Antioxidant or neurotrophic factor treatment preserves function in a mouse model of neovascularization-associated oxidative stress

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In several disease states, abnormal growth of blood vessels is associated with local neuronal degeneration. This is particularly true in ocular diseases such as retinal angiomatous proliferation (RAP) and macular telangiectasia (MacTel), in which, despite the absence of large-scale leakage or hemorrhage, abnormal neovascularization (NV) is associated with local neuronal dysfunction. We describe here a retinal phenotype in mice with dysfunctional receptors for VLDL (Vldlr−/− mice) that closely resembles human retinal diseases in which abnormal intra- and subretinal NV is associated with photoreceptor cell death. Such cell death was evidenced by decreased cone and, to a lesser extent, rod opsin expression and abnormal electroretinograms. Cell death in the region of intraretinal vascular abnormalities was associated with an increased presence of markers associated with oxidative stress. Oral antioxidant supplementation protected against photoreceptor degeneration and preserved retinal function, despite the continued presence of abnormal intra- and subretinal vessels. What we believe to be novel, Müller cell–based, virally mediated delivery of neurotrophic compounds specifically to sites of NV was also neuroprotective. These observations demonstrate that neuronal loss secondary to NV can be prevented by the use of simple antioxidant dietary measures or cell-based delivery of neurotrophic factors, even when the underlying vascular phenotype is not altered.

Introduction

Oxidative damage is associated with the pathogenesis of multiple degenerative disorders of the CNS, including Parkinson disease (1), Huntington disease (2), and Alzheimer disease (3, 4). The pathogenesis of these and other degenerative diseases is associated with oxidative stress due to multiple factors, including excessive neovascularization (NV) (5). Although the direct relationship between NV and neurodegeneration remains unclear, the presence of excess vessels may exacerbate the underlying neurodegeneration and contribute to progression of the disease (1). A growing body of evidence suggests that abnormalities in small blood vessels can have a deleterious effect on neighboring neurons. This effect may be the result of disruption of vascular/parenchymal trophic interactions (6), deleterious activity of inflammatory cells associated with NV (7), or accumulation of reactive oxygen species. Cone photoreceptors can be especially sensitive to oxidative damage, at least in part because of the stressful and naturally oxidative environment of the retina (8–10). In the presence of abnormal NV, normal oxidative stress defense mechanisms may no longer be adequate, leading to cell death.

The growth of blood vessels in the mammalian eye is highly regulated, but abnormal retinal angiogenesis is frequently associated with the most common causes of vision loss in industrialized nations (11). In a relatively uncommon, but probably underdiagnosed, form of retinal NV called macular telangiectasia (MacTel; also referred to as idiopathic parafoveal telangiectasia), intraretinal telangiectatic vessels proliferate in the central portion of the inner retina and grow into the normally avascular outer retina (12–14). In the NV form of age-related macular degeneration (AMD), abnormal vessels typically arise from the choroid and invade the subretinal space (15). However, in a subset of patients with AMD, intraretinal and subretinal NV arises from the inner retinal vessels, a condition known as retinal angiomatous proliferation (RAP) (16). Both MacTel and RAP exhibit glial cell abnormalities and varying degrees of photoreceptor degeneration associated with abnormal intra- and subretinal NV (12, 14).

Subretinal NV originating from the intraretinal vessels can be generated by blocking R-cadherin–mediated endothelial cell migratory cues (17, 18) or by overexpressing VEGF in photoreceptors (19). Similarly misdirected vascular growth occurs in the retinas of Vldlr−/− mice, which have a defective gene for the VLDL receptor (VLDLR; refs. 20–22). Aberrant retinal vessels in these mice extend from the inner retina through the photoreceptors to the subretinal space. Here we describe the retinal phenotype in eyes...
from Vldlr−/− mice and its striking similarity to that observed in human patients with RAP or MacTel, including focal disruption of photoreceptors coincident with the abnormal penetrating vessels. Using the Vldlr−/− mouse model, we also demonstrated a correlation between intraretinal vascular abnormalities, oxidative stress, and cone-dominated neuronal degeneration, an association that may also be observed in other neuronal degenerative disorders. Targeted delivery of neurotrophic factors to sites of abnormal NV or oral antioxidant supplementation significantly reduced photoreceptor degeneration and protected against visual dysfunction in Vldlr−/− mice. We hypothesize that diet-responsive, NV-induced oxidative stress may similarly link retinal angiogenesis and neuronal loss in human diseases. In addition, we demonstrated that intravitreally injected glial fibrillary acidic protein (GFAP) promoter–driven serotype 2 adeno-associated viruses (AAV-2s) targeted to activated Müller glia delivered neurotrophic factors to photoreceptors located in the outer retina via the retinal spanning Müller cell processes. Our findings reveal what we believe to be a novel treatment modality by which ocular gene therapy may be used to treat similar ocular vascular diseases with associated neuronal degeneration.

**Results**

*Characterization of abnormal retinal NV in Vldlr−/− mice.* Vldlr−/− mice appear outwardly normal, and are viable and fertile (23), but exhibit abnormalities in the retinal vasculature (20, 21). Systematic evaluation of neonatal Vldlr−/− mice revealed normal development of retinal vessels through the first postnatal week. During the second postnatal week, Vldlr−/− retinas exhibited transient hyperproliferation of the inner vascular plexus and associated astrocytes, particularly in newly vascularized regions near the retinal periphery (Figure 1, A–D). This associated astrocytosis is consistent with previous studies demonstrating a direct correlation between vessels and astrocytes in the inner retina (17, 24, 25). By the third postnatal week, vessel and astrocyte density in the superficial plexus receded to normal levels. Dramatic abnormalities were observed during formation of the deep vascular plexuses in Vldlr−/− mice. During the second postnatal week, abnormal intraretinal vessels sprouted from the deep and intermediate plexuses and penetrated through the outer nuclear layer (ONL) to the subretinal space (Figure 1, E–J). By 4 weeks of age, numerous intraretinal vessels extended to form a subretinal vascular network (Figure 1F). Choroidal vessels were normal at this time, and breaks in Bruch’s membrane and anastomoses of the intraretinal vessels with the choriocapillaris were not observed in mice less than 6 months of age, confirming the intraretinal origin of subretinal NV (Figure 1, I and J). Although the numbers of subretinal sprouts were reduced in older mice, most intra- and subretinal vessels persisted throughout the life of the Vldlr−/− mice.

The subretinal vessels were patent and perfused (Figure 2, A and B). Modest extravasation of 43-kDa fluorescein dextran was observed from a subset of subretinal vascular sprouts (Figure 2, A–C), indicating a mild dysfunction of the blood-retinal barrier normally formed by tight junctions between nonfenestrated vascular endothelial cells and close association with perivascular glia (26). Electron microscopic analysis of intra- and subretinal NV

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**Figure 1**

Dramatic abnormalities were observed during formation of the deep vascular plexuses in Vldlr−/− mice. (A and B) The developing superficial vascular plexus of Vldlr−/− retinas was hyperdense, particularly in the periphery, at P8. (C and D) Peripheral hypervascularity (isolectin GS; red) was associated with increased density of astrocytes (GFAP; green). (E) H&E-stained retinal sections from a P40 Vldlr−/− mouse showed intraretinal vessels (arrows) that originated from the inner retinal vascular plexuses and migrated through the photoreceptors to the subretinal space. GCL, ganglion cell layer; Ch, choroid. (F) These intraretinal vessels formed retinal-retinal anastomoses throughout the central two-thirds of the retina. (G) Intraretinal vessels originated from multiple sprouts off the deep (arrowheads) and intermediate vascular plexuses (arrow). (H) and (I) Fluorescein angiography (F) or isolectin GS staining (I) demonstrated the retinal origin of the intraretinal and subretinal NV. IPL, inner plexiform layer. Scale bars: 100 μm.
in Vldlr<sup>−/−</sup> mouse retinas revealed fenestrated endothelia (Figure 2D), providing an anatomic basis for the abnormal permeability observed in these vessels. Müller glial cell activation, as demonstrated by GFAP expression, was selectively associated with, but secondary to, the formation of intraretinal NV (Figure 2, E and F).

By the fifth postnatal week, the normal retinal pigment epithelium (RPE) monolayer was disrupted in areas surrounding subretinal vascular lesions, and multiple layers of RPE cells enveloped the neovascular complexes in Vldlr<sup>−/−</sup> retinas (Figure 2, G and H). These abnormalities lead to focal distortion and scarring of the retina, and were found to be associated with focal loss of photoreceptor inner and outer segments, as demonstrated by abnormal retinal morphology and an absence of red/green opsin staining in regions with subretinal NV (Figure 2, A and J). Neuronal abnormalities never preceded the appearance of intra- and subretinal NV. At P12, just prior to the onset of abnormal NV, all retinal layers were intact and identical to those of age-matched WT C57BL/6J controls when evaluated by electron and confocal microscopy (Figure 2I). Thus, neuronal abnormalities in Vldlr<sup>−/−</sup> mouse retinas appear to be the result, rather than a cause, of NV.

**Subretinal NV in Vldlr<sup>−/−</sup> retinas results from increased VEGF.** In order to gain insight into a possible relationship between VLDLR deficiency and the development of abnormal intraretinal vessels, we analyzed VLDLR expression in WT mouse retinas. The ganglion cell layer, retinal vessels, inner nuclear layer (INL), outer nuclear layer (ONL), photoreceptor inner and outer segments, and RPE were isolated using laser capture microdissection techniques. Each of these fractions is highly enriched for the indicated retinal layer, although on occasion, neighboring cells may have been inadvertently captured as a result of technical limitations with the technique. RNA was purified and analyzed by quantitative RT-PCR to assess the relative levels of VLDLR mRNA. Expression in other retinal tissues, including retinal vessels, was observed at relatively low levels (Figure 3A).
Recently it has been reported that VEGF expression is increased in Vldlr<sup>−/−</sup> mouse retinas (27). We confirmed this finding and tested for a direct correlation between VLDLR and VEGF expression in individual retinal layers at P14, when subretinal NV begins in Vldlr<sup>−/−</sup> mice. In WT mice, VEGF expression was highest in the ONL, and smaller amounts of VEGF mRNA were found in the RPE and INL (Figure 3B). In Vldlr<sup>−/−</sup> retinas, VEGF expression was upregulated compared with WT retinas more than 5-fold in the RPE and at least 2-fold in the ONL. No difference in expression levels was found in other retinal layers (Figure 3B).

It is likely that increased VEGF expression is responsible for new vessel growth. We tested this by using anti-VEGF agents to suppress subretinal NV in Vldlr<sup>−/−</sup> mice. Intravitreal injection of Macugen, an RNA aptamer that blocks VEGF<sub>165</sub>, reduced intra- and subretinal NV sprouts and the area of subretinal NV at P20 in Vldlr<sup>−/−</sup> retinas. n = 10–12 retinas per group. Veh, vehicle. (D) Combination angiostatic therapy reduced abnormal retinal NV to an even greater extent, n = 10–12 retinas per group. (E) Whole retina images of vessels, focused within the subretinal space, showed a sharp reduction in intraretinal NV after treatment with Macugen and combination angiostatics. Scale bars: 250 μm. Error bars denote SEM.

**Figure 3**
VEGF upregulation is associated with intraretinal NV in Vldlr<sup>−/−</sup> mice. (A) Most VLDLR expression in laser-captured retinal tissue from P14 C57BL6/J mice occurred in the ONL and photoreceptor segments (POS), with lower expression in the RPE and other retinal tissues (triplicate data). (B) In Vldlr<sup>−/−</sup> retinas, significant upregulation of VEGF occurred within the RPE (P < 0.0001) and photoreceptors (P < 0.05) at P14 (triplicate data). (C) Blocking VEGF<sub>165</sub> activity with intravitreal Macugen injection at P12 significantly reduced the number of intraretinal NV sprouts and the area of subretinal NV at P20 in Vldlr<sup>−/−</sup> retinas. n = 10–12 retinas per group. Veh, vehicle. (D) Combination angiostatic therapy reduced abnormal retinal NV to an even greater extent, n = 10–12 retinas per group. (E) Whole retina images of vessels, focused within the subretinal space, showed a sharp reduction in intraretinal NV after treatment with Macugen and combination angiostatics. Scale bars: 250 μm. Error bars denote SEM.
in opsin-1 expression was observed in the Vldlr−/− retinas compared with retinas of age-matched WT controls (Figure 4B). Rhodopsin expression was decreased in Vldlr−/− mouse retinas by only 20%. In 7-month-old mice, cone opsin-1 expression decreased approximately 70% in Vldlr−/− retinas compared with those of age-matched WT controls, while rhodopsin expression was reduced by 30% (Figure 4B). Thus, both rod and cone photoreceptor abnormalities were associated with the vascular phenotype in Vldlr−/− mice, with cones affected earlier and to a greater extent than rods.

Electroretinography (ERG) was performed to compare visual function in Vldlr−/− mice and age-matched WT controls. The ERGs of 6-month-old Vldlr−/− mice exhibited delayed signaling, reduced oscillatory potentials (OPs), and reduced and delayed cone responses. Amplitudes of scotopic rod-dominated responses were largely preserved. However, maximal dark-adapted B-wave implicit times in Vldlr−/− mice were consistently prolonged (Figure 4C and Table 1), implying generalized rod dysfunction. Photopic cone responses were also delayed in Vldlr−/− mice, but unlike the scotopic signals, amplitudes were significantly reduced as well (Figure 4D and Table 1). Abnormalities in the cone-specific responses to light flicker at 16 Hz were even more pronounced in the Vldlr−/− mice, with delayed implicit times as well as reduced signal amplitudes (Figure 4D, insets, and Table 1). OPs were also significantly reduced in all 6-month-old Vldlr−/− ERGs (Figure 4C, insets, and Table 1), indicating broad retinal signaling defects and suggestive of specific defects in the INL neurons.

Targeted delivery of neurotrophin-4 protects retinas from neuronal degeneration. Although we observed a highly significant reduction in the
formation of subretinal NV using combination angiostatic therapy, this effect was only transient, and subretinal NV returned to normal Vldlr–/– levels within 2–3 weeks of treatment. This is similar to clinical observations that report temporary beneficial effects of angiostatic therapy. Thus, additional strategies for protecting the retinal neurons in ocular vascular diseases are still required (32). We next evaluated the potential effects of neurotrophic factors specifically delivered to sites of retinal NV and associated retinal degeneration. We hypothesized that photoreceptor degeneration might be caused by oxidative stress generated by the presence of abnormal intra- and subretinal vessels. Metabolomic analysis comparing retinas from 4-month-old Vldlr–/– and WT mice demonstrated a significant increase in metabolites commonly associated with oxidative stress (Table 3 and Supplemental Figure 1). L-Carnitine, L-acycetlcarnitine, and their derivatives are naturally occurring antioxidants (35, 36) that are upregulated in response to increased oxidative stress (35, 37, 38). The observed increase in these metabolites in the Vldlr–/– retinas indicates an endogenous cellular response to oxidative stress. Acrolein is a commonly used marker of lipid peroxidation resulting from oxidative stress (9, 10, 39, 40). Acrolein staining was clearly greater in Vldlr–/– retinas compared with age-matched WT controls (Figure 6, A and B). In the peripheral retina, acrolein levels were similar between Vldlr–/– and WT retinas, while acrolein levels were greatly increased in the central two-thirds of Vldlr–/– retinas compared with age-matched WT controls (Figure 6C). In Vldlr–/– eyes, the vast majority of intraretinal NV occurs in the central two-thirds of the retina, whereas the peripheral retinal vasculature remains normal. Thus, increased oxidative stress measured by acrolein staining in Vldlr–/– retinas appears to be limited to regions with subretinal NV, supporting a direct relationship between subretinal NV and oxidative stress.

The transient effect of antiangiogenic treatments in the Vldlr–/– retinas rendered us unable to prevent recurrence of subretinal NV using a single angiostatic treatment, and multiple, weekly injections led to phthisis. Thus, it is not possible with this experimental model to determine whether subretinal NV directly causes the observed oxidative stress and associated neuronal degeneration. An alternative hypothesis that might explain the loss of photoreceptors in the presence of increased markers of oxidative stress would be a defect in natural oxidative stress defense mechanisms associated with VLDLR deficiency. To test this hypothesis, we used large-scale genomic analysis to examine the expression of genes encoding factors involved in protection against oxidative stress. Of 210 gene products examined, 109 were found to be expressed in the retina, and 108 of these were equally expressed in 3-week-old Vldlr–/– and WT retinas within

Table 1  
Responses to light stimuli in 6-month-old WT and Vldlr–/– mice

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Measurement</th>
<th>WT (n = 10)</th>
<th>Vldlr–/– (n = 12)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>ERG scotopic measurements</td>
<td>B-wave amplitude (µV)</td>
<td>218 ± 36</td>
<td>252 ± 28</td>
<td>0.228</td>
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<td>Low intensity</td>
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<td>128 ± 9</td>
<td>152 ± 4</td>
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<td>High intensity</td>
<td>A-wave amplitude (µV)</td>
<td>133 ± 20</td>
<td>78 ± 8</td>
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<td>B-wave implicit time (ms)</td>
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<td>B-wave implicit time (ms)</td>
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<td>110 ± 4</td>
<td>0.0008h</td>
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<td>Maximum OP</td>
<td>Amplitude (µV)</td>
<td>65 ± 18</td>
<td>21 ± 3</td>
<td>0.008h</td>
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</tbody>
</table>

Photic measurements

| 1 Hz | A-wave amplitude (µV) | 7.6 ± 0.8 | 9.5 ± 1.2 | 0.011h |
| 1 Hz | A-wave implicit time (ms) | 24 ± 2 | 32 ± 2 | 0.008h |
| 1 Hz | B-wave amplitude (µV) | 109 ± 12 | 87 ± 6 | 0.046h |
| 1 Hz | B-wave implicit time (ms) | 68 ± 7 | 89 ± 4 | 0.015h |
| 16 Hz flicker | Amplitude (µV) | 15.3 ± 1.6 | 10.4 ± 0.8 | 0.003h |
| 16 Hz flicker | Implicit time (ms) | 64 ± 4 | 79 ± 2 | 0.004h |

Values are mean ± SEM. aDark-adapted measurements performed to determine rod and mixed cone-rod function. b0.005 cd·s/m². c2 cd·s/m². d510 cd·s/m². eLight-adapted measurements performed to determine cone function. f20 cd·s/m². g10 cd·s/m². hStatistically significant difference between WT and Vldlr–/– mouse retinas.
95% confidence intervals, the single exception being 9-cis-retinol dehydrogenase (Figure 6D and Supplemental Table 1). While not absolute, these data suggest that neuronal defects in Vldlr−/− mice are not caused by a generalized failure of oxidative stress defense and support the concept of a direct relationship between excessive NV, oxidative stress, and neuronal degeneration.

Figure 5
Targeted delivery of NT4 to sites of subretinal NV protects Vldlr−/− retinas from neuronal degeneration. (A) AAV-GFAP-GFP–treated WT retinas exhibited minimal GFP expression in the inner retina and no expression in the outer retina. (B) AAV-CAG-GFP caused nonspecific GFP expression, mainly limited to the inner retina. (C and D) At 2 wk after intravitreal AAV-GFAP-GFP injection, GFP was observed in Müller glia specifically surrounding subretinal NV in P28 Vldlr−/− mouse retinas (C), which was maintained at P45, 1 mo after injection (D). (E–G) NT4 gene product was produced in Müller cells adjacent to subretinal NV in AAV-GFAP-NT4–treated Vldlr−/− retinas (E and F), resulting in accumulation of NT4 at photoreceptor segments (G). Line in G separates control, pre–immune IgG–stained retina (top) from retina stained with anti-NT4 antibodies (bottom). (H) Quantitative RT-PCR analysis of opsin-1 and rhodopsin mRNA expression, normalized to age-matched WT controls, demonstrated protective effects of AAV-GFAP-NT4 treatment. Error bars denote SEM. (I and J) ERG analysis demonstrated that AAV-GFAP-NT4 treatment attenuated loss of retinal function. Scale bars: 100 μm (A–E and G); 50 μm (F).

Antioxidant treatment protects against retinal damage in Vldlr−/− mice. If oxidative stress is associated with neuronal damage in the Vldlr−/− retina, we reasoned that treatment with commonly used antioxidants may attenuate this degeneration. Daily oral administration of an antioxidant cocktail consisting of ascorbic acid, α-lipoic acid, and reduced l-glutathione for 6 weeks dramatically reduced the lev-
els of oxidative stress in Vldlr−/− mouse retinas (Figure 7, A and B). This correlated with protection of the photoreceptors from degeneration. Furthermore, 6 weeks of antioxidant treatment initiated at 2 months of age, well after abnormal subretinal NV is established, significantly improved the expression levels of opsins and rhodopsin in the retinas of 3.5-month-old Vldlr−/− mice (Figure 7C). Opsin and rhodopsin expression levels remained similar to those of untreated 2-month-old Vldlr−/− mice, which suggests that antioxidant treatment prevented further loss of cones and rods from the time treatment was initiated. Antioxidant treatment did not have any effect on the formation and persistence of subretinal NV (Figure 7, D and E). Thus, the observed activity was not caused by prevention or elimination of NV, but rather by protection of the neurons from secondary neurodegeneration associated with NV.

Normalized ERGs were also observed in Vldlr−/− mice on an antioxidant diet compared with vehicle-treated animals. Importantly, significant preservation of retinal function was observed even when treatments were initiated at 2 months of age, an age at which intra- and subretinal NV was well established and oxidative stress was already apparent (Figure 7, F and G, and Table 4), consistent with the results obtained from the RT-PCR experiments. ERGs of animals in which treatment was initiated at an earlier age were similar to those observed in WT animals (data not shown), which indicates that early dietary measures may provide nearly full protection from neuronal degeneration in the retina. The profound protective effect of antioxidant dietary supplementation on retinal degeneration further supports a direct link between oxidative stress and neuronal degeneration in the Vldlr−/− mouse retina.

Discussion

Paucity and dysfunction of blood vessels in tissues leading to ischemia is a well-recognized pathological process that often leads to irreversible injury, particularly in neuronal tissue. At the other extreme, excessive vessels (NV) can also compromise tissue function, usually as a result of leakage, hemorrhage, or scarring caused by the abnormal new vessels. In this study, we provide evidence for yet another mechanism of injury associated with NV, even in the absence of clinically significant leakage or hemorrhage: neuronal cell death as a result of increased oxidative stress caused by proximity to the abnormal vessels.

In several diseases that can cause irreversible vision loss, such as RAP (16) and MacTel (14, 41), abnormal vessels extend from the highly vascularized inner retina into the normally avascular outer retina. The retina of the Vldlr−/− mouse exhibits vascular changes very similar to those observed in patients with MacTel and RAP and thus is a good model for studying the general relationship between NV and neurodegeneration. The new vessels observed in the human disease and the Vldlr−/− mouse exhibit relatively mild permeability defects and are accompanied by glial activation and disruption of the RPE. Another feature common to RAP, MacTel, and the Vldlr−/− retina is that the nonuniformly distributed focal vascular lesions are directly associated with neuronal degeneration separated by relatively normal regions. While other retinal degenerative models, such as rd1 or rd/s mice, have associated vascular abnormalities and neuronal loss, many of these result in uniform degeneration and relate more closely to genetic diseases, such as retinitis pigmentosa, rather than to ocular vascular disease. Taken together, these similarities support the use of the Vldlr−/− mouse as a compelling model to investigate selected human retinal diseases (e.g., MacTel) as well as other, nonocular neurodegenerative disorders with associated NV.

VEGF plays a critical role in normal retinal vascularization and retinal angiogenic diseases (42–44). In the Vldlr−/− retina, VEGF upregulation is observed in photoreceptors and RPE and partici-

### Table 2

**ERG measurements in 4-month-old WT and untreated, AAV-GFAP-GFP control–treated, and AAV-GFAP-NT4–treated Vldlr−/− mice 3.5 months after injection**

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<th>Stimulus</th>
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<th>NT4</th>
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Values are mean ± SEM. aDark-adapted measurements performed to determine rod and mixed cone-rod function. /0.005 cd/s/m² /2 cd/s/m² /10 cd/s/m² /2 Light-adapted measurements performed to determine cone function. /20 cd/s/m² /30 cd/s/m² /4Statistically significant difference between contralaterally AAV-GFAP-NT4– and AAV-GFAP-GFP–treated eyes.
pates in the stimulation of subretinal NV. Normal VLDLR expression within the avascular outer retina, and upregulation of VEGF coincident with formation of abnormal subretinal vessels in these areas of VLDLR-deficient mice, suggests a mechanism linking VLDLR with proangiogenic factors. No obvious abnormalities were observed in the choroid, suggesting potential RPE polarity of the VEGF overexpression toward the inner retina. VLDLR is a negative regulator of wnt signaling during retina development; upregulation of the wnt coreceptor LRP5/6 and increased downstream wnt signaling occurs in Vldlr−/− mouse retinas (27). VEGF is a well-known product of wnt signaling, and VLDLR may normally participate in turning off wnt signaling once retinal vascular development is complete. In the absence of VLDLR, wnt signaling persists, leading to increased levels of VEGF expression in photoreceptor and RPE layers and the angiogenic extension of abnormal intra- and subretinal vessels. Whether such a mechanism operates in diseases such as MacTel and RAP is uncertain, although anti-VEGF therapy has previously been tested in both conditions (45, 46). Initial efforts to treat RAP lesions using combination angiotostatics are also promising (47). However, in our experience with the Vldlr−/− mouse, antiangiogenic therapy is only transiently effective, subretinal NV returns within 2 weeks, and therapy must be initiated prior to NV formation. Thus, the usefulness of antiangiogenic therapy in human ocular diseases with attributes similar to those of Vldlr−/− mice may be limited.

A major finding in this study is that neuronal abnormalities and impaired retinal function developed in association with NV. We were able to prevent focal degeneration of photoreceptors in the regions of vascular abnormalities through the selective delivery of neurotrophic molecules by taking advantage of cellular changes observed in this condition. Müller glia, with cytoplasmic processes spanning the inner and outer retina, were activated in response to vascular changes such as those observed in the Vldlr−/− retina. Upon activation, these cells upregulate GFAP, and their appearance and location precisely correlate, both temporally and spatially, with subretinal NV and associated neuronal degeneration in the outer retina. By targeting the activated Müller cells and taking advantage of their retina-spanning cytoplasmic processes, NT4 was specifically delivered to sites of vascular abnormalities through the selective delivery of neurotrophic molecules by taking advantage of cellular changes observed in this condition. Müller glia, with cytoplasmic processes spanning the inner and outer retina, were activated in response to vascular changes such as those observed in the Vldlr−/− retina. Upon activation, these cells upregulate GFAP, and their appearance and location precisely correlate,
In addition, we were able to dramatically attenuate retinal degeneration in the Vldlr–/– mouse retina, supporting a link between the presence of subretinal vessels and neuronal degeneration. Vldlr–/– retinas exhibit increased levels of oxidative stress (35, 37, 38). Excessive oxygen levels can generate reactive oxygen species that cause molecular damage to lipids, proteins, and nucleic acids, which can subsequently lead to cell death unless neutralized by the antioxidant defense system (48). The retina, with its high fat content and its exposure to light, is particularly susceptible to such injury. Cone loss is associated with oxidative damage in other models of primary retinal degeneration, such as the rd1 mouse (9, 10), and oxidative damage has been linked with AMD (40). Recent studies using adaptive optics

Figure 7
Antioxidants protect retinas from degeneration and reduced function in Vldlr–/– retinas. (A and B) In retinas of antioxidant-treated 2-mo-old Vldlr–/– mice, decreased acrolein staining was observed compared with age-matched vehicle-treated controls. Panels in A are composite montages of multiple serial micrographs. Error bars denote SEM. NT, not treated; Anti, antioxidant treatment. (C–G) Vldlr–/– mice were subjected to a 6-wk treatment with vehicle or antioxidants, beginning at 2 mo of age. (C) Antioxidant treatment attenuated the loss of cones and rods, as demonstrated by normalized expression of opsin-1 and rhodopsin, respectively. n = 12 retinas per group. Error bars denote SEM. (D and E) The extent of subretinal NV in 3.5-mo-old Vldlr–/– mouse retinas was not affected by antioxidant treatment. n = 12 retinas per group. Error bars denote SEM. (F and G) ERGs were normalized after 6 wk of antioxidant treatment. Note decreased and delayed signaling in vehicle-treated Vldlr–/– mice compared with those treated with antioxidants. Scale bars: 250 μm.
Additionally, these findings also demonstrate focal photoreceptor loss associated with vascular abnormalities in diseases such as MacTel, traditionally considered a retinal vascular disease (A. Roorda, unpublished observations). We attempted to directly assess the role of abnormal NV in causing subsequent neuronal degeneration by blocking NV in the Vldlr−/− mice. However, even the most potent angiostatic combinations were only transiently effective, while the neurodegeneration was progressive over the course of several weeks to months after initiation of subretinal NV. Thus, prolonged elimination of subretinal NV was impossible without repeated injections, a process which at best leads to injury and variability in the small mouse eye, and often leads to phthisis. However, based on the strong association between subretinal NV and neurodegeneration, the correlation between subretinal NV and oxidative stress, and the neuroprotective results obtained from treatment with antioxidants, we hypothesize that increased vascularization and abnormal localization of the subretinal NV leads to increased oxidative stress and subsequent neuronal damage. No defects were found in the cellular oxidative stress defense pathways of the Vldlr−/− mice, further supporting our hypothesis.

These findings demonstrating protective effects of antioxidant treatment on neuronal degeneration associated with abnormal NV support prior epidemiological studies in humans showing that an antioxidant-rich diet protects against AMD (40) and diminishes its progression (49). However, it should be noted that only moderate treatment on neuronal degeneration associated with abnormal NV and oxidative stress, and the neuroprotective results obtained particularly when dietary supplementation is initiated after NV is established, we hypothesize that increased antioxidant- and vehicle-treated Vldlr−/− mice from each retina were normalized to β-actin. The following primers were used: cone opsin forward, 5′-GCTCCAAC-GCTCCAAC-3′; cone opsin reverse, 5′-TCACCTCAAGTGTGGCAAAG-3′; rhodopsin forward, 5′-TCATGGTCTTCGGAG-3′; rhodopsin reverse, 5′-TCACCTCCAAGTGTGGCAAAG-3′; and β-actin. The following primers were used: cone opsin forward, 5′-CAAGGCCCTTTGGCAATTGTA-3′; cone opsin reverse, 5′-GCTCCAAC-3′; rhodopsin forward, 5′-CATCGTCTTGGAG-GATTCA-3′; rhodopsin reverse, 5′-TACCTCAAGTGTGGCAAAG-3′; also demonstrate focal photoreceptor loss associated with vascular abnormalities in diseases such as MacTel, traditionally considered a retinal vascular disease (A. Roorda, unpublished observations). We attempted to directly assess the role of abnormal NV in causing subsequent neuronal degeneration by blocking NV in the Vldlr−/− mice. However, even the most potent angiostatic combinations were only transiently effective, while the neurodegeneration was progressive over the course of several weeks to months after initiation of subretinal NV. Thus, prolonged elimination of subretinal NV was impossible without repeated injections, a process which at best leads to injury and variability in the small mouse eye, and often leads to phthisis. However, based on the strong association between subretinal NV and neurodegeneration, the correlation between subretinal NV and oxidative stress, and the neuroprotective results obtained from treatment with antioxidants, we hypothesize that increased vascularization and abnormal localization of the subretinal NV leads to increased oxidative stress and subsequent neuronal damage. No defects were found in the cellular oxidative stress defense pathways of the Vldlr−/− mice, further supporting our hypothesis.

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These findings demonstrating protective effects of antioxidant treatment on neuronal degeneration associated with abnormal NV support prior epidemiological studies in humans showing that an antioxidant-rich diet protects against AMD (40) and diminishes its progression (49). However, it should be noted that only moderate levels of protection were observed in these epidemiological studies, particularly when dietary supplementation is initiated after NV is already present. Such dietary measures may be a simple and effective method of preventing neuronal cell loss associated with certain retinal vascular diseases such as MacTel and RAP. In instances of associated neuronal degeneration with substantial ERG abnormalities, gene therapy using intravitreally injected AAV-2 vectors encoding neurotrophic transgenes may also serve to preserve visual function. These findings using the Vldlr−/− mouse retina model also support other studies that demonstrate benefits of antioxidant-rich diets in relation to other neurodegenerative disorders of the CNS (50). Thus, our present findings may also have application to other neurodegenerative disorders, such as Huntington disease, Parkinson disease, and Alzheimer disease. Further studies will be required to better understand the link between vascular abnormalities such as those observed in retinal NV, oxidative stress, and neuronal degeneration.

### Methods

**Retina dissection and staining.** All animal studies were approved by the animal protocols review committee at The Scripps Research Institute. Vldlr−/− mice (23, 51) were obtained and backcrossed in the C57BL/6J background for at least 10 generations. WT mice were normal, nonknockout C57BL/6J mice. Retinas were dissected and prepared for whole mounts or sectioning as previously described (17). For preparation of retinal cross-sections, dissected retinas were fixed flat with 4 radial relaxing incisions, placed in 4% PFA, and incubated at 4°C overnight. Retinas were then placed in 20% sucrose at 4°C for 4 hours and embedded in Tissue-Tek OCT compound (Sakura FineTechnical) for cryosectioning.

Fluorescently conjugated isoelectin GS from Grifonia simplicifolia (Invitrogen) or antibodies against collagen IV (Chemicon), GFAP (DAKO), and red/green opsin (Chemicon) were used for immunohistochemistry. For analysis of vascular leak, FITC-dextran (43,200 MW; Sigma-Aldrich) was perfused through the left ventricle of deeply anesthetized mice using 150 μl of 50 mg/ml solution in PBS.

**Electron microscopy analysis of Vldlr−/− retinas.** Eye cups containing the retina and choroid from each mouse were fixed in 4% paraformaldehyde plus 1.5% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C followed by rinsing in 0.1 M Na cacodylate buffer for 1 hour. The eye cups were then postfixed in 1% OsO4 in 0.1 M cacodylate buffer for 2 hours, followed by another 1-hour wash and dehydration with graded ethanol solutions. Samples were incubated overnight in a 1:2 mixture of propylene oxide and Epox/Araldite (Sigma-Aldrich) and then placed in 100% resin followed by embedding. The blocks were sectioned and used for high-magnification electron microscopy analysis.

**Laser capture.** All steps were carefully performed using RNase-free conditions. Whole eyes were isolated without fixation and embedded in OCT. After serial sectioning, laser capture microdissection was performed using an Arcturus PixCell II Laser Capture Microdissection microscope. The ganglion cell layer, INL, ONL, photoreceptor inner/outer segments, and retinal pigment epithelium were isolated separately from histologically stained retinas. Segments highly enriched for vessel fragments were also isolated after a brief stain with isoelectin GS (Invitrogen). RNA was isolated from the microdissected retina using microRNA kits (Qiagen) and, after ensuring the quality of each RNA preparation, was reverse transcribed into cDNA for analysis by quantitative RT-PCR.

**Quantitative RT-PCR analysis.** Retinas were dissected under RNase-free conditions, and each retina was immediately lysed in separate tubes containing RLT Plus buffer (Qiagen) with β-mercaptoethanol (Sigma-Aldrich). RNA was isolated using the RNeasy plus mini kit (Qiagen) protocol, which incorporates a genomic DNA elimination step. Total RNA (0.5 μg from each retina) was reverse transcribed into cDNA using the Quantitect reverse transcription kit (Qiagen). SYBR green–based real-time quantitative RT-PCR was performed using the iCycler machine (BioRad) to determine mRNA expression levels of various gene products. Expression levels from each retina were normalized to β-actin.

**Primers.** The following primers were used: cone opsin forward, 5′-CAAGGCCCTTTGGCAATTGTA-3′; cone opsin reverse, 5′-GCTCCAAC-3′; rhodopsin forward, 5′-CATCGTCTTGGAG-GATTCA-3′; rhodopsin reverse, 5′-TACCTCAAGTGTGGCAAAG-3′;
VLDDL forward, 5′-TTTCTAGCTCATCTTCTTGAC-3′; VLDDL reverse, 5′-CTGACCACTGTAATTATGTCG-3′; VEGF forward, 5′-GGAGACCTCTTCCAGGAGCCT-3′; VEGF reverse, 5′-GGCGATTTAGCAGCA-GATATAAGAA-3′. For β-actin normalization, the Mm_Actb_2_5G_Sa

tiTect Primer Assay was used (Qiagen).

Treatment of murine eyes with antiangiogenics. Intraocular injections of Macu
gen, combination angiostatics, or vehicle controls were performed at P12, just prior to formation of the subretinal vascular sprouts in the Vldr−/− retinas. All intraocular injections were performed using a Hamilton syringe fitted with a 33-gauge needle (Hamilton), injecting 0.5 μl of solutions containing 1.5 μg Macugen (3.0 mg/ml; Pfizer), a triple angiostatic combination, or PBS vehicle. The triple combination solution consisted of Macugen (3.0 mg/ml), integrin αβ3, and αβ3 antagonist EMD472523 (20 mg/ml; Merck), and T2-TrpRS (0.5 mg/ml; Angiosyn). T2-TrpRS is a fragment of human TrpRS that has previously been shown to have angiostatic activity (30). The retinas were

ted in 2 steps. First, human NT4 cDNA was isolated from a human cDNA
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ERG. After overnight dark adaptation, mice were anesthetized under dim red light by intraperitoneal injection of 15 mg/kg ketamine and 7 mg/kg xylazine. Full-field ERGs were recorded from the corneal surface of each eye after pupil dilation (1% tropicamide and 2.5% phenylephrine) using a gold loop electrode together with a mouth reference and tail ground electrode, similar to previously published methods (52). A computerized system with an electronically controlled Ganzfeld dome was used (Espion software (Silicon Genetics). Genes related to oxidative stress pathways were analyzed for retinal expression, and only those above background in WT or Vldr−/− retinas with statistically consistent results amongst the replicates were analyzed further. The expression levels for studies that used contralateral eyes to compare various treatments were derived using a standard Student’s t test with a 1-tailed distribution, and 2 sample equal variance for treatment groups using unpaired eyes or paired values for studies that used contralateral eyes to compare various treatments. A P value less than 0.05 was considered significant.

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