Light-Induced Retinal Degeneration Correlates with Changes in Iron Metabolism Gene Expression, Ferritin Level, and Aging

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PURPOSE. Retinal degeneration is associated with iron accumulation in several rodent models in which iron-regulating proteins are impaired. Oxidative stress is catalyzed by unbound iron.

METHODS. The role of the heavy chain of ferritin, which sequences iron, in regulating the thickness of the photoreceptor nuclear layer in the 4- and 16-month-old wild-type H ferritin (HFe+/−) and heterozygous H ferritin (HFe+/−) mice was investigated, before and 12 days after exposure to 13,000-lux light for 24 hours. The regulation of gene expression of the various proteins involved in iron homeostasis, such as transferrin, transferrin receptor, hephaestin, ferroportin, iron regulatory proteins 1 and 2, hepcidin, ceruloplasmin, and heme-oxynase 1, was analyzed by quantitative (q)RT-PCR during exposure (2, 12, and 24 hours) and 24 hours after 1 day of exposure in the 4-month-old HFe+/− and HFe+/− mouse retinas.

RESULTS. Retinal degeneration in the 4-month-old HFe+/− mice was more extensive than in the HFe+/− mice. Yet, it was more extensive in both of the 16-month-old mouse groups, revealing the combined effect of age and excessive light. Injury caused by excessive light modified the temporal gene expression of iron-regulating proteins similarly in the HFe+/− and HFe+/− mice.

CONCLUSIONS. Loss of one allele of H ferritin appears to increase light-induced degeneration. This study highlighted that oxidative stress related to light-induced injury is associated with major changes in gene expression of iron metabolism proteins. (Invest Ophthalmol Vis Sci. 2011;52:1261–1274) DOI:10.1167/iovs.10-5705

Iron is an essential element for cell survival and is a cofactor of various enzymes.1 However, it can generate free radicals and is harmful when present in excess. Homeostatic mechanisms maintain iron levels by regulating the proteins involved in iron import (transferrin and transferrin receptor), storage (ferritins), and export (ferroportin, ceruloplasmin, and hephaestin), through various central regulators, including iron regulatory proteins 1 and 2, hepcidin, human hemochromatosis protein (HFE), hemopexin, and hemoglobin. Iron and several proteins involved in its homeostasis have been reported in various retinal layers.2–7 A specific iron cycle involving phagocytosis of outer segment (OS) tips by retinal pigmented epithelium (RPE) cells and transferrin-dependent redistribution of iron to photoreceptors has been described.8 Retinal cell dysfunction has been recently associated with deregulation of gene expression of hemopexin,9 human hemochromatosis protein,5 hemoglobin 1, and ferroportin 1.10 However, the role of ferritin has not been directly investigated, despite reports that its level increases with age in various animal models, as in Hfe−/− mice,11 in cytomegalovirus-infected retina,12 and in RPE–choroid complex and neural retina of aged rodent.13,14 It was also reported that inadequate ferritin expression was found in hemopexin null mice brain.14 Most of the retinal degeneration occurring in these animal models appears with aging, which reflects additional factors such as age-dependent oxidative stress. In addition, age-related macular degeneration (AMD) is associated with iron accumulation15 and high levels of ferritin and ferroportin in the retina.16

The role of the ferritin complex is to bind iron in a nontoxic and readily available form.17 It consists of two functionally and genetically different chains, termed heavy (H) and light (L). H and L ferritins are present in all the outer retinal layers.2–7 A specific iron cycle involving phagocytosis of OS tips by RPE cells and transferrin-dependent redistribution of iron to photoreceptors has been described.8 Retinal cell dysfunction has been recently associated with deregulation of gene expression of hemopexin,9 human hemochromatosis protein,5 hemoglobin 1, and ferroportin 1.10 However, the role of ferritin has not been directly investigated, despite reports that its level increases with age in various animal models, as in Hfe−/− mice,11 in cytomegalovirus-infected retina,12 and in RPE–choroid complex and neural retina of aged rodent.13,14 It was also reported that inadequate ferritin expression was found in hemopexin null mice brain.14 Most of the retinal degeneration occurring in these animal models appears with aging, which reflects additional factors such as age-dependent oxidative stress. In addition, age-related macular degeneration (AMD) is associated with iron accumulation15 and high levels of ferritin and ferroportin in the retina.16

To evaluate the stress related to light-induced injury is associated with major changes in gene expression of iron metabolism proteins.
mRNAs of iron-regulating proteins—H ferritin, L ferritin, transferrin, transferrin receptor, ceruloplasmin, ferroportin, hephaestin, hepcidin, and iron-regulatory proteins—as well as that of the antioxidative stress protein heme oxygenase-1 at various times after illumination. We found substantial time-dependent variations in the expression of genes of several proteins involved in iron homeostasis.

**METHODS**

### Animals

HFt/−/− and HFt/+ mice of both genders (an equal number of males and females) and in an albino BALB/c genetic background were provided by Carole Beaumont (Faculté Bichat). They were screened for the presence of one or two allele(s) by PCR. All mice were fed a standard laboratory diet and tap water ad libitum and were maintained in a temperature-controlled room at 21°C to 23°C with a 12-hour/12-hour light–dark photoperiod. Experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with policies on animal use in neuroscience research. They were approved by each author’s institutional animal care review board.

### Mouse Light-Induced Injury

Four- and/or 16-month-old HFt/−/− and HFt/+ mice were dark adapted, exposed to light (13,000 lux) in nonreflective cages for 24 hours, and killed 12 days after exposure to light. Their eyes were then enucleated and fixed (see the following section) for histologic analyses. Also, for histologic and molecular analyses, some 4-month-old enucleated and fixed (see the following section) for histologic analy-

### Preparation of Retinal Extracts

The eyes were enucleated, and the neural retinas were extracted and immediately stored in RNA stabilization reagent (Qiagen, Courtaboeuf, France) at −80°C for qRT-PCR analysis.

For enzyme-linked immunosorbent assays (ELISAs), the mice were perfused through the left cardiac ventricle with 1× PBS. Then, the eyes were enucleated and dissected to isolate the neural retinas. They were immediately frozen in liquid nitrogen and stored at −80°C.

### Analysis by qRT-PCR

Neural retinas were homogenized with a motorized pestle (Dutsch, Issy les Moulineaux, France) in lysis buffer and RNA was isolated on a mini column (RNeasy; Qiagen). First-strand cDNA was obtained by a standard reverse transcription (RT) reaction (Invitrogen). Next, 150 ng RNA was reverse transcribed for 50 minutes at 42°C with 200 U of reverse transcriptase (SuperScript II; Invitrogen) and oligo-dT. One microliter of RNase H was added to each sample and incubated for 20 minutes at 37°C before target cDNA amplification. A qPCR master mix was prepared to a final volume of 18 L SYBR green qPCR master mix (Platinum Super-Mix-UD; Invitrogen) and olio-dT. One microliter of cDNA was added to each sample and incubated for 20 minutes at 37°C before target cDNA amplification. A qPCR master mix was prepared to a final volume of 18 µL:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Beta-actin</td>
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<td>5' CCA CAG GAT TCC ATA CCC AAG A 3'</td>
</tr>
<tr>
<td>H ferritin</td>
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<td>5' CTT GGT CAC CCA GGT CTT TA 3'</td>
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<tr>
<td>L ferritin</td>
<td>5' TGG CCA TGG AGA AGA ACC TGA ATC 3'</td>
<td>5' CCA CAG TCC TCA CTT AAG A 3'</td>
</tr>
<tr>
<td>Transferrin</td>
<td>5' GGA CGC CAT GAC TTT GGA TG 3'</td>
<td>5' GGC ATG ACA GGC ACT AGA C 3'</td>
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<tr>
<td>Transferrin receptor</td>
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<td>5' CTT GCC GGC AAC ACC AGC A 3'</td>
</tr>
<tr>
<td>Iron-regulatory protein 1</td>
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<td>5' TAG CTC GGT CAG GAA TGG AGC A 3'</td>
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<td>Iron-regulatory protein 2</td>
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<td>5' ACG CTT ACT TGC CTC AGG TGG TTT 3'</td>
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<td>Ferroportin</td>
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<td>5' CAG CAA CTG TGT CAG GAT CAA 3'</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>5' GGG AGG CTT CTA GCA GCA TCA 3'</td>
<td>5' GCA CTT GGG CAT CAC GAT 3'</td>
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<tr>
<td>Hepcidin</td>
<td>5' GCC TGG GCA CAG AGA CTG AT 3'</td>
<td>5' GGT GTG AGG AAA GAG CAT GAC TG 3'</td>
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<tr>
<td>Heme oxygenase 1</td>
<td>5' GCC ACC AAG GAG GTA CAC AT 3'</td>
<td>5' GCT TGC TGC GCT CTA CTC CC 3'</td>
</tr>
</tbody>
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Determination

Neural retinas were incubated in lysis buffer (15 mM Tris [pH 7.9], 60 mM KCl, 15 mM NaCl, 2 mM EDTA, and 0.4 mM phenylmethylsulfonyl fluoride [PMSF]; Perbio Science, Brebières, France). After four successive freeze–thaw cycles (liquid nitrogen–room temperature), the lysates were centrifuged at 1000g for 10 minutes. The supernatants were then stored at −20°C.

Ferritin concentration was determined by ELISA specific for the H and L chains, as previously described. Briefly, microtiter plates were coated with 1 μg polyclonal antibody specific for mouse H or L ferritin. Soluble mouse tissue homogenate or standard ferritin was diluted in PBS-Tween-BSA (50 mM sodium phosphate [pH 7.4], 150 mM NaCl, 0.05% vol/vol Tween-20, and 1% BSA) and added to the plates. The presence of ferritin was revealed by incubation with the same antibody labeled with horseradish peroxidase. Peroxidase activity was developed with o-phenylenediamine dihydrochloride (Sigma). Recombinant mouse H and L subunits were used as ferritin standards. Supernatants were used to determine the ferritin content. Protein concentrations were determined with the BCA protein assay kit (Pierce Biotechnology, Brebières, France), with BSA used as the standard. Results are reported in nanograms per milliliter of supernatant.

Histology

Enucleated eyes from mice were fixed with 4% paraformaldehyde (LADD; Inland Europe, Conflans-sur-Lanterne, France) and 0.5% glutaraldehyde (LADD) in PBS for 2 hours. After fixation, the samples were washed, dehydrated, transferred to the infiltration solution in a resin embedding kit (Historesin; Leica), and incubated overnight at 4°C. Embedded samples were semithin sections on a microtome (RM 2055; Leica Jung). The sections were mounted on slides coated with gelatin, stained for 2 minutes with 1% toluidine blue solution, analyzed by microscope (Aristoplan; Leica), and photographed with a digital camera (Leica) with identical exposure settings, so that the images could be compared.

Measurement of Nuclear Layer Thickness

We studied retinal morphology in the 4- and 16-month-old Hft+/+ and Hft−/− mouse retinas. The outer nuclear layer (ONL) thickness was measured at 200-μm intervals in both superior and inferior poles and averaged to give a single value.

We used two histologic protocols for retinal analysis after exposure to light. First, the ONL thickness was measured in similar central regions: from 100 to 600 μm of the optic nerve head, in both superior and inferior poles in retinas of 4-month-old Hft+/+ and Hft−/− mice exposed to light for 24 hours and killed 1 day later. Second, the thicknesses of the ONL and inner nuclear layer (INL) were measured at 200-μm intervals on each side of the optic nerve and across the whole retina in the 4- and 16-month-old Hft+/+ and Hft−/− mice killed 12 days after being exposed to light for 24 hours.

For each protocol, three separate sections collected from two to six eyes from separate animals were measured. Results were averaged to give a single value.

Statistical Analyses

Results are presented as the mean ± SEM. We used the Mann-Whitney U test to evaluate the significance of the differences between control and illuminated Hft+/+ and Hft−/− mice. For qRT-PCR data analysis, Hft+/+ mice were compared to Hft−/− mice, unless the bracket on the graph indicates another comparison. Two-way ANOVA was used to analyze retinal nuclear layer thickness after illumination ( Prism; GraphPad, San Diego, CA). P < 0.05 was considered statistically significant. We performed three or four replicates for each experimental condition.

Results

Comparative Analysis of 4- and 16-Month-Old Hft+/+ and Hft−/− Mouse Retinas

Histologic Analysis. We performed histologic analysis of the retinas of Hft+/+ mice and control Hft−/− littermates. There were no gross regional differences in the retinal structures (Figs. 1A–D). Nevertheless, the ONLs in the 4- and 16-month-old Hft−/− mice was slightly but significantly less thick than those in the Hft+/+ mice (7.7% in the 4-month-old mice and 9.9% in the 16-month-old mice). The ONL was also thinner (by 25%) in both the 16-month-old Hft+/+ and Hft−/− than in the 4-month-old mice (Fig. 1E) reflecting a similar age-related cell loss.

ampfiltration was performed on a real-time PCR system (model 7300; ABI). The following conditions were used: 95°C for 10 minutes (denaturation); 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (amplification and quantification); and 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds (melting curve). Crossing points for each transcript (the point at which the fluorescence is significantly higher than background fluorescence) were determined for subsequent analysis.

ELISA for Tissue H and L Ferritin Subunit Determination

We studied retinal morphology in the 4- and 16-month-old HFt−/− retina in the 4- and 16-month-old HFt−/− eyes from separate animals were measured. Results were averaged to measured at 200-
Ferritin Proteins and mRNAs. We used qRT-PCR to assay the corresponding mRNAs in the neural retina (Figs. 2A, 2B). H ferritin mRNA was less abundant in the HFt+/− mice than in the HFt+/+ mice at all ages studied (4 months, 52.7%; 16 months, 45.9%; Fig. 2A). However, the amounts of L ferritin mRNA were similar in the 4- and 16-month-old HFt+/− and

![Graph showing qRT-PCR quantification of H ferritin mRNA](image)

![Graph showing qRT-PCR quantification of L ferritin mRNA](image)

**Figure 2.** Ferritin proteins and mRNAs in 4- and 16-month-old HFt+/+ and HFt+/− mice. (A, B) Quantification by qRT-PCR of H (A) and L (B) ferritin mRNAs in lysates of retinas from 4- and 16-month-old HFt+/+ and HFt+/− mice. (C, D) Quantification by ELISA of H (C) and L (D) ferritin proteins in lysates of retinas from 4- and 16-month-old HFt+/+ and HFt+/− mice. In the 4- and 16-month-old HFt+/− mice, the amounts of H ferritin protein and mRNA were nearly half those in the HFt+/+ mice. L ferritin protein was more abundant in the 4-month-old HFt+/+ than HFt+/− mice, but was similar in the 16-month-old HFt+/+ and HFt+/− mice. Data are the mean ± SEM. *P < 0.05; **P < 0.01. (E-M) Immunolocalization of H (F-I) and L (J-M) ferritin on frozen sections of retina from 4- and 16-month-old HFt+/+ and HFt+/− mice. (E) Omission of the primary antibody was used as the control. (F-I) H ferritin in the HFt+/+ (F, H) and HFt+/− (G, I) mouse retina from 4- and 16-month-old (F, G) and 16-month-old (H, I) mice. (J-M) L ferritin in the HFt+/+ (J, L) and HFt+/− (K, M) mouse retina from 4-month-old (J, K) and 16-month-old (L, M) mice. In the HFt+/+ and the HFt+/− mice, the signals for H and L ferritin were in the photoreceptor inner segments (IS), ONL, OPL, and INL and in the inner top of the IPL. Sections were counterstained with DAPI (blue). Scale bar, 100 μm.
HFt\(^{+/+}\) mice (Fig. 2B). The concentration of H ferritin protein in the 4-month-old HFt\(^{+/−}\) mice was 37.0% of that in the HFt\(^{+/+}\) mice. Similarly, the concentration of H ferritin protein in the 16-month-old HFt\(^{+/−}\) mice was 44.5% lower than in the HFt\(^{+/+}\) mice (Fig. 2C), indicating that there was no age effect. Conversely, L ferritin protein was significantly more abundant (168.0%) in the 4-month-old HFt\(^{+/−}\) mice than in the 4-month-old HFt\(^{+/+}\) mice. However, the amount of L ferritin in the 16-month-old HFt\(^{+/−}\) mice was similar in the WT mice (Fig. 2D). The amount of L ferritin mRNA and protein decreased in the HFt\(^{+/−}\) mice between 4 and 16 months (Figs. 2B, 2D). That a significant increase in L ferritin occurred only at 4 months seems to indicate a mechanism of compensation that was not present at 16 months.

We used immunostaining with cryosections to test whether the location of H and L ferritin in the retina was modified in the 4- and 16-month-old HFt\(^{+/−}\) and HFt\(^{+/+}\) mice (Figs. 2E–M). H ferritin was found throughout the retina, with the strongest signals in the RPE, photoreceptor inner segments, ONL, outer plexiform layer (OPL), inner nuclear layer (INL), and in the inner top of the inner plexiform layer (IPL; Figs. 2F–I). With the same exposure time, H ferritin gave the same pattern in the HFt\(^{+/−}\) mice as in the HFt\(^{+/+}\) mice. H ferritin had the same distribution as H ferritin in the HFt\(^{+/−}\) mice, but was nevertheless present (Figs. 2G, 2I). L ferritin had the same distribution as H ferritin (Figs. 2J–M). Immunodetection of mitochondrial ferritin gave the same pattern in the HFt\(^{+/−}\) and HFt\(^{+/+}\) mice at both developmental stages studied. Mitochondrial ferritin was present in all the layers of the retina, with the strongest immunolabeling in the RPE and photoreceptor inner segment ellipsoids (data not shown).

Iron-Regulating Protein Expression. We assayed the mRNAs for several proteins important in regulating iron homeostasis in the 4- and 16-month-old HFt\(^{+/+}\) and HFt\(^{+/−}\) mouse retinas: ferroportin, transferrin, transferrin receptor, ceruloplasmin, hephaestin, hepcidin, and iron regulatory proteins 1 and 2. Ferroportin mRNA was significantly more abundant in the 16-month-old mice than in the 4-month-old mice (by 35% in the HFt\(^{+/+}\) mice and 37% in the HFt\(^{+/−}\) mice), but was unaffected by genotype (data not shown). The amounts of other mRNAs for iron-regulating proteins were not modified by either the age or the genotype of the mice (data not shown).

Although the concentration of H ferritin in the HFt\(^{+/−}\) mice was half that in the HFt\(^{+/+}\) mice, the thickness of the ONL in the retina was only slightly affected. There were no other differences in the amounts of mRNAs for the iron-regulating proteins between the HFt\(^{+/+}\) and the HFt\(^{+/−}\) mice.

Thus, the allelic diminution of H ferritin does not appear to affect retinal histology and iron-regulating protein expression during normal aging. To test whether the response to oxidative stress of the retina was affected by H ferritin level, we used light-induced retinal degeneration experiments.

**Figure 3.** Retinal modification in 4- and 16-month-old HFt\(^{+/+}\) and HFt\(^{+/−}\) mice 12 days after illumination. (A, B) Measurement of the ONL thickness, every 200 μm, throughout the retina of 4-month-old (A) and 16-month-old (B) HFt\(^{+/+}\) and HFt\(^{+/−}\) mice exposed or not exposed to light for 24 hours and killed 12 days later. The decrease in ONL thickness after exposure to light was much higher in the 4-month-old HFt\(^{+/+}\) than HFt\(^{+/−}\) mice at both poles, especially toward the periphery. The decrease in ONL thickness was more apparent in the 16-month-old HFt\(^{+/+}\) and HFt\(^{+/−}\) mice exposed to light than in the unexposed HFt\(^{+/+}\) and HFt\(^{+/−}\) mice. (C, D) Measurement of the INL thickness, every 200 μm, throughout the retinas of 4-month-old (C) and 16-month-old (D) HFt\(^{+/+}\) and HFt\(^{+/−}\) mice illuminated or not illuminated for 24 hours and killed 12 days later. Solid gray line: HFt\(^{+/+}\) control mice; solid black line: HFt\(^{+/−}\) control mice; dashed gray line: HFt\(^{+/+}\) illuminated mice; dashed black line: HFt\(^{+/−}\) illuminated mice. Data are the mean ± SEM of results from 6 to 18 independent sections. ***P < 0.0001; ns: not significant.
Comparison of Light-Induced Retinal Degeneration in 4- and 16-Month-Old HFT+/+ and HFT+/- Mouse Retinas

We used two different experimental protocols to compare the light sensitivity of the HFT+/+ and the HFT+/- mice. The first was to study retinal structure after 24 hours of illumination. For retinal histology analyses, the mice were killed 12 days after the end of illumination. For evaluating modifications to several iron homeostasis proteins and mRNA, the mice were killed 1 day after illumination. For the second protocol, the mice were exposed for 2, 12, and 24 hours and killed immediately. Iron-regulating protein mRNAs were assayed after these various times of exposure.

Histologic Analysis of the Effects of Light on 4- and 16-Month-Old HFT+/+ and HFT+/- Mice 12 Days after Illumination. After 24 hours of dark adaptation, the 4- and 16-month-old HFT+/+ and HFT+/- mice were subjected to 13,000 lux white light for 24 hours and then returned to a 12-hour/12-hour light–dark photoperiod for 12 days. The illumination caused photoreceptor death characterized by a decrease in ONL thickness in the retinas of the 4- and 16-month-old HFT+/+ and HFT+/- mice (Figs. 3A, 3B). However, INL thickness was unaffected (Figs. 3C, 3D). In the 4-month-old mice, exposure to light caused a decrease in ONL thickness over most of the retina, with the largest effect in the central part. The thinning of the ONL was greater in retinas from the HFT+/- mice than in those from HFT+/+ illuminated mice. In the central part of the retinas (1000 μm from each pole), the mean decrease in ONL thickness compared with that in the nonilluminated controls was 93% in the HFT+/- mice and 60% in the HFT+/+ mice. The effects of light decreased (ONL thickness increased) with the distance from the optic nerve. Light had significantly different effects on HFT+/+ and HFT+/- mouse retinas in each pole, between 1000 and 2200 μm from optic nerve. ONL thickness in the HFT+/- mouse retinas was 45.0% of that in the HFT+/+ retinas in the superior pole and 49.6% in the inferior pole (Fig. 3A). Illumination led to a significant reduction of ONL thickness in the entire retina in the 16-month-old HFT+/+ and HFT+/- mice (84.7% in the HFT+/+ mice and 78.0% in the HFT+/- mice; Fig. 3B).

The same protocol of illumination as that used for the 4-month-old mice led to retinal degeneration so massive in the 16-month-old mice that we were unable to detect any difference between mice of the two genotypes.

To describe further the effect of H ferritin haploid deficiency in the HFT+/- light-exposed mice, we concentrated our study on the 4-month-old HFT+/+ and HFT+/- controls 1 day after the same light treatment.

Effects of Light on the 4-Month-Old HFT+/+ and HFT+/- Mouse Retinas 1 Day after Illumination. Histologic Analysis. We tested the difference in photoreceptor loss 1 day after illumination compared with that after 12 days, by measuring the ONL thickness. The animals were subjected to the same exposure to light, but were allowed only 1 day of 12-hour/12-hour light–dark photoperiod recovery before analysis. After treatment, the photoreceptor nuclei layer was not regular, showing several folds. RPE monolayer was disorganized, with hypertrophic cells compared with that in the nonilluminated controls. The disturbances of the ONL and RPE were more pronounced in the HFT+/- than in the HFT+/+ mice (Figs. 4A, 4B). Mean ONL thickness in the exposed HFT+/- and HFT+/- mice was significantly lower (41.0%) than in the unexposed controls. However, there was no difference between the two genotypes (Fig. 4C). Thus, the H ferritin deficiency in the HFT+/- mice was not associated

![Figure 4.](https://example.com/four.png)
FIGURE 5. Apoptotic cells, membrane lipid peroxidation, and glial activation in the 4-month-old Hft<sup>+/+</sup> and Hft<sup>++/++</sup> mice 1 day after 24 hours of exposure to 13,000-lux light. (A-D) TUNEL staining of retinal sections from the Hft<sup>++/++</sup> (A, B) and the Hft<sup>++/++</sup> (C, D) mice not exposed (A, C) or exposed (B, D) for 24 hours and killed 1 day later. (E, H) 4-HNE immunostaining of retinal sections from the Hft<sup>++/++</sup> (E) and the Hft<sup>++/++</sup> (H) nonilluminated mice. (F, G, I, J) 4-HNE staining of retinal sections from the Hft<sup>++/++</sup> (F, G) and the Hft<sup>++/++</sup> (I, J) illuminated mice. (F, I) Enlargement of the inner part of the retina from the Hft<sup>++/++</sup> and the Hft<sup>++/++</sup> illuminated mice. (G, J) Enlargement of the outer part of the retina from Hft<sup>++/++</sup> and Hft<sup>++/++</sup> illuminated mice. 4-HNE immunostaining was very weak in the Hft<sup>++/++</sup> and Hft<sup>++/++</sup> nonexposed mice. In illuminated mice, the 4-HNE staining was stronger in IPL of Hft<sup>++/++</sup> than Hft<sup>++/++</sup> mice. (K, L) GFAP staining of the retinas of Hft<sup>++/++</sup> mice in control conditions (K) and exposed to light (L). (M, N) Iba1 staining of the retinas of the Hft<sup>++/++</sup> mice in control conditions (M) and exposed to light (N). Müller glial cell were activated (GFAP) and microglia cells (Iba1) migrated to the outer retina after exposure to light in the Hft<sup>++/++</sup> and Hft<sup>++/++</sup> two mice. Sections were counterstained with DAPI (blue). Scale bar, 50 μm.
with greater immediate susceptibility of photoreceptors during the initial light insult.

**Light-Induced Injury Marker Analysis.** Exposure to light induces oxidative stress. We examined several markers of processes associated with exposure to light: apoptosis, lipid peroxidation, glial activation, and microglia migration. We used TUNEL staining to study apoptosis 1 day after exposure to light. Most ONL nuclei in the retinal center were TUNEL positive in both the HFt+/+ and HFt+/- mice, in agreement with our findings for ONL thickness (Figs. 5B, 5D). 4-HNE is a product of lipid peroxidation, a specific marker of oxidative stress in the retina. We used an antibody against GFAP to investigate unexposed HFt+/- and HFt+/- immunostaining was observed throughout the retinal layers of the HFt+/+ and HFt+/- illuminated mice, but was clearly more intense in the ONL and IPL of the illuminated HFt+/+ than in the HFt+/-/+ mouse retinas (compare Figs. 5E, 5F to Figs. 5I, 5J). We used an antibody against GFAP to investigate glial activation in the retina of the HFt+/+ and HFt+/-/+ mice after illumination. Labeling after illumination was strong in Müller glial cells and astrocytes, inside the ganglion cell layer (GCL) throughout the retina (Figs. 5K, 5L). Since GFAP staining was identical for cells and astrocytes, inside the ganglion cell layer (GCL) through the ONL, IPL, and GCL to the ONL and RPE. Activation of microglial cells is characterized by a round morphology without extensions (Figs. 5M, 5N). Since no gross differences in microglial cell distribution or number were seen between the HFt+/+ and the HFt+/-/+ mice, only images of stained retinas of HFt+/+ mice are shown.

**H and L Ferritin Proteins and mRNAs.** We assessed the direct effects of exposure to light (24 hours) on ferritin expression 1 day after illumination (Figs. 6A-D). H ferritin mRNA was significantly more abundant in the exposed than in the unexposed HFt+/+ and HFt+/-/+ mice (Fig. 6A), but L ferritin mRNA was unchanged after illumination in both mouse retinas (Fig. 6B). Illumination had no effect on the amount of H ferritin protein, but L ferritin protein was significantly (twofold) more abundant after illumination than in the controls (Figs. 6C, 6D). Immunolocalization experiments indicated that the distributions of H and L ferritin proteins in the retinas of mice were unaffected by exposure to light (data not shown). Thus, illumination decreased the availability of H ferritin, which may be compensated by an increase in L ferritin protein content. This strongly suggests that the illumination protocol used in these experiments modified the dynamics of iron storage in ferritin protein. We therefore studied various iron-regulating protein gene expression.

**Profile of mRNAs for Other Iron-Regulating Proteins.** We studied mRNAs for several proteins (transferrin, transferrin receptor, ceruloplasmin, hephaestin, ferroportin, hepcidin, and iron regulatory proteins 1 and 2) involved in iron homeostasis 1 day after 24 hours illumination in the HFt+/+ and HFt+/-/+ mice. Transferrin mRNA was significantly more expressed in illuminated retinas than control (2.74- and 1.71-fold for HFt+/+ and HFt+/-, respectively; Fig. 7A). Light significantly decreased transferrin receptor mRNA in the HFt+/+ and HFt+/-/+ mice by 80% (Fig. 7B). The mRNAs for the two ferroxidases ceruloplasmin and hephaestin, which export iron from cells, were compared after exposure to light. The amount of mRNA for ceruloplasmin increased significantly, by nearly fourfold in the HFt+/+ and threefold in the HFt+/-/+ mice, whereas hephaestin mRNA significantly decreased by 29% in the HFt+/+ mice and by 27% in the HFt+/-/+ mice (Figs. 7C, 7D). Ferroportin mRNA was unaffected by light treatment in the HFt+/+ and HFt+/-/+ mice (Fig. 7E). Hepcidin mRNA was significantly decreased by exposure to light (by 4.0- and 3.6-fold) in the HFt+/+ and HFt+/-/+ mouse retinas, respectively (Fig. 7F). The mRNAs for iron regulatory proteins 1 and 2 were both 50% downregulated after illumination in the HFt+/+ and HFt+/-/+ mouse retinas (Figs. 7G, 7H). Thus, for all these iron-regulating proteins, the light-exposure protocol had substantial effects on the patterns of expression of their

![Figure 6](https://example.com/Figure6.png)
mRNAs, but the effects were independent of the H ferritin genotype. We tested different initial illumination periods to study the timing of these effects.

**Progressive Illumination Time Protocol: Effects of Light on the 4-Month-Old Mouse Retina Immediately after Various Times of Illumination**

Hft<sup>+/+</sup> and Hft<sup>−/−</sup> mice were exposed to 2, 12, and 24 hours of light, and the retinas were analyzed immediately thereafter. We assayed various mRNAs and proteins involved in iron metabolism: H and L ferritin, transferrin, transferrin receptor, ferroportin, ceruloplasmin, hephaestin, hepcidin, and iron-regulatory proteins 1 and 2.

**H and L Ferritin mRNA and Protein Profiles.** In the Hft<sup>+/+</sup> and Hft<sup>−/−</sup> mouse retinas, H ferritin mRNA abundance was not affected after 2 hours of illumination, but had significantly decreased (60%) after 12 hours and 24 hours. (Fig. 8A).

We assayed various mRNAs and proteins involved in iron metabolism: H and L ferritin, transferrin, transferrin receptor, ferroportin, ceruloplasmin, hephaestin, hepcidin, and iron-regulatory proteins 1 and 2.

**Figure 7.** mRNAs for iron-regulating proteins in 4-month-old Hft<sup>+/+</sup> and Hft<sup>−/−</sup> mouse retinas 1 day after exposure for 24 hours to 13,000-lux light. (A-H) Quantification by qRT-PCR of mRNAs for transferrin (A), transferrin receptor (B), ceruloplasmin (C), hephaestin (D), ferroportin (E), hepcidin (F), iron-regulatory protein 1 (G), and iron-regulatory protein 2 (H) in lysates of retinas from Hft<sup>+/+</sup> and Hft<sup>−/−</sup> mice killed 24 hours after 24 hours of light treatment and from the nonexposed controls. The light treatment increased the amounts of transferrin and ceruloplasmin mRNAs, whereas it decreased those of transferrin receptor, hephaestin, hepcidin, iron-regulatory protein 1, and iron-regulatory protein 2 in the 4-month-old Hft<sup>+/+</sup> and Hft<sup>−/−</sup> mouse retinas. mRNA for ferroportin was unaffected. (□) Hft<sup>+/+</sup> mice; (■) Hft<sup>−/−</sup> mice. Data are the mean ± SEM. *<i>P</i> < 0.05; **<i>P</i> < 0.01.
protein amounts in the HFt+/+ and HFt−/− mice (Fig. 8C). In this short period of illumination, L ferritin mRNA and protein levels were not modified in the two mouse strains (Figs. 8B, 8D).

Profiles of mRNAs for Other Iron-Regulating Proteins. The mRNAs for transferrin, transferrin receptor, hephaestin, and ferroportin were rapidly activated after 2 hours of illumination and then returned to their initial level or lower after 12 hours of exposure (Figs. 9A–D). Ceruloplasmin mRNA increased steadily by 12 hours and further by 24 hours of exposure (Fig. 9E). Hepcidin mRNA was less abundant at 12 hours after exposure in both the HFt+/+ and the HFt−/− mice (Fig. 9F). After 2 hours of illumination, there was no significant difference in iron regulatory protein 1 mRNA between exposed and unexposed mouse retinas. However, after longer periods of illumination (from 12 hours), iron regulatory protein 1 mRNA levels decreased in the HFt+/+ and HFt−/− mice (Fig. 9G). Iron regulatory protein 2 mRNA was significantly upregulated after 2 hours of illumination in the HFt+/+ and HFt−/− mice, but in both type of mouse after 12 hours of illumination, iron regulatory protein 2 mRNA was less abundant than in the control condition and after 2 hours of illumination (P ≤ 0.05; Fig. 9H).

The mRNA for the antioxidative stress protein heme-oxygenase 1 started to increase at 12 hours, and it remained high thereafter; the mRNA for ceruloplasmin showed a similar pattern (Fig. 9I). Finally, there was no significant difference between the HFt+/+ and HFt−/− mice in levels of all the iron-regulating proteins mRNAs.

The effect of light on the expression of iron-regulating proteins is summarized in Supplementary Figure S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5705/-/DCSupplemental. The hypotheses that iron increases after light-induced stress is based on publications that describe iron accumulation during retinal degeneration. The iron homeostasis modifications, even after the end of exposure to light, caused us to suppose that the iron level was increased. However, it is difficult at this time to determine in what tissue or cell compartment this increase took place.

Several iron-regulating protein mRNAs were increased rapidly after the beginning of the exposure. An extensive light-exposure period correlated with the increase of reactive oxygen species formation and modified expression of other iron-regulating protein mRNAs. At the end of illumination, the iron level should continue to increase due to cell death. Thus, the iron homeostasis cycle was mobilized at each step of oxidative stress, which was enhanced by illumination.

**DISCUSSION**

Iron accumulates in the outer retina during normal aging, and in some retinal degenerative animal models. Recently, age-dependent and gender-specific changes in mouse retina and RPE/choroid iron levels by strain have been reported. A proton-induced x-ray emission study showed accumulation of iron in the RPE and photoreceptors during aging in C57Bl/6J mice. To investigate the role of ferritin, the main iron-regulating protein, we used a mouse strain with a haploid deletion of the ferritin gene (HFt−/−). These HFt−/− mice had 63% less H ferritin than did the WT control mice. This deficit was unaffected by the duration of exposure. The amounts of L ferritin mRNAs and proteins in two mice were unaffected during light treatment. The amounts of H ferritin mRNAs in the HFt+/+ and HFt−/− mice decreased after 12 hours of light treatment, whereas H ferritin protein was unaffected by the duration of exposure. The amounts of H ferritin mRNAs and proteins in two mice were unaffected during light treatment. The amounts of H ferritin mRNAs in the HFt+/+ and HFt−/− mice decreased after 12 hours of light treatment, whereas H ferritin protein was unaffected by the duration of exposure. The amounts of H ferritin mRNAs and proteins in two mice were unaffected during light treatment.
after illumination, the ONL was thicker in the 4-month-old HFt^{+/+} and HFt^{−/−} mice than in the HFt^{+/+} mice. However, the massive retinal degeneration in the central part of the retina in the 16-month-old mice (which have normally lost 20% of the ONL thickness by this age) after treatment with light was similar in the HFt^{+/+} and HFt^{−/−} mice.

FIGURE 9. mRNAs for iron-regulating proteins in retinas from 4-month-old HFt^{+/+} and HFt^{−/−} mice after 2, 12, and 24 hours of light treatment. (A–I) Quantification by qRT-PCR of mRNAs for transferrin (A), transferrin receptor (B), hephaestin (C), ferroportin (D), ceruloplasmin (E), hepcidin (F), iron-regulatory protein 1 (G), iron-regulatory protein 2 (H), and heme-oxygenase 1 (I) in lysates of retina from the 4-month-old HFt^{+/+} and HFt^{−/−} mice, immediately after 2, 12, and 24 hours of light treatment and from the nonexposed controls. The amounts of mRNAs for transferrin, transferrin receptor, hephaestin, ferroportin, and iron-regulatory proteins 1 and 2 increased after 2 hours of exposure to light and decreased to the initial level after 12 and 24 hours. mRNA for ceruloplasmin increased, whereas that of hepcidin decreased with duration of exposure. The amount of heme-oxygenase 1 mRNA increased after 12 hours of illumination. No difference was detected between the mRNA levels in the HFt^{+/+} and HFt^{−/−} mice. (□) HFt^{+/+} mice; (■) HFt^{−/−} mice. Data are the mean ± SEM. *P < 0.05; **P < 0.01; ns: not significant.
In normal retinal physiological conditions or under the moderate stress associated with normal retinal aging, the iron-sequestering capacity of the half-normal amounts of H ferritin is sufficient. Indeed, a minimum of one or two H subunits in ferritin polymers is thought to be sufficient to allow formation of ferritin molecules, which have the capacity to sequester iron.40 Overexpression of L ferritin can compensate for the deficiency of H ferritin. L ferritin contributes to the nucleation of iron cores in the ferritin molecule.40 Recently, it has been shown that the conditional deletion of H ferritin in mouse liver had no immediate or long-term effect unless the animals were previously overloaded with iron. Thus, it is the combined effect of iron excess and aging that is toxic in these animals with totally inactivated H ferritin expression.40 This effect is in accordance with our results on the combined role of aging and iron metabolism with exposure to light in the retina. The capacity of the aged retina to respond to additional stress may depend on the cells' ability to accumulate more L ferritin, a property that is higher in young than in old retinas (Figs. 2B, 2D).

The retinal degeneration in the 16-month-old HFt+/− and HFt−/− mice 12 days after illumination was too great for them to be useful in these studies, and so we used only 4-month-old mice for subsequent analyses. We studied management of iron by iron-regulating protein during exposure to light (2, 12, or 24 hours) and 1 day after the end of 24 hours of exposure. The illumination protocol that we used revealed different time courses of modifications of the expression of mRNA for the various iron-regulating proteins (see Supplementary Fig. S1, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.10-5705/DCSupplemental).

At the beginning of illumination (2 hours), the mRNAs for transferrin mainly, but also for transferrin receptor, hephaestin, ferroportin, and iron regulatory proteins 1 and 2 were increased. Thus, the retinas reacted quickly to oxidative stress-caused, light-induced injury by regulating the mRNA level of these iron metabolism proteins. Moreover, photoreceptor phagocytosis is under the control of the circadian rhythm; such disturbance by aberrant illumination triggers modification of OS phagocytosis by the RPE,49,50 decreasing iron recycling throughout. Transferrin, synthesized by RPE and Müller glial cells,7,51 and its receptor must be increased for the egress of free iron to occur across the retina from the photoreceptor layers into the circulation.52 Ferroportin is a protein that exports iron from cells and is coupled to a ferrooxidase hephaestin. Hephahesin converts iron in a form available for transferrin binding.

Effects on ceruloplasmin and hepcidin were dependent on the duration of illumination. As the duration of exposure to light increases, oxidative stress and therefore free reactive iron increase, and the need for ceruloplasmin to oxidize the iron increases. Reduced hepcidin expression should promote iron export by increasing ferroportin on the cell surface.53 The modification of ceruloplasmin and hepcidin gene expression over the same period as heme-oxygenase 1 suggests that there may be a later stress response of these proteins to light.26,54

After exposure to light, the central part of HFt+/− retinas was more susceptible than that of the HFt−/− retinas to oxidative stress, as revealed by the presence of 4-HNE and TUNEL. After light treatment, iron flux in the retina was modified as early as 1 day after 24 hours of illumination. Iron was transformed into a bioavailable form by ceruloplasmin, which was increased and was chelated by transferrin, which also increased. The decrease in hephaestin mRNA and the absence of modification of ferroportin mRNA showed a reduction in iron exportation. However, iron export was always essential, since mRNA of hepcidin was decreased. These observations thus confirm a drastic change in iron metabolism, as a response of retinal cells against damage from light-induced stress.

The excess iron flux in the retina after exposure to excessive light mobilizes retinal cells, including microglia and Müller glial cells. Indeed, Müller glial cells are activated after light-induced degeneration,55 and microglia are recruited from the inner retina55 through the release of chemokines produced by photoreceptors and Müller glial cells.55,56 Activation of Müller glial cells may be part of the early response to light-induced injury. Indeed, they produce and secrete transferrin51,57 and ceruloplasmin,51,58 which are increased after exposure.

This study demonstrated the difference in transcription and posttranscription regulation of H and L ferritin. During and even after exposure, H ferritin mRNA was decreased, whereas its protein level was unmodified. The decrease started at 12 hours of illumination, at the same time that mRNA of heme-oxygenase 1 increased. H ferritin, like heme-oxygenase 1, has antioxidant-responsive element (ARE) sequences in its gene, sensitive to oxygen species, which represses H ferritin and enhances heme-oxygenase 1 transcription.59 L ferritin mRNA was unmodified during and after exposure, but its protein was significantly elevated after exposure to light. The iron regulatory proteins are sensitive to high iron levels, and binding iron regulatory element (IRE) sequences on the mRNA of L ferritin enhances its transcription. We noted that iron regulatory protein 2 mRNA increased after 2 hours of illumination.

We can suppose that the increase in iron reacts with iron regulatory protein and enhances L ferritin protein for rapid nucleation of iron in the ferritin complex. Thereafter, high oxidative stress, starting at 12 hours of illumination, decreases mRNA of H ferritin to avoid overexpression (by iron regulatory protein) and to maintain H ferritin level at a steady threshold. Indeed, H ferritin overexpression is known to result in reduced cell growth and iron deficiency.59,60 This may also explain why the H ferritin level does not change dramatically in brain samples from iron-fed mice, despite significant changes in the H/L ratio.61

In conclusion, we demonstrated that iron-regulating proteins were able to manage photo-oxidation resulting from light-induced injury as early as the beginning of illumination. The response is regulated after exposure to light, so as to allow retinal remodeling and management of excess iron.

The early oxidative stress (heme-oxygenase 1 expression) during exposure was similar in both mice. Nevertheless, 24 hours after illumination, the oxidative stress (4-HNE staining) was increased in the HFt−/− mice, resulting in more photoreceptor cell death (decrease in photoreceptor nuclear layer thickness) in this mouse 12 days after illumination. The sensitivity of the HFt+/− mouse retina to excessive light seems to be due to a decreased capacity to manage iron, rather than to more oxidative stress. Even the modification of gene expression of iron-regulating proteins (which are similar in both mouse groups) did not induce redistribution of iron across the retina cells.

Therefore, therapeutic strategies involving the reduction of photo-oxidative stress and thus iron toxicity in various retinal diseases including AMD may be beneficial to retinal health. This outcome may be obtained by the use of iron chelators62 or by modulating iron homeostasis mechanisms.

References

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