Increased Frataxin Levels Protect Retinal Ganglion Cells After Acute Ischemia/Reperfusion in the Mouse Retina In Vivo

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PURPOSE. The mitochondrial protein frataxin (FXN) is highly expressed in metabolically active tissues and has been shown to improve cell survival in response to oxidative stress after ischemia. Retinal ischemia/hypoxia is a complication of ocular diseases such as diabetic retinopathy and glaucoma. There are no effective therapeutic approaches currently available. This study was performed to evaluate the neuroprotective effects of FXN after acute retinal ischemia/reperfusion in vivo.

METHODS. Retinal ischemia/reperfusion was induced in adult wild-type and FXN-overexpressing mice by transient elevation of intraocular pressure (IOP) for 45 minutes. Expression of FXN was evaluated by quantitative (q)RT-PCR and Western blot analysis between 6 and 48 hours after ischemia. Retinal ganglion cell (RGC) survival was determined with immunofluorescent staining and fluorescence microscopy 14 days after lesion. Expression of hypoxia-inducible factors *Hif-1α* and *Hif-2α* and of oxidative stress markers heme oxygenase-1 (*Hmox1*), glutathione peroxidase 1 (*Gpx1*), superoxidase dismutase 1 and 2 (*Sod1*, *Sod2*), and catalase was evaluated by qRT-PCR.

RESULTS. Endogenous FXN levels were upregulated for up to 24 hours after retinal ischemia in vivo. Retinal ganglion cell survival was significantly improved in FXN-overexpressing mice 14 days after ischemia. Expression of antioxidative enzymes *Gpx1*, *Sod2*, and catalase was significantly increased in FXN-overexpressing mice after lesion.

CONCLUSIONS. Retinal FXN levels are increased in response to ischemia. Furthermore, elevated FXN levels had a clear neuroprotective effect as shown by increased ganglion cell survival after acute retinal ischemia/reperfusion. Frataxin's neuroprotective effect was associated with an upregulation of antioxidative enzymes. The data suggest that FXN induces neuroprotection by decreasing oxidative stress.

Keywords: frataxin, retinal ischemia, retinal ganglion cell, antioxidants

Frataxin (FXN) is a nuclear-encoded mitochondrial protein highly conserved among eukaryotes. The mouse gene (*Frda*) encodes a 207–amino acid protein showing 73% amino acid identity to its human counterpart.[1] It is abundantly expressed in metabolically active tissues such as liver, skeletal and cardiac muscle, and brain.[1] In the retina of normal mice, immunoreactivity to FXN is found in the external and internal plexiform layers, the ganglion cell layer, and the inner nuclear layer.[2] Frataxin deficiency is closely related to Friedreich ataxia (FRDA), a neurodegenerative disease associated with abnormal influx of iron into the mitochondria, which increases the susceptibility of the nervous system to oxidative stress.[3–8] Furthermore, complete absence of FXN leads to early embryonic lethality in transgenic mice. Conditional FXN knockout strains for striated muscles and neuron/cardiac muscles reproduce progressive pathophysiological and biochemical features of the human disease and demonstrate time-dependent intramitochondrial iron accumulation.[9] The abnormal use of iron leads to changes in metabolism and increased production of harmful free radicals.[10,11] Although the biological functions of FXN are still not fully understood, the protein serves important functions in iron homeostasis and biogenesis

of iron–sulphur clusters in active sites of the complexes I and II of the mitochondrial electron transport chain and aconitase.[12,13] Cells that are deficient in FXN appear to be less efficient at generating natural antioxidant factors.[14] There is evidence indicating that increasing FXN reduces the effects of oxidative stress and increases cell survival after oxidative stress–induced cell death.[3,4,15,16]

Retinal ischemia/hypoxia is a complication of ocular diseases such as diabetic retinopathy, retinopathy of prematurity, and glaucoma.[17] A hallmark of the pathologic alteration in retinal ischemia is the generation of excessive reactive oxygen species (ROS) during reperfusion, which is involved in neuronal cell death.

To date, the effect of ischemia on FXN levels and of FXN overexpression on oxidative stress levels and neuronal survival after acute retinal ischemia/reperfusion has not been evaluated.

The aims of this study were to determine in vivo the effect of a transient ischemia/reperfusion on the expression of retinal FXN, whether increased FXN levels improve retinal ganglion cell (RGC) survival after an ischemic lesion, and the effect of increased FXN on the expression of antioxidative enzymes after ischemia.

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MATERIALS AND METHODS

Animal Guidelines

Male wild-type C57BL/6J and FXN-overexpressing mice, weighing 24.9 ± 2.4 g, aged 3 months, were used. Transgenic animals overexpressing FXN were generated by breeding homozygous mice expressing the *Cre* gene under the transcriptional control of a human cytomegalovirus (CMV) minimal promoter[18] (Deleter, kindly provided by Christian Hübner, Department of Human Genetics, Jena University Hospital, Germany) to heterozygous mice carrying a CMV-driven human FXN cDNA preceded by a loxP-flanked stop cassette (B6 Rosa 26 Frataxin, developed by Michael Ristow, Department of Health Sciences and Technology, Swiss Federal Institute of Technology, Zürich, Switzerland). Cre-mediated excision of the stop cassette leads to expression of human frataxin (hFXN) and of green fluorescent protein (GFP). Hereafter, transgenic mice are referred to as Deleter B6 FXN.

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, and were also approved by the local Animal Care Committee. Animals were kept in standard cages in groups of five on a 14-hour light/10-hour dark cycle, with food and water available ad libitum, temperature ranging from 22°C to 25°C, and humidity ranging between 55% and 60%. Mice were randomized for each experimental setup into a nonlesioned group (C57BL/6 $n = 28$; Deleter B6 FXN $n = 14$) and an ischemia group (C57BL/6 $n = 55$; Deleter B6 FXN $n = 16$).

Transient Retinal Ischemia

Transient retinal ischemia (TRI) was induced by elevating the intraocular pressure above systolic pressure.[19,20] Briefly, mice were anesthetized with an intraperitoneal injection of 5% chloral hydrate in phosphate-buffered saline (PBS; 500 mg/kg body weight; Fluka, Seelze, Germany). After application of topical anesthesia (oxybuprocaine-hydrochloride 4 mg/mL; Bausch & Lomb GmbH, Feldkirchen, Germany), the anterior chamber of the right eye was cannulated with a 30-gauge needle connected to an elevated normal saline reservoir. Intraocular pressure (IOP) was elevated above systolic pressure for 45 minutes. For sham procedures, a needle attached to a saline reservoir was inserted into the anterior chamber, but pressure was not increased. Intraocular pressure increase and maintenance were evaluated during the procedure using an induction/impact tonometer (TonoLab; Tiolat Ltd., Helsinki, Finland). This method has been validated for the mouse eye.[21] One drop of antibiotic solution (ofloxacin; Bausch & Lomb GmbH, Berlin, Germany) was applied topically to the treated eye after cannulation. After the corresponding period of ischemia, the needle was withdrawn from the anterior chamber, and the IOP was normalized. Treated eyes were covered with eye ointment (Panthenol; Jenapharm, Jena, Germany) and inspected daily. Animals with signs of inflammation or iatrogenic cataract were excluded from further analysis.

In order to determine the appropriate duration of ischemia, we evaluated RGC survival after 30, 45, or 60 minutes of ischemia. An ischemic duration of 30 minutes induced a slight loss of RGCs, whereas 60 minutes of ischemia resulted in less than 15% of RGCs surviving the insult. Therefore, we chose 45 minutes of retinal ischemia to perform all further analyses. Our results are in agreement with previous studies.[19,20]

Immunostaining and Evaluation of RGC Numbers

In order to evaluate RGC survival following ischemia, animals were killed with an overdose of chloral hydrate (30%) 14 days after TRI. For immunostaining, eyes were enucleated and retinae were removed and fixed by immersion in 4% paraformaldehyde for 20 minutes. After washing in PBS, retinal whole mounts were flattened by making incisions from the periphery halfway to the optic nerve to form four symmetric lobes and permeabilized with 0.3% Triton X-100 for 45 minutes at room temperature (RT). Blocking was achieved by incubation with 3% bovine serum albumin (BSA) and 10% normal donkey serum (NDS) in PBS supplemented with 0.3% Triton X-100 for 2 hours at RT. After nonspecific binding was blocked, the tissue was incubated with primary antibody raised against the RGC specific transcription factor Brn3a (1:300; Santa Cruz, Heidelberg, Germany) in 2% NDS overnight at 4°C. After washing with PBS, retinae were incubated with corresponding secondary antibody (Molecular Probes, Leiden, The Netherlands) in 10% NDS for 1 hour at RT. Specificity of the staining was tested by incubation without primary antibody.

Retinal ganglion cell survival was evaluated on retinal whole mounts 14 days post ischemia. Labeled RGCs were counted in single fields at three different retinal eccentricities in each of the four whole-mount lobes (1/6, 3/6, and 5/6 from retinal radius; 0.093 mm^2 each, 12 fields in total) by means of fluorescence microscopy according to Schmeer et al.[22] The number of surviving RGCs was expressed as number of cells per square millimeter, and cell counts were given as percentage of corresponding contralateral and unlesioned eyes.

Quantitative Polymerase Chain Reaction (qRT-PCR) Analysis

Unlesioned and ischemic animals were killed with a chloral hydrate overdose (30%) 6, 12, 24, and 48 hours after lesion. Eyes were enucleated and retinae were shock frozen in liquid nitrogen. Isolation of mRNA was carried out using the RNeasy micro kit (Qiagen, Hilden, Germany), and equal amounts of total retinal mRNA were reverse transcribed into cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Schwerte, Germany). Quantitative RT-PCR amplification of murine frataxin (*mFxn*), *hFXN*, hypoxia-inducible factors 1 and 2 (*Hif-1α, Hif-2α*), glial fibrillary acidic protein (*Gfap*), and the antioxidative stress markers heme oxygenase-1 (*Hmox1*), glutathione peroxidase 1 (*Gpx1*), and superoxide dismutase 1 and 2 (*Sod1* and *Sod2*), as well as catalase (*Cat*), was carried out with Brilliant II SYBR Green QPCR Mastermix (Agilent Technologies, Santa Clara, CA, USA) using the primer sequences depicted in the Table. After denaturing at 95°C for 10 minutes, 40 amplification cycles were carried out as follows: denaturation at 95°C for 60 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds. The housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine–guanine phosphoribosyltransferase (*Hprt*) were used as reference genes. To determine the relative expression ratio (fold change) of the target genes, the analysis introduced by Pfaffl et al.[23] was applied.

Western Blotting

In order to evaluate changes in the protein levels of retinal FXN in Deleter B6 FXN mice and C57BL/6 mice following increased IOP, we performed Western blotting. Animals were killed with an overdose of chloral hydrate (30%) 12 and 48 hours after

TABLE. Quantitative RT-PCR Primer Sequences for mRNAs Analyzed in the Study, Together With Their Expected Product Length, GenBank Accession Number, and Reference

mRNA		Primer Sequence	Product Size	Accession No.	Reference
hFXN	fw	5′ CCTTGCAGACAAGCCATACA 3′	150 bp	NM_000144.4	41
	re	5′ CCACTGGATGGAGAAGATAG 3′			
mFxn	fw	5′ CCTGGCCGAGTTCTTTGAAG 3′	152 bp	U95736.1	42
	re	5′ GCCAGATTTGCTTGTTTGG 3′			
Gpx1	fw	5′ GGGACTACACCGAGATGAACGA 3′	197 bp	NM_008160	43
	re	5′ ACCATTCACTTCGCACTTCTCA 3′			
Sod1	fw	5′ GTCCGTCGGCTTCTCGTCT 3′	163 bp	NM_011434.1	44
	re	5′ CACAACTGGTTCACCGCTTG 3′			
Sod2	fw	5′ ATTAACGCGCAGATCATGCA 3′	161 bp	NM_013671.3	43
	re	5′ TGTCCCCCACCATTGAACTT 3′			
Cat	fw	5′ GCAGATACCTGTGAACTGTC 3′	229 bp	NM_009804.2	45
	re	5′ GTAGAATGTCCGCACCTGAG 3′			
Hmox1	fw	5′ GGTGATGGCTTCCTTGTACC 3′	155 bp	NM_010442.2	46
	re	5′ AGTGAGGCCCATACCAGAAG 3′			
Hif-1α	fw	5′ CAACGTGGAAGGTGCTTCA 3′	242 bp	NM_010431.2	
	re	5′ CGGCTCATAACCCATCAACT 3′			
Hif-2α	fw	5′ AGGTCTGCAAAGGACTTCGG 3′	162 bp	NM_010137.3	
	re	5′ CAAGTGTGAACTGCTGGTGC 3′			
Gfap	fw	5′ AGAAAGGTTGAATCGCTGGA 3′	176 bp	NM 010277.3	47
	re	5′ GCCACTGCCTCGTATTGAGT 3′			
Hprt	fw	5′ TGACACTGGTAAAACAATGCA 3′	94 bp	NM_013556.2	48
	re	5′ GGTCCTTTTCACCAGCAAGCT 3′			
Gapdh	fw	5′ AGGTCGGTGTGAACGGATTTG 3′	123 bp	NM_001289726.1	49
	re	5′ TGTAGACCATGTAGTTGAGGTCA 3′			

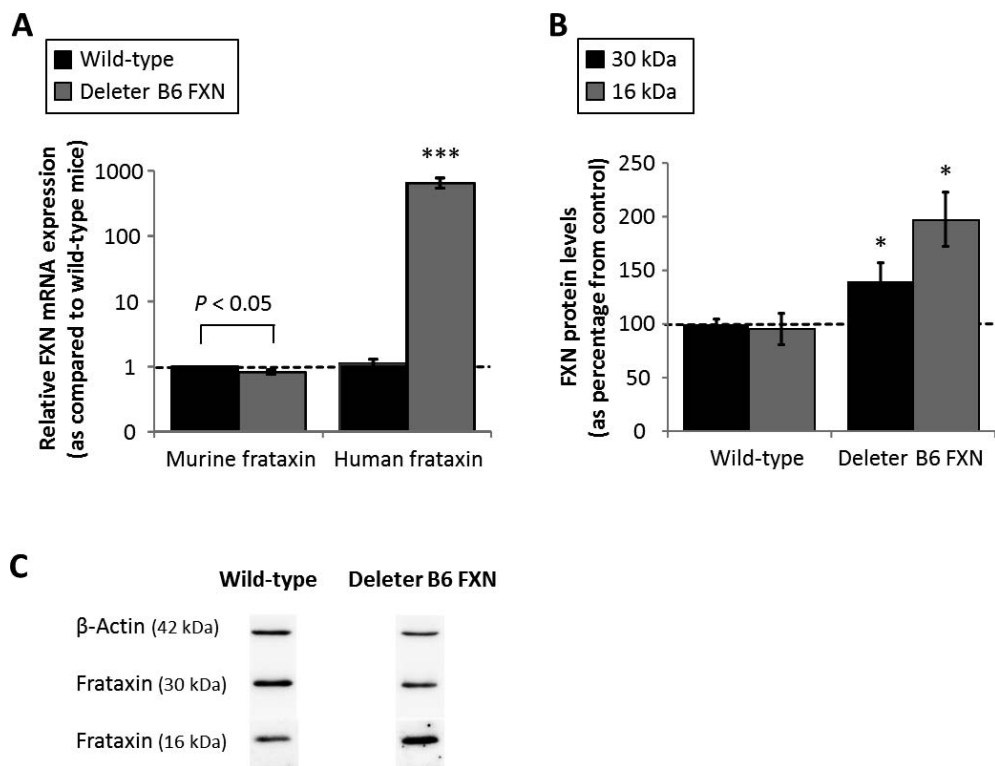fw, forward primer; re, reverse primer.

FIGURE 1. Overexpression of human frataxin in the retina from Deleter B6 FXN mice. (A) Relative expression of human frataxin (hFXN) mRNA was determined by quantitative polymerase chain reaction, normalized against *Gapdh* and *Hprt* mRNA levels and reported as fold increase of expression in wild-type C57BL/6 mice. The *y*-axis is depicted in logarithmic scaling. *Bars* represent the mean ± SEM; *dashed line* represents 1.0 ratio, ***$P < 0.001$ versus naïve C57BL/6 control; $n = 6$. (B) Frataxin protein levels of precursor frataxin form (30 kDa) and mature frataxin form (16 kDa) were determined by densitometric analysis of Western blotting in wild-type C57BL/6 and Deleter B6 FXN mice. Values are expressed as percentage of naïve C57BL/6 mice and normalized against β-actin. *Bars* represent mean ± SEM; *dashed line* represents 100%, $n = 4$, *$P < 0.05$ versus C57BL/6. (C) Representative Western blots showing precursor and mature bands for frataxin in wild-type and Deleter B6 FXN naïve mice.

lesion and eyes were enucleated. Retinae were dissected, harvested in RIPA lysis buffer (Santa Cruz), homogenized, and then centrifuged at 10,000g for 10 minutes at 4°C. Protein concentration was determined using the Quickstart Bradford Protein Assay Kit 3 (BioRad, München, Germany). Proteins (10–20 μg) were loaded onto an SDS 12% polyacrylamide gel. After electrophoresis, proteins were transferred onto poly-vinylidene fluoride (PVDF) membranes (GE Healthcare, Wuppertal, Germany). Nonspecific binding was blocked with 5% skim milk in Tween/Tris-buffered saline (TBS-T). Membranes were incubated overnight at 4°C with primary antibodies raised against FXN (1:500; Santa Cruz) and β-actin (1:2000; Abcam, Cambridge, UK) prepared in 2% skim milk in TBS-T. Membranes were washed with TBS-T and incubated with a 1:5000 dilution of horseradish peroxidase (HRP)–conjugated secondary antibody for 60 minutes. Protein bands were visualized using an enhanced chemiluminescence reaction kit (Immun-Star WesternC Chemiluminescence Kit, Bio-Rad), photographed with Fujifilm LAS-3000 Imager (Fuji Photo Film, Düsseldorf, Germany), and analyzed with ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij; Version 1.46). Measurements were done in quadruplicate. Each band was normalized against the corresponding β-actin band. Changes in protein expression were expressed as percentage from unlesioned control levels.

Statistical Analysis

All results are expressed as the mean ± standard error of the mean (SEM). Each group consisted of at least five animals. For RGC survival analysis, statistical significance was assessed using 1-way ANOVA followed by Holm-Sidak post hoc analysis by comparing wild-type control mice and treatment groups. For relative gene expression values (ratio), 1-way ANOVA followed by Holm-Sidak post hoc analysis was applied between treatment groups and control. Two-way ANOVA followed by Holm-Sidak post hoc analysis was applied for comparison of lesion effects between mice strains. For Western blot analysis, results were compared by 2-way ANOVA followed by Holm-Sidak post hoc analysis comparing wild-type and transgenic control mice and ischemic groups. SigmaPlot version 13.0 (Systat, Erkrath, Germany) was used, and the level of significance was set at $P < 0.05$.

RESULTS

Frataxin mRNA and Protein Levels Are Increased in the Retina of FXN-Overexpressing Mice

In Deleter B6 FXN mice, differentiation between mFXN and hFXN was possible only at the mRNA level. Primers were specifically designed for the sequence corresponding to the N-terminal region of the protein, which shows the highest diversity between species, whereas the antibody for Western blotting was directed against the C-terminal mitochondrial sequence, which is highly conserved between eukaryotes.

Human FXN expression was 650 times higher than endogenous mouse FXN ($P < 0.001$). Importantly, in the presence of hFXN, mRNA expression of endogenous mouse *Fxn* was decreased by 20% (1.00 ± 0.01 fold change versus 0.81 ± 0.05 fold change, $P < 0.05$) (Fig. 1A). In order to evaluate changes at the protein level, we measured levels of FXN precursor and mature forms (30 and 16 kDa, respectively). Relative levels of the 30-kDa form were 39 ± 12% higher compared to those in wild-type mice ($P < 0.05$), whereas the 16-kDa form was increased by 97 ± 20% ($P < 0.05$) (Fig. 1B).
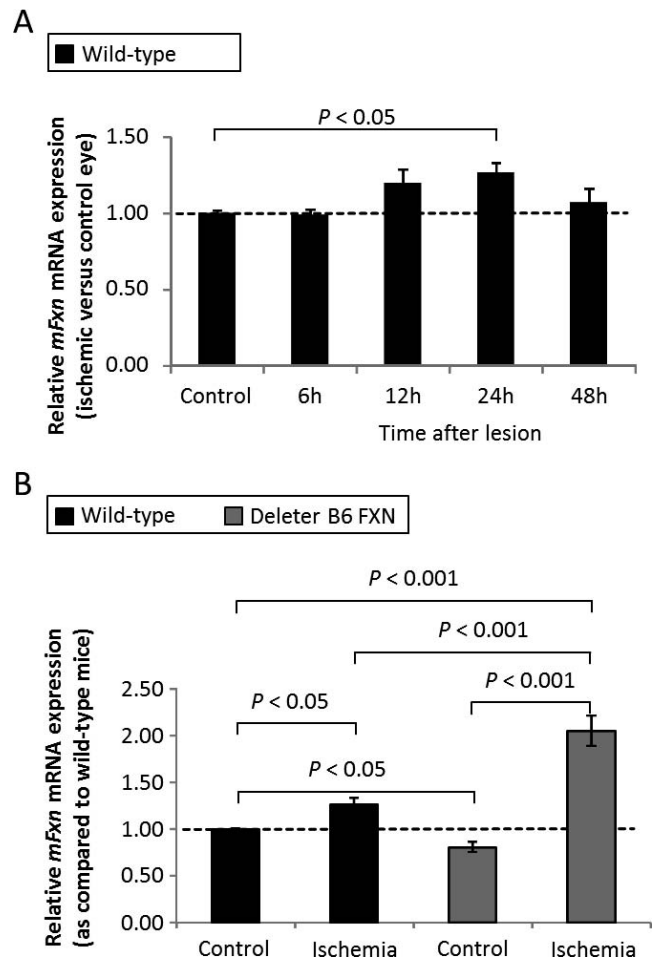
FIGURE 2. Effect of acute retinal ischemia/reperfusion on mRNA levels of frataxin, depicted as the ratio of treated versus naïve control eyes. Frataxin mRNA was determined by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt* mRNA levels. (**A**) Relative expression of murine frataxin in wild-type mice at different times of reperfusion. (**B**) Relative expression of murine frataxin in wild-type and Deleter B6 FXN mice as compared to naïve control, after 24 hours of reperfusion. *Bars* represent the mean ± SEM; *dashed lines* represent 1.0 ratio, $n = 5$ to 6. P values as depicted versus unlesioned wild-type C57BL/6 control.

Levels of FXN mRNA and Protein Are Increased in the Retina After Transient Ischemia/Reperfusion

Frataxin mRNA levels in wild-type mice were significantly increased by 26% after lesion, reaching a peak 24 hours after ischemia (1.26 ± 0.07-fold increase, $P < 0.05$), and decreased again to basal levels after 48 hours of reperfusion (Fig. 2A). In transgenic mice, *mFxn* expression showed a 2-fold increase compared to wild-type mice (2.05 ± 0.16 vs. 1.26 ± 0.07 fold change, $P < 0.001$) (Fig. 2B) after ischemia. Human *FXN* expression did not change after lesion (data not shown).

In order to evaluate the effect of ischemia on FXN levels, we determined protein content of the precursor and mature forms of FXN by means of Western blotting.

As shown in Figure 3A, the precursor form was not increased in wild-type mice for up to 48 hours after lesion compared to unlesioned control levels (100 ± 10% vs. 102 ± 11% and 112 ± 17%, NS). Protein levels for the mature form of FXN significantly increased 12 hours after lesion (100 ± 8% vs. 208 ± 27% $P < 0.01$) (Fig. 3B) and remained elevated for up to 48 hours after lesion (100 ± 8% vs. 216 ± 30% $P < 0.01$). In
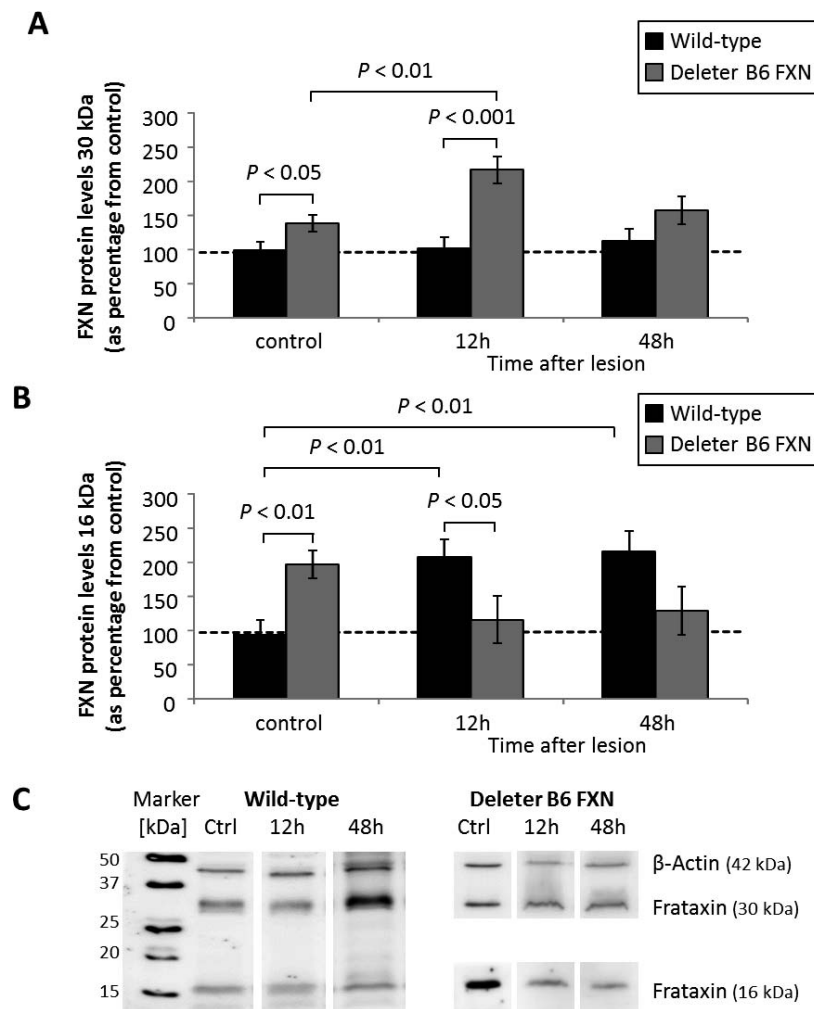
FIGURE 3. Effect of acute ischemia/reperfusion on protein levels of frataxin 12 and 48 hours after lesion. Densitometric analysis of (**A**) precursor frataxin (30 kDa) and (**B**) mature frataxin (16 kDa) protein levels by Western blotting in naïve and ischemic retinae. Values are expressed as percentage from unlesioned wild-type control retinae (100%). *Bars* represent mean ± SEM; *dashed line* represents 100%, $n = 5$, *P* values as depicted versus unlesioned wild-type C57BL/6 control. (**C**) Representative Western blots showing precursor and mature bands for frataxin.

transgenic mice, levels of the FXN precursor form were increased 12 hours after lesion compared to those in unlesioned animals (139 ± 12% vs. 217 ± 20%, $P < 0.01$, Fig. 3A), whereas the mature form of FXN did not change (197 ± 20% vs. 117 ± 35%, NS, Fig. 3B). Furthermore, levels of the mature form of FXN were decreased compared to those in wild-type mice 12 hours (208 ± 27% vs. 117 ± 35%, $P < 0.05$) after ischemia (Fig. 3B).

Retinal Ganglion Cell Survival Is Significantly Increased in FXN-Overexpressing Mice After Acute Ischemia/Reperfusion

Retinal ganglion cell density was analyzed in wild-type and transgenic mice at different retinal eccentricities (1/6, 3/6, and 5/6 from retinal radius). Values for the number of cells were normalized against the corresponding contralateral eye. Ganglion cell numbers were indistinguishable between unlesioned wild-type and transgenic mice ($P > 0.05$, data not shown). Therefore, contralateral eyes from each strain were used to normalize for the number of surviving RGCs after lesion.

Fourteen days after lesion, the percentage of surviving RGCs was significantly reduced both in wild-type (32.8 ± 5.2%; $n = 6$) and in transgenic mice (65.2 ± 4.5%, $n = 5$) compared to the corresponding unlesioned control animals ($P < 0.05$). However, FXN-overexpressing mice had a 32.4% higher number of RGCs surviving after lesion compared to wild-type mice ($P < 0.001$, Fig. 4).

Expression of Hypoxia-Inducible Factor and Antioxidative Markers Is Increased in FXN-Overexpressing Mice After Lesion

To determine whether increased antioxidative capacity might be involved in FXN-mediated neuroprotection, we evaluated the gene expression of antioxidative enzymes *Hmox1*, *Gpx1*, *Sod1* and *2*, and *Cat* by means of qRT-PCR. Expression analysis was performed at 24 hours of reperfusion to match FXN peak expression induced by the ischemic lesion.

In wild-type mice, expression levels of *Hmox1*, *Gpx1*, and *Sod1* were significantly increased after lesion (Ho-1: 9.21 ± 1.74-fold increase, $P < 0.001$, Fig. 5A; *Gpx1*: 1.33 ± 0.06-fold increase, $P < 0.001$, Fig. 5B; *Sod1*: 1.12 ± 0.06-fold increase, $P < 0.05$, Fig. 5D), whereas *Cat* expression was decreased (0.74
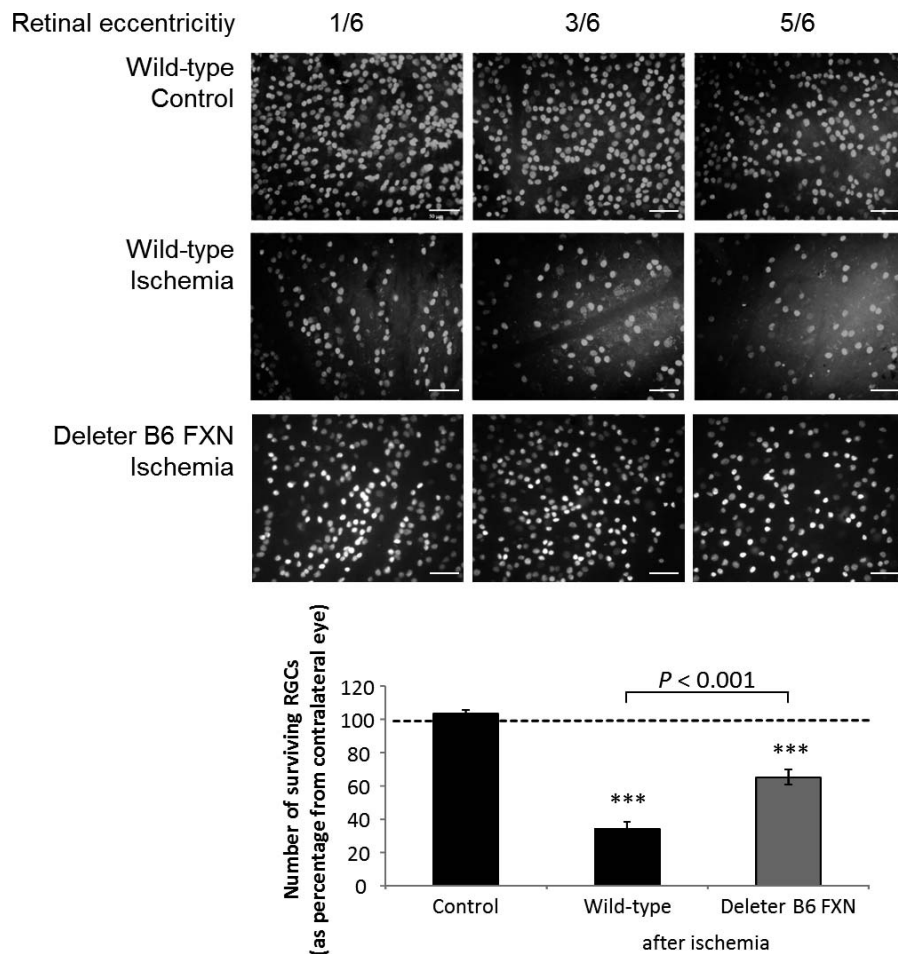
FIGURE 4. Effect of frataxin overexpression on RGC survival 14 days after acute retinal ischemia/reperfusion. (A) Images of retinal whole-mount preparations from lesioned and unlesioned wild-type and Deleter B6 FXN animals at various retinal eccentricities (1/6, 3/6, 5/6 from retinal radius) are shown. Images were taken with a fluorescent microscope (×40). *Scale bars* represent 50 μm. (B) Analysis of surviving RGCs after acute retinal ischemia. Numbers of RGCs are expressed as percentages from contralateral eyes. Cell counts were determined 14 days after the insult. *Bars* represent the mean ± SEM; *dashed line* represents 100%, $n = 5$–9. ***$P < 0.001$ versus unlesioned C57BL/6 control.

± 0.02-fold decrease, $P < 0.001$, Fig. 5C) compared to unlesioned controls.

In Deleter B6 FXN mice, expression of *Hmox1* (4.45 ± 1.53), *Gpx1* (1.66 ± 0.1), *Sod1* (1.25 ± 0.05), Sod2 (1.45 ± 0.1), and *Cat* (1.39 ± 0.04) was significantly higher compared to the corresponding unlesioned control 24 hours after lesion ($P < 0.001$) (Figs. 5A–E).

Gene expression 24 hours after lesion changed significantly between wild-type and Deleter B6 FXN mice for *Gpx1* (1.33 ± 0.06 vs. 1.66 ± 0.1-fold increase, $P < 0.001$), *Sod2* (1.13 ± 0.02 vs. 1.45 ± 0.1-fold increase, $P < 0.001$), *Hmox1* (9.21 ± 1.74 vs. 4.45 ± 1.53-fold decrease, $P < 0.05$), and *Cat* (0.74 ± 0.02 vs. 1.39 ± 0.04-fold increase, $P < 0.001$) (Figs. 5A–E).

Expression of *Gfap* significantly increased after lesion in wild-type and Deleter B6 FXN mice (5.85 ± 0.27 and 4.51 ± 0.28, $P < 0.001$). Although the *Gfap* increase in transgenic mice was slightly lower compared to wild-type, this was not statistically significant ($P = 0.081$, Fig. 5F).

Expression levels of both *Hif-1α* and *Hif-2α* were not changed after lesion in C57BL/6 wild-type mice but significantly increased in Deleter B6 FXN (*Hif-1α* 1.08 ± 0.04 vs. 1.54 ± 0.04 and *Hif-2α* 0.92 ± 0.03 vs. 1.37 ± 0.05 fold change, $P < 0.001$) (Figs. 6A, 6B).

DISCUSSION

Summary of Results

In the present work, we found an upregulation of the mitochondrial enzyme FXN both at the mRNA and at the protein level after retinal ischemia, indicating its involvement in the response to lesion in the adult mouse eye in vivo. Importantly, we found increased RGC survival after ischemia in transgenic mice overexpressing FXN. Improved cell survival correlated with an increase in gene expression of antioxidative-related enzymes.

Frataxin Overexpression Increases RGC Survival After Acute Ischemia/Reperfusion In Vivo

Here, we show that FXN levels are upregulated after acute retinal ischemia/reperfusion with a peak at 24 hours of reperfusion. To our knowledge, this is the first study showing an involvement of FXN in the response to ischemia/reperfusion in the central nervous system in vivo. Our findings are in agreement with other studies reporting FXN upregulation in response to hypoxic stress in vitro.[24,25] Upregulated FXN expression is also found in several tumor cell lines in response to hypoxic stress and promotes tumor
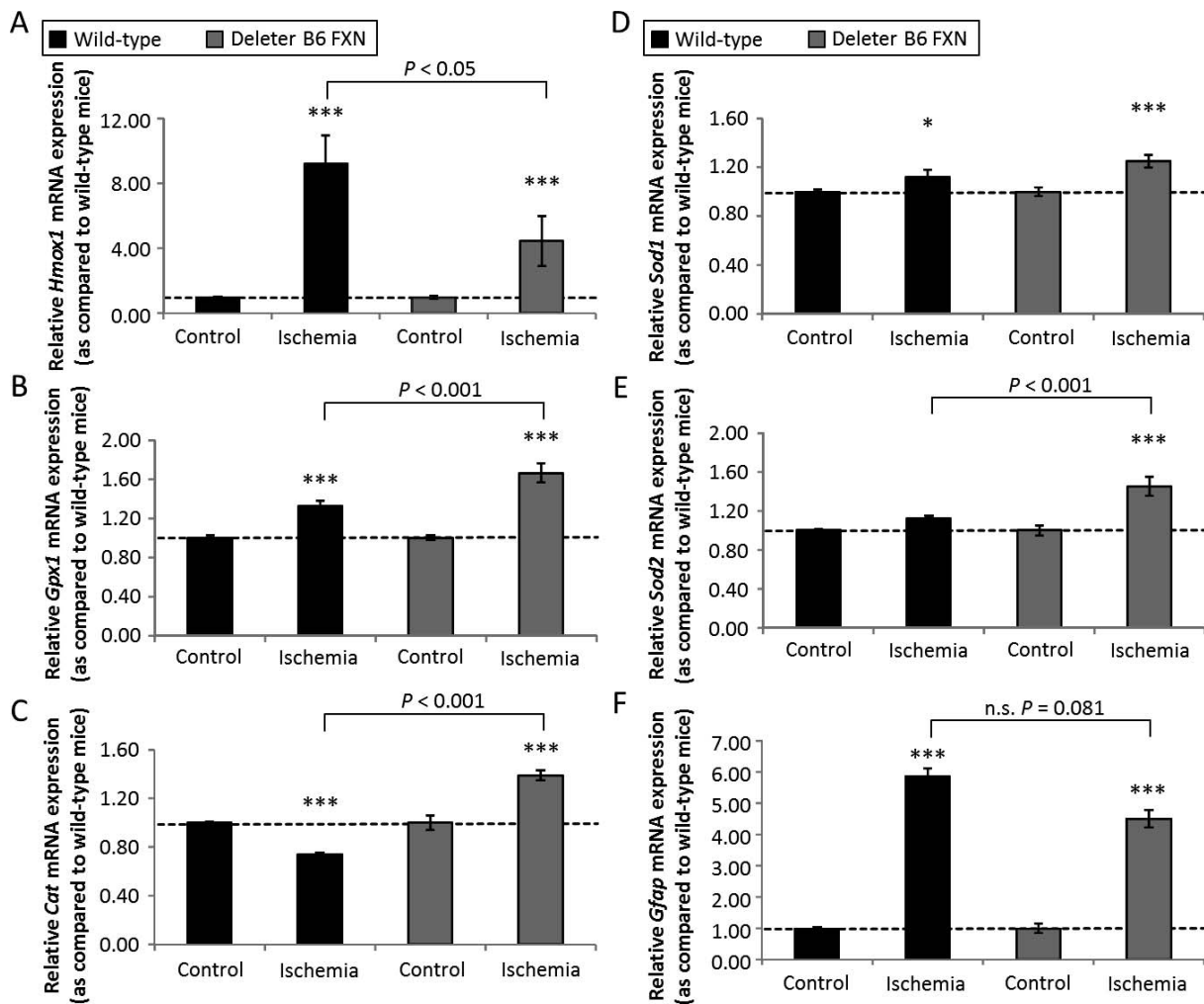
FIGURE 5. Effect of acute retinal ischemia/reperfusion on mRNA levels of (A) *Hmox1*, (B) *Gpx1*, (C) *Cat*, (D) *Sod1*, (E) *Sod2*, and (F) *Gfap*, depicted as the ratio of treated versus naïve corresponding control eyes. mRNA levels were determined 24 hours after ischemia by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt* mRNA levels. *Bars* represent the mean ± SEM; *dashed lines* represent 1.0 ratio, $n = 5$ to 6, ***$P < 0.001$ versus corresponding unlesioned control.

cell survival and progression.[25] The role of increased FXN levels following retinal ischemia is not clear, but could be part of a response mechanism aimed to overcome lesion-induced iron dysregulation and increased oxidative stress. Recently, an increase in FXN levels was found to have a cardioprotective effect against ischemia/reperfusion.[26] In order to evaluate the effect of increased FXN on neuronal survival following acute retinal ischemia/reperfusion in vivo, we used a conditional mouse model overexpressing FXN under the control of a ubiquitous active promoter. Mice constitutively expressing the full-length hFXN cDNA have been already described and do not show any signs of ataxia or other obvious abnormalities.[27] High levels of FXN were revealed in all major organs affected in FRDA, including the pancreas, brain, skeletal muscle, and the heart.[28] In our mouse model, we found that prelesion overexpression of hFXN and not lesion-induced expression leads to enhanced neuronal cell survival in the retina after acute ischemia/reperfusion in vivo. Our results are in support of other studies showing that FXN delivered prelesion is neuroprotective in different brain regions.[3,16] Frataxin-mediated neuroprotection might involve a preconditioning effect and an induction of long-lasting retinal ischemic tolerance, as has been shown after treatment with the iron chelator deferroxamine.[29] As already mentioned, FXN plays an important role in mitochondrial iron homeostasis.[12,13] In wild-type mice, we found increased levels of FXN mature protein after lesion, whereas the precursor form was not elevated. In contrast, although levels of the precursor protein were significantly increased in transgenic as compared to wild-type mice, the mature protein was not changed after lesion. This might suggest that in wild-type mice FXN is converted into mature protein after lesion, whereas in transgenic mice maximum levels of the mature protein have already been reached before lesion. Therefore, no further increase takes place after lesion, and the precursor form accumulates in the cell. The reason for the lack of increase of FXN's mature form after ischemia in transgenic animals, compared to wild-type, is not clear and needs to be further evaluated. Importantly, translation of FXN mRNA into protein as well as enzyme maturation has been shown to be tightly regulated.[30]

Frataxin Overexpression Leads to an Increased Antioxidative Response

Since oxidative stress is critically involved in cell damage after retinal ischemia, we evaluated the expression levels of
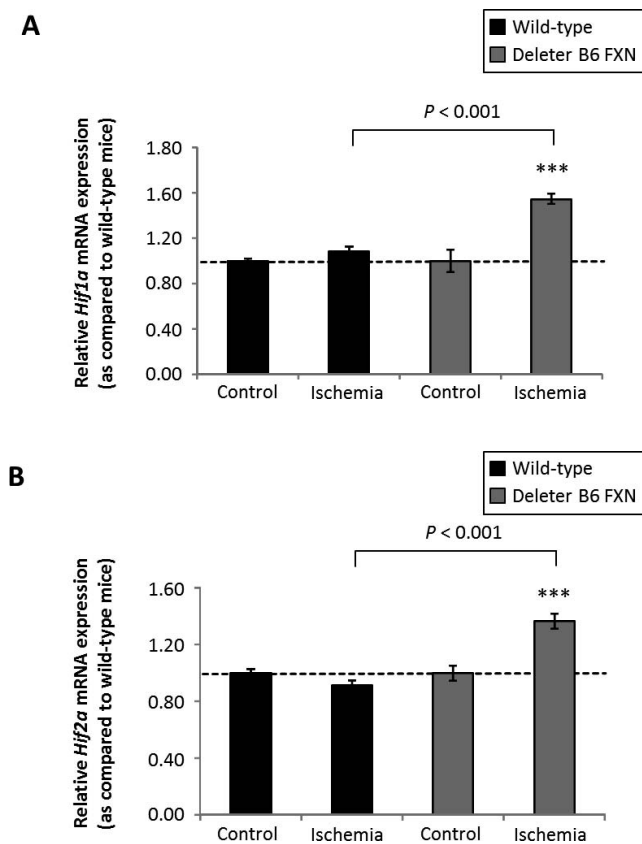
A

B

FIGURE 6. Effect of acute retinal ischemia/reperfusion on mRNA levels of (**A**) *Hif-1α* and (**B**) *Hif-2α*, depicted as the ratio of treated versus naïve corresponding control eyes. mRNA levels were determined 24 hours after ischemia by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt* mRNA levels. *Bars* represent the mean ± SEM; *dashed lines* represent 1.0 ratio, $n = 5$ to 6, ***$P < 0.001$ versus corresponding unlesioned control.

antioxidant enzymes including *Gpx1*, *Sod1*, *Sod2*, *Cat*, and *Hmox1*, all known to be involved in the response to ischemia. It is known that protective effects mediated by FXN overexpression involve several antioxidant mechanisms and activation of mitochondrial energy metabolism.[15,27,28,31] Moreover, Shoichet et al.[15] showed that FXN itself lacks classical antioxidant properties similar to Gpx, Sod, and Cat. In particular, FXN overexpression enhances Gpx1 activity, increases cellular glutathione content, and upregulates Cat, at least in vitro.[4,15,28,32] In our study, following lesion, expression of *Gpx1*, *Sod2*, and *Cat* was significantly enhanced in mice overexpressing FXN compared to wild-type mice. Interestingly, Cat, which is a potent scavenger of H_2O_2 and provides a powerful antioxidant defense in the retina, was found to be reduced after ischemia in wild-type animals, in agreement with previous studies in rats.[33] Increase in Sod2 and Cat has been previously shown to be neuroprotective in the retina; in particular, intravitreal injection of AAV-Sod2 and AAV-Cat protected RGCs and inner neurons from ischemia/reperfusion injury.[34,35] Glutathione peroxidase 1 has been shown to inhibit cell death and, interestingly, glial activation following experimental stroke in mice.[36]

Expression of *Hmox1* was increased 24 hours after lesion, both in wild-type and in FXN-overexpressing mice; however, it was significantly lower in transgenic mice compared to wild-type mice. The reason for this lower *Hmox1* increase in transgenic mice is not clear. Heme oxygenase-1, which has

been shown to protect RGCs after ischemia,[37] is known to be induced in Müller cells by oxidative stress 6 hours after reperfusion and peaks at 12 to 24 hours after lesion.[38] The lower *Hmox1* expression might reflect changes in glial activity in transgenic mice; however, since expression analysis was performed in whole retinal lysates, it is not possible to draw conclusions about cell-specific responses. The trend to a lower *Gfap* expression in transgenic animals, although not significant, could also be indicative of changes in glial response due to FXN overexpression.

Therefore, increased expression of antioxidative markers seems to be involved in the improved survival of RGCs after retinal ischemia/reperfusion found in our study. A possible role of glia cells in mediating neuroprotective effects, as suggested by the regulation of some of the markers evaluated, will be further addressed in a future study.

Levels of Hypoxia-Inducible Factors Hif-1α and Hif-2α Are Elevated in the Retinae of FXN-Overexpressing Mice After Ischemia

Hypoxia-inducible factors (HIFs) are the principal regulators of the transcriptional response to hypoxia. These factors regulate the expression of genes containing the conserved hypoxia-responsive element (HRE). In particular, mouse FXN promoter possesses an HRE and its expression is controlled by *Hif-2α*.[24] Furthermore, Sod2 is also regulated by *Hif-2α*.[39] We measured expression levels of the hypoxia-inducible factors *Hif-1α* and *Hif-2α* in both unlesioned retinae and 24 hours after ischemia. We found that expression levels for both factors were similarly increased in FXN-overexpressing mice, but not in wild-type mice at this time point. Mowat et al.[40] found that in the retina, HIFs show an expression peak already 2 hours after ischemia and reach basal levels 24 hours after lesion. Increased expression of HIFs in transgenic mice 24 hours after lesion further supports our assumption that FXN-mediated neuroprotection is due to a preconditioning-induced ischemic tolerance. Interestingly, in the mouse retina, *Hif-2α* expression has been reported to be limited to Müller cells and that of *Hif-1α* to neurons.[40]

Taken together, our findings show that increased FXN expression is part of the cellular response mechanism to ischemia in the retina. The role of the FXN increase after lesion in the normal retina is still not clear, but might constitute an attempt to overcome damage caused by iron dysregulation and increased oxidative stress after retinal ischemia/reperfusion. Reduced levels of the active form of FXN in transgenic mice after ischemia suggest that the protective effects observed are due to a preconditioning mechanism, and not to a FXN-mediated postlesional effect. The increased neuronal survival elicited by FXN overexpression involves an upregulated antioxidative mechanism after acute retinal ischemia/reperfusion in vivo.

Treatments aiming to modulate FXN levels might constitute therapeutic targets to induce ischemic tolerance and reduce neuronal damage caused by mitochondrial dysfunction after ischemia/reperfusion in the retina.

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