Chapter 11

HYPOXIC PRECONDITIONING AND ERYTHROPOIETIN PROTECT RETINAL NEURONS FROM DEGENERATION

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Abstract: Reduced tissue oxygenation stabilizes the alpha-subunit of the transcription factor hypoxia-inducible factor-1 (HIF-1). This leads to the induction of a number of hypoxia responsive genes. One of the best known HIF-1 targets is erythropoietin that exerts neuroprotective effects on ischemia-related injury in the brain. Thus, pre-exposure to low environmental oxygen concentrations might be exploited as a preconditioning procedure to protect tissues against a variety of harmful conditions. We present recent work on neuroprotection of retinal photoreceptors induced by hypoxic preconditioning or by systemically elevated levels of Epo in mouse plasma.

Key Words: hypoxia-inducible factor-1, apoptosis, photoreceptor, blinding disease, overexpression of EPO

INTRODUCTION

Reduced oxygenation triggers several adaptive responses in mammals including man. At the cellular level, low oxygen supply induces expression of a variety of oxygen-regulated genes such as erythropoietin (Epo), vascular endothelial growth factor (VEGF) and several glycolytic enzymes. Once 'loaded' with the products of hypoxically activated genes, the organism is able to cope with hypoxia. Expression of oxygen-dependent genes is transient and the return to normoxic conditions will gradually reduce the intra- and extracellular concentrations of induced proteins to pre-hypoxic levels. Upon re-oxygenation, however,

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there is a given period of time in which the tissue is still 'loaded' with gene products that were upregulated during the antecedent hypoxic period. If re-exposed to hypoxia, these tissues are better protected against hypoxic injury compared to non-preexposed ones. We call this concept *hypoxic preconditioning* (Grimm et al; submitted).

It is conceivable that hypoxic preconditioning of cells, tissues, animals or even patients has a clinical potential (reviewed in (16)). We propose that the full set of oxygen-dependent genes will include some that can provide tissue- or cell-protective effects. This protection may not be limited to hypoxic/ischemic insults but may also protect against other stimuli that otherwise might harm an organism. It is therefore desirable to identify such protective genes or gene products that have a potential as therapeutical drugs. We show that hypoxic preconditioning increases the resistance of neuronal tissue against harmful insults and that Epo is one of several putative factors involved in this protection.

THE MOLECULAR RESPONSE TO HYPOXIA IS VERY FAST

Many reviews on the oxygen-sensing mechanism and the involved hypoxia-inducible factors and prolyl hydroxylases have been published recently (24, 25, 53, 54). In brief, hypoxic exposure of cells leads to the accumulation of the α-subunit of the hypoxia-inducible factor-1 (HIF-1). Heterodimerization of HIF-1α with its partner HIF-1β (also termed ARNT for arylhydrocarbon receptor nuclear translocator) leads to the formation of the HIF-1 complex that binds to the hypoxic response element that is present in all HIF-target genes described so far (3) and enhances their transcription. While hypoxia quickly stabilizes HIF-1α protein, normoxia leads to immediate degradation of HIF-1α (28). Stabilization of HIF-1α is highly efficient also *in vivo*: upon exposure of mice to 6% oxygen, we observed accumulation of HIF-1α protein in several organs within 60 minutes (58). This observation implies that hypoxic preconditioning for less than one hour might be sufficient to provide tissue protective effects.

EPO PROVIDES NEUROPROTECTION IN THE BRAIN

Until recently, the function of Epo was thought to be restricted to erythropoiesis. Binding of Epo to its receptor (EpoR) present on erythroid progenitor cells was shown to repress apoptosis, thereby allowing red blood cells to mature (27). However, we and others discovered expression of Epo and EpoR in the brain (13, 39, 59). The presence of the blood-brain-barrier made a systemic erythropoietic function of brain-derived Epo unlikely and suggested a local role of Epo in the brain by binding to local EpoR's. In analogy to the kidney, Epo gene expression in brain is regulated in an oxygen-dependent manner. Considering that Epo is a HIF-1-target gene, we were not surprised to find elevated Epo protein levels in the hypoxic brain (58).

Why do we need Epo in the brain? Sasaki and co-workers reported for the first time that Epo protects neurons from ischemic damage *in vivo* (51) and many subsequent animal stroke models confirmed that Epo protects against this ischemic brain injury (reviewed in (40)). By now, several reports have enlarged the possible use of Epo in ischemic-related injuries (including spinal cord) and the involved protective mechanisms have been summa-

rized recently (17, 18, 38). At the same time, Ehrenreich and co-workers (14) pioneered the first clinical trial applying rhEpo on patients suffering from acute stroke. They reported that Epo-treated patients showed markedly enhanced neurological recovery and an improved clinical outcome. The fact that no side effects of Epo therapy were identified makes the therapeutic use of Epo in ischemia-related neuronal injuries very promising.

TRANSGENIC MICE OVEREXPRESSING HUMAN EPO

Encouraged by the beneficial effects of Epo in the brain of stroke patients and animal models we started to study the potential neuroprotective capacity of Epo in the retina by three different approaches: i) by upregulation of Epo (among other factors) through hypoxic preconditioning, ii) by application of rhEpo and iii) by transgenic overexpression of hEpo. For the latter approach, a transgenic mouse line was generated that expresses human Epo cDNA driven by the human platelet-derived growth factor (PDGF) B-chain promoter. This promoter directs expression of the transgene preferentially, but not exclusively, to neuronal cells. The resulting transgenic mouse line termed tg6 showed a dramatic increase of cerebral and plasma Epo levels, the latter leading to excessive erythrocytosis with hematocrit values of up to 90% (50, 55, 62). Unexpectedly, blood pressure was not elevated nor was the cardiac output altered in tg6 mice. Despite concomitant activation of the endothelin system (46), elevated NO-levels observed in tg6 mice led to a generalized vasodilation (50). In concert with regulated blood viscosity (61), the observed vasodilation protected tg6 animals from cardiovascular complications.

EPO IS OVEREXPRESSED IN THE TRANSGENIC RETINA

The transgene caused more than 20-fold elevated levels of Epo in the retina (21). The high levels of Epo did not influence retinal development, and the adult retina showed normal morphology and thickness without obvious alterations, and also a normal content and regeneration of visual pigments (21). However, the retinal vessels were massively dilated, in particular the veins (Fig. 1A). The functional analysis was performed to answer the question whether enhanced hematocrit would lead to a reduction (e.g. via a reduced capillar flow) or an enhancement of the electrophysiological activity. It turned out that retinal function was enhanced in tg6 mutant mice. Both rod and cone system function as measured by the scotopic (Fig. 1B) and photopic (not shown) electroretinogram (ERG) were increased in comparison to normal, especially at higher stimulus intensities (Fig. 1B). The exact reason for the enlarged ERGs is not clear but presumably involves alterations in the blood and oxygen supply to the retina and/or the retinal pigment epithelium (RPE), caused by the high hematocrit values, vasodilation and altered blood viscosity in tg6 mice. Alternatively, subtle changes in retinal architecture, especially in the inner nuclear layer (INL) and Muller cells may contribute to the increased b-wave amplitudes. Such potential structural changes in the tg6 retina may not be detectable in histological sections examined by conventional light microscopy. A more detailed analysis of the tg6 retina with respect to the observed functional alterations is currently in progress. Although these experiments are not yet completed, we used the tg6 mouse to test the potential benefit of increased Epo

levels on the survival of retinal cells in mouse models of induced and inherited retinal degeneration.

Light is considered a risk- or cofactor for many human retinal degenerations (10-12, 57, 60). Furthermore, degeneration in many animal models is accelerated by light and light-induced retinal degeneration in wildtype mice has been used to study pathophysiological mechanisms in the retina (8, 9, 33, 44, 52, 63, 66). Short exposure of mice to high levels of white light induces a synchronized burst of apoptosis in a large number of photoreceptors. This process leads to cell death, loss of retinal function and blindness (47, 48, 66). Since apoptosis is the final common death pathway in most human retinal diseases, this system can be used to study signaling pathways and apoptotic cascades in the retina with respect to human pathology.

Many transgenic and spontaneously arised mouse lines exist that represent models for inherited retinal degenerations (22). Results obtained in the induced system can be tested in some of these models especially to analyze effectivity of therapeutical approaches like gene therapy, implantation of prosthetic devices and neuroprotective treatments (see below).

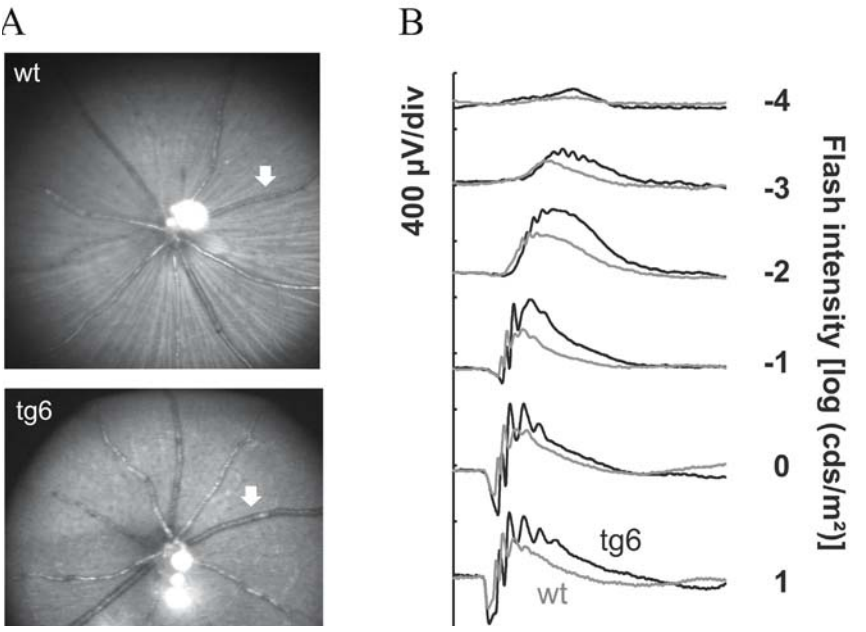


Figure 1. Functional and morphological *in vivo* analysis of tg6 mutant mice (see Ref (26) for methodological details on the scanning laser ophthalmoscope (SLO) and ERG analysis). A) Analysis of retinal vessels *in vivo* by SLO reveals dilatated retinal vessels in the tg6 mouse. The comparison between wt and tg6 mice shows that particularly the veins have a massive increase in diameter (arrows). B) Enlargement of ERG amplitudes in tg6 mice. Both the negative (a-wave) and positive (b-wave) portion of the signals is increased.

WHY THE RETINA?

The retina is a highly specialized and easily accessible 'outpost' of the brain designed to convert light into an electrical signal that can be interpreted by the brain. This demanding task is carried out by the different cells of the retina with photoreceptors being the light-absorbing cells. The extreme specialization of these cells renders them highly vulnerable and their physical and physiological integrity is easily disturbed by environmental factors as well as by gene mutations.

Many human blinding diseases exist in which cells of the retina, in particular photoreceptors, are lost through an apoptotic cell death. As a consequence, the retina degenerates leading to partial or complete loss of vision. In the gene for the visual pigment rhodopsin, over a hundred mutations are known that lead to Retinitis Pigmentosa (RP). To date, no effective treatment exists for most degenerative diseases of the retina. Strategies to develop therapies include retinal prosthesis, stem cell transplantation, gene therapy, surgery and anti-apoptotic or neuroprotective measures to interfere with the cell death program. To inhibit cell death in various models of retinal degeneration, several anti-apoptotic factors have been used with different success. Tested factors included members of the Bcl-2 family of proteins, growth factors and cytokines (66). Similarly, several protocols of preconditioning, including hyperthermia, food deprivation, light exposure or injury of the retina were shown to protect the retina against degeneration (1, 5, 30, 35-37, 41-43, 67). Likewise, ischemic preconditioning conferred protection against apoptotic stimuli like ischemia-induced death of ganglion cells and light-induced photoreceptor degeneration (6, 37, 69).

HIF-1α IS UPREGULATED DURING POSTNATAL DEVELOPMENT OF THE RETINA

The mouse retina fully develops during the first three weeks after birth. This postnatal development is characterized by the final differentiation of the various retinal cell types, the expression of the visual pigment and the development of the retinal vasculature. Until retinal vessels have fully developed, the retina may experience reduced oxygen concentrations. Using RT-PCR, we show that during this critical period of retinal development as well as in the adult mouse retina, both HIF-1α and HIF-1β/ARNT are expressed at constantly high levels (Fig. 2A). HIF-1α protein, however, can be detected at high levels only during early postnatal development (Fig. 2B). Concomitant with the development of the retinal vessels of the inner retina, HIF-1α protein levels decrease, presumably because the retina becomes more and more oxygenized. High levels of HIF-1 transcription factor during postnatal retinal development most likely secures proper retinal vasculogenesis by regulating expression of proteins involved in angiogenesis like VEGF. In the adult retina, HIF-1α is barely detectable. Nevertheless, the genes for both HIF-1 subunits are still highly expressed suggesting that the retina may be able to quickly react to any reduction in oxygen tension. Stabilization of HIF-1α with subsequent differential regulation of HIF-1 target genes in reduced oxygen conditions may be an important neuroprotective mechanism in the adult retina. This may particularly be necessary during nighttime, when the retina has the highest energy demand and oxygen conditions are borderline hypoxic in the retina.

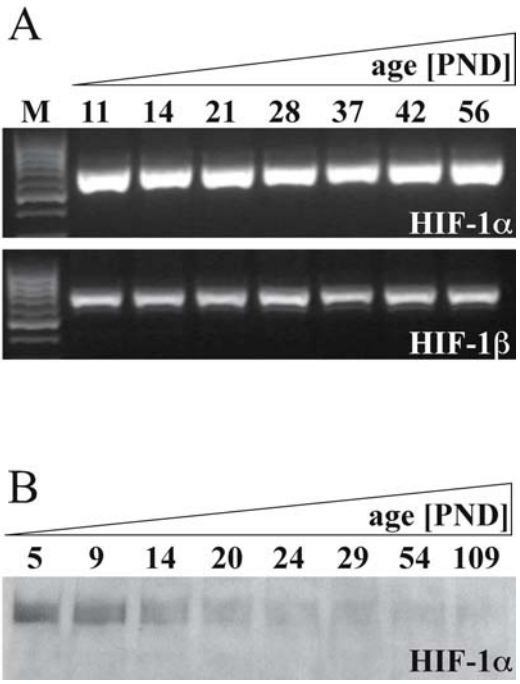


Figure 2. HIF-1α and HIF-1β expression during retinal development. A) Total RNA was prepared from isolated retinas at different post-natal days (PND) as indicated. RNA was reverse transcribed and amplified using specific primers for HIF-1α and HIF-1β, respectively. B) Total protein was prepared from isolated retinas at different post-natal days as indicated. HIF-1α levels were tested by Western blotting using a specific antibody (4).

NEUROPROTECTION IN THE RETINA THROUGH HYPOXIC PRECONDITIONING AND HIF-1 ACTIVATION.

We intended to prepare the adult mouse retina to cope with a strong apoptotic stimulus by a short exposure to low environmental oxygen concentrations. We then analyzed the molecular response of the retina to this hypoxic preconditioning and tested the neuroprotective capacity of this treatment (20). Hypoxic preconditioning stabilized HIF-1α in the retina and levels of retinal HIF-1α protein followed a dose-response function: exposure to 6% and 10% oxygen for 6 hours strongly stabilized HIF-1α, while exposure to 14% oxygen had an intermediate effect on HIF-1α levels, and 18% and 21% (normoxia) conditions did not cause any notable stabilization of the transcription factor. As noticed in other tissues, HIF-1α was quickly destabilized upon reoxygenation and protein levels reached basal values already after 1 hour in normal room air (20). The functionality of the stabilized HIF-1α protein was shown by the increased expression of HIF-1 target genes in the retina. Of particular interest was the strong induction of erythropoietin (up to 10-fold) (20).

Hypoxic preconditioning induced a neuroprotective response in the retina rendering photoreceptors resistant against exposure to high levels of white light, a strong apoptotic insult (48, 66). Without preconditioning, exposure to 5'800 lux of white fluorescent light for one hour was sufficient to induce apoptosis in a large number of photoreceptors leading to cell loss and retinal degeneration. Photoreceptors of mice preconditioned by hypoxic exposure, however, were completely protected against the light insult when exposed after

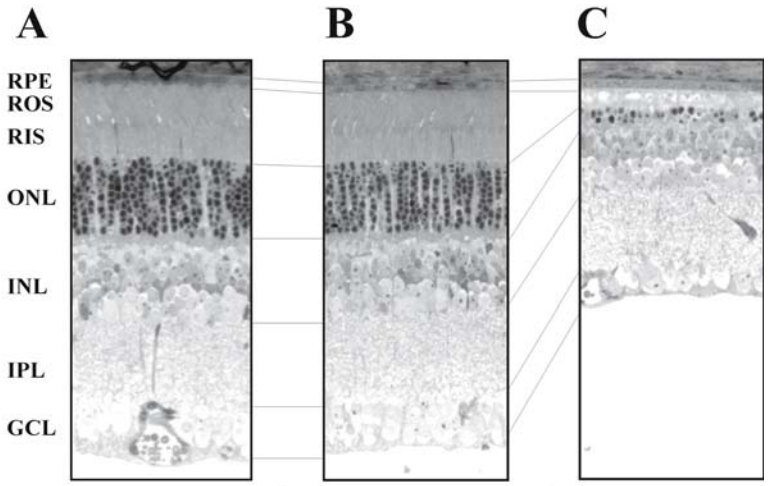
a 4-hour period of reoxygenation (Fig. 3A). Importantly, not only retinal morphology was protected but also retinal function as determined by ERG recordings (20). After 16 hours of reoxygenation, photoreceptors were again susceptible to light damage and light exposure resulted in photoreceptor loss and thinning of the outer nuclear layer (ONL) as well as of the layer of rod inner (RIS) and rod outer segments (ROS). All other layers including the RPE remained remarkably intact (Fig. 3B). The transient nature of the neuroprotective effect indicates that that the protective factors induced by hypoxic preconditioning are short-lived and implies that these factors might be deactivated immediately upon reoxygenation to restore a physiological retinal environment. Neuroprotection induced by hypoxic preconditioning most likely was dependent on the transcription factor HIF-1 since protection correlated directly with retinal levels of HIF-1α and inversely with oxygen concentrations during the preconditioning period (20).

Other potential protective mechanisms involve stress-mediated protection (67) or severely affected rhodopsin regeneration in the visual cycle (31, 56, 68). Light damage as applied here utilizes signaling cascades involving the activation of the transcription factor AP-1 (23, 64, 67). Stress was shown to inhibit AP-1 DNA binding via the activation of the glucocorticoid receptor (GR) in the retina (67). Although hypoxic preconditioning indeed imposed a stress on the animals leading to an activation and nuclear translocation of the glucocorticoid receptor (GR) immediately at the end of the hypoxic period, nucleo-cytoplasmic distribution of GR was normal and indistinguishable form normoxic controls after the 4-hour period of reoxygenation that preceded light exposure (20). Furthermore, light exposure of hypoxic preconditioned mice strongly activated AP-1 activity without leading to photoreceptor apoptosis. This suggests that protection was not mediated through the inhibition of AP-1 and that hypoxic preconditioning interrupts the apoptotic signaling cascade downstream of AP-1. Another important parameter for light induced retinal degeneration is the speed of the visual cycle. Upon photon absorption 11-cis retinal is photoisomerized to all-trans retinal in the visual pigment rhodopsin. Before rhodopsin can absorb another photon, 11-cis retinal has to be regenerated from all-trans retinal in the visual cycle, a complex reaction cascade involving a multitude of enzymes in both the RPE and the photoreceptors. Slowing or blocking the visual cycle renders the retina resistant against light damage due to the reduced number of photon absorptions by rhodopsin (31, 56, 65, 68). It has been shown that hypoxic exposure alters rhodopsin regeneration in mice (45) as well as dark adaptation in humans (2, 32, 34). However, after 4 hours of reoxygenation – at the timepoint of light exposure – rhodopsin regeneration was comparable in preconditioned and control mice (20). Neuroprotection against light toxicity might therefore not be mediated by alterations in the visual cycle but rather by factors differentially regulated by the hypoxic period. Such factors may not be stably expressed under normoxic conditions and their production might depend on oxygen-sensing molecules like HIF-1.

EPO AS NEUROPROTECTIVE AGENT IN THE RETINA

One of the most prominent genes induced by hypoxia via HIF-1 is Epo. Using real-time PCR, we showed that Epo was strongly upregulated in the retina upon hypoxic exposure (20). In addition, Epo receptor was found to be expressed on photoreceptor cells (20) and retinal ganglion cells (29). Epo protects neuronal cells from apoptotic cell death in a variety

of models for neuronal damage (15) including the retina (29). We therefore tested whether Epo is responsible for the retinal protection observed after hypoxic preconditioning. Recombinant human Epo (rhEpo) was injected intraperitoneally into normal mice before or after light exposure. This treatment reduced light damage susceptibility of the mice, suggesting that Epo-mediated activation of the Epo receptor directly induces protection of the visual cells. However, protection by rhEpo was less complete than the protection observed after hypoxic preconditioning. It is possible that the limited protective capacity of rhEpo was due to a poor translocation of the protein across the blood-retina barrier leading to an insufficient availability of Epo in the retina. However, even the 20-fold increased retinal Epo levels in tg6 mice did not induce the same complete protection as observed after hypoxic preconditioning (21). This result suggests that pretreatment with low oxygen induces several factor(s) (Epo being one of them) that may need to act in concert to fully protect photoreceptors against a light-induced injury.



Prec	6h	6h	6h
Reox	4h	4h	16h
Light	No	Yes	Yes

Figure 3. Hypoxic preconditioning induces a transient neuroprotection of the retina against light-induced photoreceptor apoptosis. All BALB/c mice were preconditioned (Prec) for 6h with 6% oxygen, reoxygenized (Reox) for 4 hours (A, B) or 16 hours (C) prior to exposure to 1 hour of 5'800 lux of white light (Light; B, C). Retinal morphology was assayed on plastic embedded sections by light microscopy 10 days after light exposure. A) Retinal morphology of a control mouse without light exposure. B) Retinal morphology of a light exposed mouse preconditioned with hypoxia followed by a 4-hour reoxygenation period. The retina was completely protected against light induced apoptosis. C) Retinal morphology of a light exposed mouse preconditioned with hypoxia followed by a 16-hour reoxygenation period. The retina was susceptible to light induced apoptosis. Retinal degeneration and loss of photoreceptor cells is indicated by the thinning of the outer nuclear layer (ONL), rod inner segments (RIS) and rod outer segments (ROS). The other layers of the retina were not affected by the exposure to light. Shown are representative sections of at least 4 different animals. RPE: retinal pigment epithelium, INL: inner nuclear layer, IPL; inner plexiform layer, GCL: ganglion cell layer.

EPO IN MODELS OF INHERITED DEGENERATION OF PHOTORECEPTORS

Many mouse models of inherited retinal degeneration exist (22). To test the therapeutic potential of Epo, we used two models: the retinal degeneration mouse 1 (rd1) as a model for autosomal recessive Retinitis Pigmentosa (RP) and the VPP transgenic mouse resembling an autosomal dominant form of RP. The rd1 mouse carries a spontaneous insertion of a retrovirus in the gene encoding the β-subunit of phosphodiesterase leading to aberrant splicing and a null phenotype (7, 66). The lack of phosphodiesterase activity increases levels of cGMP in photoreceptors resulting in constantly open ion channels and, as a consequence, elevated intracellular Ca^{2+} levels. Either the elevated cGMP and/or the high levels of Ca^{2+} might be the cause of the observed early onset (around post natal day 10) and rapid degeneration of the photoreceptor cell layer. Already ten days after onset of degeneration, retinas of rd1 mice are mostly devoid of rods.

The VPP mouse expresses a transgene encoding a mutant form of rod opsin with three amino acid substitutions at the N-terminal end of the protein (19). One of these mutations causes the substitution of prolin at residue 23 by histidine (P23H). This mutation is the most frequent mutation found in opsin of human RP patients and accounts for about 10% of autosomal dominant RP in the USA.

Two approaches were used to investigate the potential neuroprotective capacity of Epo in inherited retinal degeneration: i) recombinant human Epo (rhEpo) was systemically applied by intraperitoneal (ip) injections into rd1 and VPP mice every other day before onset and during the first phase of degeneration. ii) using classical breeding schemes, both mouse models for inherited retinal degeneration were combined with the transgene in tg6 that expresses Epo at high levels especially in neuronal tissue including the retina (see above). However, neither the transgene nor the systemic application of rhEpo protected the rd1 or the VPP mouse against retinal degeneration (21). Protection of photoreceptors in the rd1 and the VPP mouse may therefore require other or additional factors. We are currently attempting to identify the postulated (see above) additional neuroprotective molecules induced by hypoxic preconditioning. Once identified, it will be important to test these factors in the inherited models. Recently, it has been suggested that only systemically but not locally produced Epo is neuroprotective in the retina (49). Since the tg6 mice seem to produce most of their Epo locally in neuronal tissue, this observation might additionally explain the weaker neuroprotective capacity – as compared to hypoxic preconditioning – of the transgene against light toxicity. Additional experiments have to be conducted in order to establish the physiological and neuroprotective role of Epo produced locally in the retina.

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