ischemic conditions. (**B**) Outcome measures from recordings of ganglion cell activity measured on individual electrodes (minipig retina). The corresponding full-field light stimulus is shown at the *top*. *Left*: Light responses are evident from the modulation of the spike density. *Middle*: In the case of spontaneous activity, the spiking activity was not modulated by the stimulus. *Right*: On this electrode, only electrical noise is visible but no spiking activity.

human retina. As the inner retinal layers are supplied directly by the central retinal artery,[17,18] ganglion cells are particularly affected by retinal artery occlusion. We used multielectrode arrays (MEAs) to record spontaneous and light-evoked activity of ganglion cells in the isolated retina after various durations of ischemia at 37, 21, and 4°C, thereby evaluating the influence of ischemia as well as the potentially protective effect of hypothermia on the functionality of the retina. Our results demonstrate a strong protective effect of hypothermia, prolonging ganglion cell survival to at least 50 hours, the longest time tested so far.

Materials and Methods

Pieces from isolated retina of postmortem human donors or of minipigs (Fig. 1A) were placed on flat 60-electrode MEAs,[19] and the spiking activity of ganglion cells was qualitatively assessed for each electrode (Fig. 1B). Before their activity was recorded, minipig eyes were left intact and stored for a defined duration ("ischemia duration") at specific temperatures (37°C, 21°C, and 4°C). In our experiments, we therefore induced global ischemia of the retina. Global ischemia is a more severe manipulation than the ischemia experienced by patients during central artery occlusion. In the clinical case, the outer retina (photoreceptors and bipolar cells) is normally still supplied by an independent capillary system. The inner retina, in particular ganglion cells, is directly affected by the stroke. The capacity of ganglion cells to survive ischemic conditions is therefore of particular clinical interest. Our experiments, which produce global ischemia in the retina, therefore probably overestimate the effects on the outer retina (e.g., light responses). Details for the experimental procedures are given in the Supplementary Methods.

Results

Human Retinal Ganglion Cells Can Survive Long Periods of Ischemia

We measured ganglion cell activity of six postmortem human retinas (six donors) on flat MEAs. Eyes were obtained between 12 and 27 hours post mortem (Supplementary Table S1; Fig. 2, measurements from human tissue are represented by black circles). For each retina, the time spans between death, enucleation of the eye, and preparation of the retina are listed in Supplementary Table S1. Unfortunately, no information was available about temperature conditions before enucleation of the eye, that is, the temperature at which the body of the donor was stored or the time at which the body was brought into a mortuary refrigerator. None of the postmortem human retinas showed responses to light stimuli, and in retinas from two donations (12 and 15 hours post mortem) ganglion cell activity was completely absent. We were able to record ganglion cell spiking activity in the other four retinas, with ischemic durations of 12.5, 23.5, and 27 hours. Ganglion cell activity was apparent on 52% to 83% of the recording electrodes. These results were surprising because previous reports showed that retinal activity ceased completely after 4 hours of ischemia.[7]

In order to investigate this phenomenon in more detail, we performed a series of experiments with minipig eyes to test the survival of ganglion cells under controlled conditions.

Ganglion Cell Activity Ceases Within 4 Hours of Ischemia at 37°C

We first tested the survival of ganglion cells when ischemia was experienced at 37°C. Twelve minipig eyes were exposed to ischemic conditions for 0.5 to 5 hours. Figure 2 shows the fraction of electrodes of the MEA on which ganglion cell spiking activity was detectable (Fig. 2, orange disks; Supplementary Table S2 lists these results together with the detailed experimental conditions). Spiking activity (Fig. 2A, orange disks) remained visible on most recording electrodes (93%) for up to 1.5 hours of ischemia at 37°C and sharply dropped to 0% to 12% of electrodes after 2 hours of ischemia. In a single retina, we had activity on 44% electrodes after 3.25 hours of ischemia. After 4 hours, we found ganglion cell activity only on one electrode.

Next we counted only the electrodes on which ganglion cell spiking activity was modulated by light stimuli (Fig. 2B, orange disks; Supplementary Table S2), which would indicate functional neural circuits from photoreceptors to bipolar cells to ganglion cells, including their synaptic machinery. Absence of light responses, on the other hand, would indicate that at least one step in this neural processing chain has been compromised. We found light-evoked activity under control conditions, that is, when the retina was immersed in physiological solution immediately after enucleation (no ischemia) and after short ischemic durations of up to 1 hour on 49% to 83% of the electrodes. With ischemic duration of 1.5 hours and longer, light responses completely vanished, with the exception of responses on a single electrode (3%) after 3 hours of ischemia.

Overall, we found a fast decay of ganglion cell activity with increasing duration of ischemia at 37°C. Most light responses ceased after 1 hour and overall ganglion cell spiking activity strongly decreased within 1.5 hours. These results are fully consistent with previous reports by Hayreh et al.[7,14] measured in monkeys. Our recordings from ischemic pig retina stored at

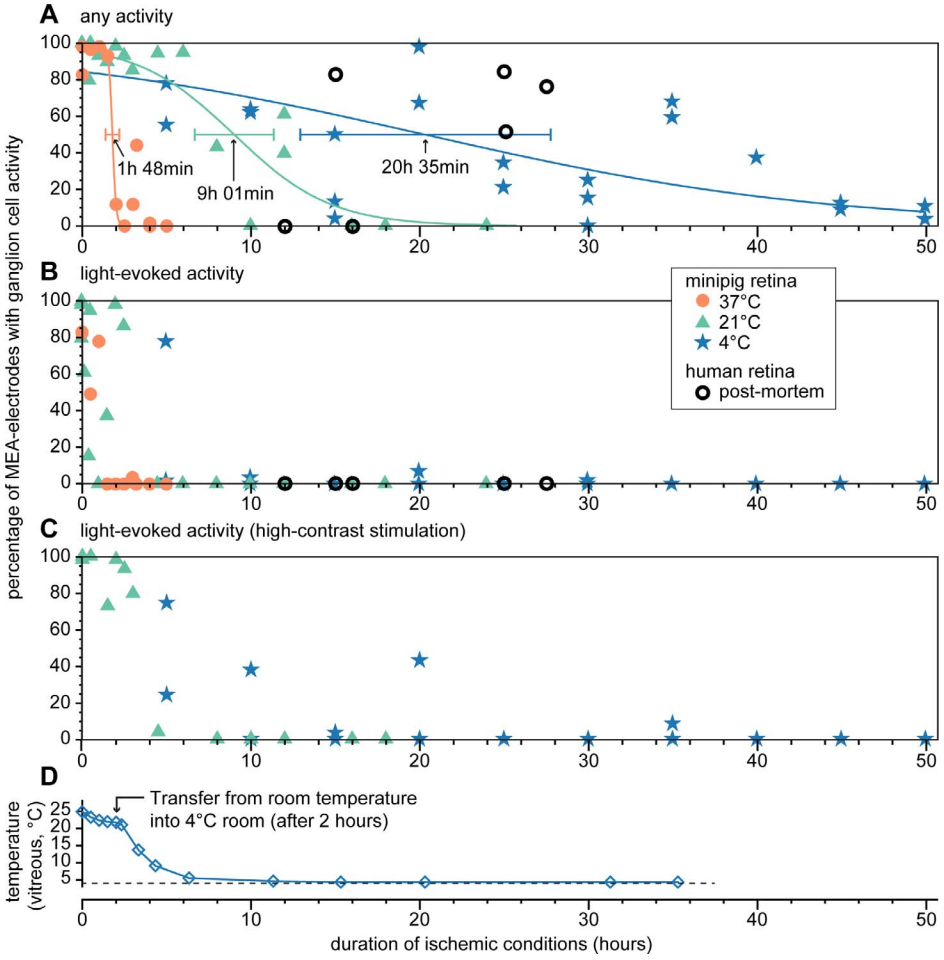

Figure 2. Ganglion cell activity in postmortem human retina and in minipig retina after ischemia at various temperatures. (**A**) Ganglion cell spiking activity after ischemia at 37°C (*orange disks*), 21°C (*green triangles*), and 4°C (*blue stars*). Data from postmortem human retinas (*black circles*) are shown for comparison. Values are fractions of multielectrode array (MEA) electrodes with any activity (light-driven or spontaneous) for different ischemia durations. For each temperature, data were fitted by a logistic model (see Materials and Methods). *Horizontal lines* indicate 99% confidence interval of the ischemic durations after which ganglion cell activity dropped and was recorded on only 50% of MEA electrodes. Note that confidence intervals do not overlap; therefore, differences among temperature conditions are highly significant. (**B**) Fraction of electrodes with light-modulated ganglion cell spiking activity. Other conventions were as described in (**A**). (**C**) Light responses of minipig ganglion cells to high-contrast light stimuli. After ischemia at 21 and 4°C, minipig ganglion cells still exhibited substantial light responses after 3 hours (21°C) and 20 hours (4°C). Occasional responses were detectable after 35 hours (4°C) of ischemia. (**D**) Progression of vitreal temperature inside a minipig eye treated with the 4°C ischemia protocol.

37°C could thus not explain why ganglion cells in postmortem human retina survived almost 30 times longer.

Hypothermia (21°C) Prolongs Ganglion Cell Survival

Because the temperature of postmortem human retina quickly approached room temperature, we hypothesized that hypothermia might be responsible for the long survival time observed in postmortem human retina. Therefore, we repeated the experiments with minipig eyes while storing the enucleated bulbi at 21°C instead of 37°C. Otherwise, the experimental conditions were identical.

We tested ischemic durations between 0 and 24 hours using 20 minipig eyes. Ganglion cell spiking activity (Fig. 2A, green triangles; Supplementary Table S3) remained detectable after up to 12 hours of ischemia at 21°C (activity of up to 61% of electrodes), with normal levels of activity remaining even after an ischemic time of 6 hours (>85% of electrodes). Ganglion cell spiking activity was undetectable only in later measurements, after 16 to 24 hours.

Abundant light-modulated ganglion cell activity could be detected for up to 2.5 hours of ischemia (on 80%–100% of electrodes) (Fig. 2B, green triangles; Supplementary Table S3). At 3 hours, we found an abrupt cessation of light responses. Overall, the preservation of light responsiveness was 2.5-fold prolonged at 21°C compared to 37°C.

In summary, we found a slower decay of ganglion cell activity under conditions at 21°C than under those at 37°C. The longer survival time of ganglion cells at 21°C was statistically significant. We fitted a logistic model to the data recorded at 37°C and 21°C (see Supplementary Methods). The orange and green curves in Figure 2A show the best fit, suggesting that ganglion cell activity would decline and be detectable on only half of the MEA electrodes after 1:48 hours at 37°C, but only after approximately 9 hours at 21°C. The horizontal bars in Figure 2A show the corresponding 99% confidence intervals for these time estimates. However, despite the significantly longer survival time of minipig ganglion cells at 21°C, activity

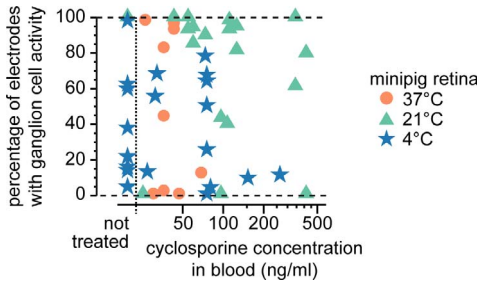

FIGURE 3. Retinal activity is not correlated with cyclosporine concentration. Spontaneous ganglion cell activity (see Fig. 2A) shown as a function cyclosporine concentration (ng/mL) measured in blood samples. Values below 25 ng/mL were below detection threshold; lower values are considered 0 (in sham-treated animals and in one treated animal).

was present for more than twice as long in postmortem human retina (up to 27 hours).

Long Retinal Ganglion Cell Survival by Slow Cooling to 4°C

The human donor's body might have been stored in the mortuary refrigerator for some time, and the donor's bulbi were subsequently stored in the refrigerator between enucleation and preparation of the retina. We could therefore assume that the retinas of postmortem donors had been exposed to temperatures as low as 4°C. We performed an additional experimental series aimed at mimicking these conditions.

Here, the minipig eyes were first kept for 2 hours at 21°C and then slowly cooled to 4°C. Figure 2D shows the progression of the temperature measured inside one minipig eye by a temperature probe that we inserted into the vitreous. Otherwise, this bulbus was treated in the same way as the other eyes in this experimental series, and we expected that these retinas were exposed to a very similar temperature progression. Directly after enucleation of the eye, the vitreal temperature was at 25°C. During 2 hours at room temperature, temperature decreased to 22°C. Subsequently the eyes were stored in a 4°C coldroom, where the temperature gradually decreased and reached a stable 4.3°C at all measured time points after 15:20 hours.

The influence of such strong hypothermia was tested using eyes of 22 minipigs. We observed ganglion cell spiking activity for all ischemic durations between 5 and 50 hours (in 5-hour steps) (Fig. 2A, blue stars; Supplementary Table S4). For ischemic durations of 10 to 40 hours, activity was present on 25% to 98% of the electrodes and decreased to 4% to 13% for longer ischemic times (45–50 hours). After 50 hours, the longest ischemic duration measured so far, we found activity on 11% of electrodes; therefore, the maximal ganglion cell tolerance for ischemia at 4°C might not have been reached yet. The logistic model suggests that, on average, ganglion cell activity is recordable on only 50% electrodes after approximately 20.5 hours (Fig. 2A, blue curve), with 99% confidence intervals for this estimate spanning 13 to 28 hours of ischemia at 4°C. Therefore, the reduction of temperature to as little as 4°C had a significant, further beneficial effect on ganglion cells survival time.

When we considered the relatively abrupt disappearance of light responses at 37 and 21°C after less than 3 hours of ischemia, it was surprising to find clear light responses on 78% of the electrodes even after 5 hours of ischemia at 4°C (Fig. 2B, blue stars). For longer ischemic durations than 5 hours, only few light responses were found (on 3% of electrodes after 10 hours, 7% after 20 hours, 2% after 30 hours). However, the number of electrodes with light responses for such long ischemic durations varied strongly from one retinal piece to another.

In a subset of experiments at 21 and 4°C, we added a high-contrast light stimulus. In response to such a strong contrast, abundant light responses could be recorded even after 3 hours (21°C) and 35 hours (4°C), respectively (Fig. 2C).

In summary, the number of electrodes on which we observed activity in human retina was comparable to minipig data at 10 to 30 hours of ischemia under the 4°C condition. Furthermore, loss of light responses was further delayed to more than 5 hours (with low contrast stimulus) and 35 hours of ischemia (with high contrast stimulus), suggesting that the whole neural circuit from photoreceptors to bipolar cells to ganglion cells, including their synaptic machinery, was still functional.

Schultheiss et al.[20] demonstrated that cyclosporine has a dose-dependent neuroprotective effect that enhances the survival of rat ganglion cells in culture when cyclosporine concentrations exceed 1 μg/mL. The minipigs used for our study had been under cyclosporine treatment; however, we found that the spread of ganglion cell activity was equally large in the retinas of sham-treated pigs (no cyclosporine, data points to the left of the dashed vertical line in Fig. 3) as in treated pigs. There was no correlation between the number of electrodes with spiking activity and the level of cyclosporine in blood samples ($P = 0.74$, Spearman rank test) (Fig. 3).

DISCUSSION

In this study, we examined the survival of retinal ganglion cells during ischemia as a function of ischemia duration and temperature. Recordings using MEAs allowed us to monitor the spiking activity and therefore vitality of ganglion cells directly. We also tested whether spiking activity of ganglion cells was modulated by light stimuli. Preservation of light responses would indicate that the retina as a whole is still functional. Spontaneous ganglion cell activity without light responses, on the other hand, demonstrates that at least some ganglion cells are still alive and able to produce action potentials, while the upstream retinal circuits are impaired at the level of the photoreceptors and/or bipolar cells. In the clinically relevant case of a central retinal artery occlusion, the nutrient supply to the inner retina, in particular ganglion cells, is disrupted, while the upstream retinal structures (photoreceptors and bipolar cells) are usually still supplied by an independent capillary system. Therefore, the survival of ganglion cells is of upmost importance in acute cases of a central retinal artery occlusion. However, in case of postmortem human tissue used in our study, the ischemia is global. In our experimental conditions, in contrast to the clinical situation during central artery occlusion, not only was the inner retinal circulation interrupted, but also the choroidal circulation.

For human retina, we defined the ischemia duration as the time between the death of the donor and exposure of the retina to enriched medium. Generally, for postmortem retina, the temperature to which the retina is exposed after death will slowly decrease from body temperature to room temperature, until the body is brought into a mortuary refrigerator. For the tissue samples that we obtained, we were unfortunately not able to recapitulate the exact times and temperature conditions at which the body had been stored. After enucleation, however, all eyes have been stored in a refrigerator (2–8°C) for 1 to 17 hours before we obtained the retina for electrophysiological recordings. Under these conditions, human retinal ganglion cells survived for up to 27 hours of ischemia, with ganglion cell activity observed on a large proportion of electrodes (52%–84%).

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We found no ganglion cell activity in two human retinal donations with ischemia durations of 12:35 and 15:10 hours. In case of the donation 12:35 hours after death, this might be explained by the medical history of the donor, which included chemotherapy. For the second nonactive retina, we have no further information about the donor's history. Nevertheless, we were able to detect spontaneous ganglion cell activity in four retinal donations with very long ischemic durations of 12 to 27 hours. These results were in sharp contrast to published findings showing that the ganglion cells are dead after at most 4 hours of ischemia.[8] To examine this phenomenon more closely, we developed a minipig eye model to study global retinal ischemia under controlled conditions.

We enucleated minipig eyes immediately after death and stored the intact bulbi for defined times at specific temperatures. In the first experimental condition we set the storage temperature to 37°C to mimic physiological conditions. In agreement with earlier results of Hayreh et al.,[14] we found no detectable activity of ganglion cells after 4 hours of ischemia, with ganglion cell spiking activity continuously decreasing with increasing duration of ischemia. Even though the tissue was supplied with nutrients during the preparation of the retina and during the MEA recording (which lasted 20–30 minutes) ganglion cell responses did not recover. This implies that the retina is irreversibly damaged by such long durations of nutrient deprivation. Hayreh et al.[7] used stereoscopic color fundus photography and fluorescein fundus angiography to characterize the degeneration of the retinal nerve fiber layer in rhesus monkeys. In those studies, the retinal central artery was occluded for 97 to 300 minutes. A retinal occlusion for less than 100 minutes led to no evidence for a morphologic damage, while an occlusion of more than 240 minutes produced total or an almost total optic nerve atrophy and nerve fiber damage.[7] Intermediate ischemic durations between 100 and 240 minutes led to variable results. Taken together, our data are fully consistent with these results.

At 37°C, we were able to record light responses on 78% of electrodes after 1 hour of ischemia. In all measurements with longer ischemic durations, no light responses could be found. Preserved light responses depend on intact light perception by the photoreceptors and on preserved synaptic transmission between photoreceptors, bipolar cells, and ganglion cells. If the nutrient supply (i.e., circulation) is restored within this time window of 1 hour, one can assume that the prospects for a restoration of visual function are still quite high. The retinal tolerance to short ischemic durations has also been shown by Zhao et al.[16] in rat eye. There, the retinal arteries were occluded for 17 minutes, followed by reperfusion. Retinal activity measured by electroretinography ceased during occlusion but recovered within 48 hours. Retinal ganglion cell death after acute retinal ischemia was also investigated by Lafuente et al.[17] in rat retinas by ligature of the ophthalmic vessels. In response to 30 to 45 minutes of ischemia, the authors observed a continuous decrease in the number of ganglion cells for up to 14 days, with approximately half of the cells surviving. Increasing the duration of ischemia to 60 to 120 minutes reduced the survival rate to 25% to 1% measured after up to 90 days. This supports our finding that short ischemic durations of less than 1 hour at 37°C are less harmful, whereas durations of more than 1 hour can cause severe damage.

Still, these findings could not explain why the postmortem ganglion cells survived such long periods of ischemia. In further experiments, we lowered the temperature during ischemia to 21°C and even 4°C, as we expected that hypothermia might have a protective effect. Zilis et al.[21] reported that extreme cooling with 2°C fluid can lead to electroretinographic changes and retinal detachments. Therefore, and for better comparison with postmortem human retina, we ensured slow cooling during our 4°C experiments. Lowering the temperature indeed led to a pronounced increase in retinal cell survival times. While retinas were at 37°C, light responses remained for only 1 hour, and they were detectable after up to 2.5 hours at 21°C (3 hours for high-contrast stimuli) and after up to 5 hours at 4°C (35 hours for high-contrast stimuli). The ischemic duration after which spontaneous ganglion cell activity could still be measured increased to 12 hours of ischemia at 21°C and 50 hours at 4°C, the longest time we have tested so far. Together with our results from postmortem human retina, this suggests that a reduction of temperature is highly beneficial. The overall level of spontaneous activity in the postmortem human retinal tissue, recorded between 15 and 27 hours post mortem, was comparable to the activity in minipig retinas measured after 2 to 3 hours of ischemia at 21°C or after 20 to 40 hours at 4°C. Our results at 21°C and 4°C thus confirm the positive effect of hypothermia on the retinal tolerance of ischemia. These results are in accordance with the study of Faberowsky et al.,[22] who could show a clear decrease in cell damage in the rat retina (measured by counting nonpyknotic nuclei) after 2 hours of ischemia while cooling with an ice pack.

Finally, we kept some human retinal pieces of the donations obtained at 12 and 23 hours post mortem in organotypic tissue culture. Measurements repeated after 72 to 96 hours showed a comparable level of spontaneous activity, as recorded directly after receiving the tissue (data not shown). This confirms that the ganglion cells were still viable after ischemia for at least several days. However, our data do not allow drawing conclusions about the long-term survival of ganglion cells, further studies will be necessary to investigate this in more detail.

Hypothermia appears to be a key factor for the survival of the retina under ischemic conditions and should be considered in clinically acute cases. In general, stronger cooling, if slowly applied,[21] appears to increase chances of regeneration. It remains open how long hypothermia prolongs retinal survival under ischemic conditions in an in vivo situation. Nevertheless, hypothermia is so far the only neuroprotective treatment which extends the ischemic tolerance and which would currently be applicable in a clinical case.

How could hypothermia be practically achieved? Once in the hospital, the retina can be cooled with neuroprotective irrigation solution.[23] Before reaching the hospital, however, noninvasive first-aid measures would be desirable, especially considering the narrow time window which is available until reperfusion of the retina has to be achieved. We suggest that it may be beneficial to cool the affected eye with an ice pack. Medical emergency personnel should be trained and made aware of the potential benefits of cooling. However, it remains to be investigated how well external cooling spreads to the retina.

Apart from the clinical implications, our study also shows that postmortem human retina, because of its long survival times due to "naturally" occurring hypothermic conditions, is suitable for basic scientific questions dealing with retinal function, ranging from histologic examinations to physiological studies, and from viral expression studies to optogenetic treatment for blindness.

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