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# Transducin1, Phototransduction and the Development of Early Diabetic Retinopathy

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**PURPOSE.** Recent evidence suggests that retinal photoreceptor cells have an important role in the pathogenesis of retinal microvascular lesions in diabetes. We investigated the role of rod cell phototransduction on the pathogenesis of early diabetic retinopathy (DR) using *Gnat1*<sup>-/-</sup> mice (which causes permanent inhibition of phototransduction in rod cells without degeneration).

**METHODS.** Retinal thickness, oxidative stress, expression of inflammatory proteins, electroretinograms (ERG) and optokinetic responses, and capillary permeability and degeneration were evaluated at up to 8 months of diabetes.

**RESULTS.** The diabetes-induced degeneration of retinal capillaries was significantly inhibited in the *Gnat1*<sup>-/-</sup> diabetics. The effect of the *Gnat1* deletion on the diabetes-induced increase in permeability showed a nonuniform accumulation of albumin in the neural retina; the defect was inhibited in diabetic *Gnat1*<sup>-/-</sup> mice in the inner plexiform layer (IPL), but neither in the outer plexiform (OPL) nor inner nuclear (INL) layers. In *Gnat1*-deficient animals, the diabetes-induced increase in expression of inflammatory associated proteins (iNOS and ICAM-1, and phosphorylation of IκB) in the retina, and the leukocyte mediated killing of retinal endothelial cells were inhibited, however the diabetes-mediated induction of oxidative stress was not inhibited.

**CONCLUSIONS.** In conclusion, deletion of transducin1 (and the resulting inhibition of phototransduction in rod cells) inhibits the development of retinal vascular pathology in early DR.

Keywords: retina, photoreceptors, microvasculature, *Gnat1*, phototransduction

Diabetic retinopathy (DR) is a leading cause of acquired blindness, but its pathogenesis and reason for the relatively unique susceptibility of the retinal vasculature to diabetes-induced injury remains unclear. Recently, several cell types that participate in vision and are unique to the eye (photoreceptor cells and RPE) have been implicated in having key roles in the pathogenesis of DR.<sup>1–7</sup> We investigated the possibility that phototransduction, a unique function of photoreceptor cells, has a role in the development of DR.

Phototransduction is a G-protein signaling cascade in photoreceptor outer segments that converts incoming light stimuli into an electrical response.<sup>8</sup> When struck by a photon, 11-*cis* retinal in photoreceptor outer segments undergo photoisomerization to *all-trans* retinal, leading to the activation of transducin and cyclic GMP (cGMP) phosphodiesterase. This, in turn, leads to the closure of cGMP-gated ion channels, and hyperpolarization of the photoreceptor cells.<sup>9–11</sup>

Recent studies showed that an inhibitor of RPE65 and the visual cycle (retinylamine<sup>12,13</sup>) inhibited the diabetes-induced degeneration of the retinal vasculature, as well as increased retinal vascular permeability, oxidative stress, and inflammation,<sup>14</sup> thus suggesting that processes stimulated by light

enhance the development of the lesions that characterize early DR. In contrast, however, treatment with retinaldehydes restores the diabetes-induced defect in retinal rod function and inhibits production of superoxide in diabetes.<sup>15</sup> Since light activates phototransduction and the visual cycle, we investigated the role of phototransduction in the pathogenesis of the retinopathy using a mutant mouse model deficient in phototransduction in rod cells (*Gnat1*<sup>-/-</sup> mice).

## MATERIALS AND METHODS

All procedures involving animals were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice in which the rod transducin  $\alpha$ -subunit (*Gnat1*<sup>-/-</sup>) was knocked out (on the C57Bl/6 background) were provided originally by Janis Lem (Tufts University, Boston, MA, USA). In the retina, this gene is expressed only in rod photoreceptor cells. Some C57Bl/6J mice were housed in continuous darkness



for 2 months as a nongenetic control for the effects of eliminating phototransduction on retinal production of superoxide. These chronically dark-adapted animals and their controls that experienced the normal day/night cycle were killed in the morning.

Animals were housed in a ventilated microisolator with free accessibility to food and water. Diabetes was induced at 2 to 3 months of age by intraperitoneal injection of a freshly prepared solution of streptozotocin in citrate buffer (55 mg/kg body weight [bw] for 5 consecutive days). Female mice have been recognized to be resistant to the diabetogenic effects of streptozotocin, so only males were used in this study. Insulin was given as needed (0–0.2 units subcutaneously 0–3 times a week) to inhibit weight loss, while still allowing hyperglycemia. Following streptozotocin, the milieu of the entire body changes rapidly, and the new steady-state takes days to weeks to stabilize; thus, blood glucose concentration was not measured until at least 7 days after the final administration of streptozotocin. Blood glucose was determined with a portable glucose meter, using blood collected from the tail vein under nonfasting conditions. The onset of diabetes was defined as three consecutive measures of blood glucose over 275 mg/dL. Hemoglobin A1c (HbA1c) was measured every 2 to 3 months as reported previously.<sup>16</sup>

### Vascular Histopathology

After 8 months of diabetes (D) or in age-matched nondiabetic (N) controls (10 months old), one eye was fixed in formalin. The retina then was isolated, washed, and digested in elastase as we reported previously.<sup>16</sup> When totally cleaned of neural cells, the isolated vasculature was floated out flat on a glass microscope slide, dried overnight, stained with hematoxylin and periodic acid-Schiff, dehydrated, and coverslipped. Degenerate (acellular) capillaries were quantitated in 6 to 7 field areas corresponding to the mid-retina ( $\times 200$  magnification) in a masked manner. Acellular capillaries (reported per square millimeter of retinal area) were identified as capillary-sized vessel tubes having no nuclei anywhere along their length.

### Leakage of Albumin into Neural Retina

Accumulation of the blood protein, albumin, in the neural retina was measured as reported previously.<sup>5,17,18</sup> At 8 months of diabetes, sterile FITC-BSA (50  $\mu\text{g}/\mu\text{L}$ ) in phosphate buffered saline (NaCl, 0.138 M; KCl, 0.0027 M; pH 7.4) was injected into the tail veins of mice (100  $\mu\text{g}/\text{g}$  bw). After 20 minutes, mice were euthanized, and one retina fixed in ice-cold 4% paraformaldehyde and then frozen in optimal cutting temperature compound (O.C.T.) in isopentane on dry ice after infusion with sucrose. Retinal cryosections were cut and viewed by fluorescence microscopy. Leakage of albumin was estimated from measurements of FITC-BSA in the inner plexiform (IPL), inner nuclear (INL), and outer plexiform (OPL) layers of the neural retina using computer-assisted microscopy. Permeability in diabetes is expressed as the ratio of FITC-BSA concentration in neural retina relative to that of in plasma.

### Ultrahigh-Resolution Spectral-Domain Optical Coherence Tomography (SD-OCT) Imaging

SD-OCT (Bioptigen, Durham, NC, USA) was used for in vivo imaging of mouse retinas. Mice were anesthetized by intraperitoneal injection of 10  $\mu\text{L}/\text{g}$  dilute ketamine/xylazine (16.5/1.65 mg/mL). Pupils were dilated with 1% tropicamide. Five pictures were acquired in the B-scan mode and used to construct each final averaged image. Thickness of the retina

and ONL was measured at distances of 150, 300, and 450  $\mu\text{m}$  from the optic nerve.

### Superoxide Generation

Superoxide generation and oxidative stress were assessed by two independent methods: (1) lucigenin-induced bioluminescence and (2) dichlorofluorescein (DCF) staining of unfixed cryosections. For the lucigenin-induced bioluminescence method, freshly isolated retinas were incubated in 200  $\mu\text{L}$  Krebs-Hepes buffer, pH 7.2, with 5 or 25 mM glucose for 5 minutes at 37°C in 5%  $\text{CO}_2$ . Luminescence indicating the presence of superoxide was measured 5 minutes after addition of 0.54 mM (final concentration) lucigenin.<sup>16</sup> Luminescence intensity is reported in arbitrary units per mg protein.

For the DCF method, eyes were collected, immediately embedded in O.C.T. compound, and fresh frozen using liquid nitrogen vapor. Sections (12  $\mu\text{m}$ ) were cut, and while still frozen, slides were transferred to ice cold acetone for 10 minutes at  $-20^\circ\text{C}$ . Slides then were warmed to room temperature for 20 minutes for acetone to evaporate. Next, sections were washed in PBS 3 times 5 minutes. Subsequently (from here on, work is done in dark), sections were submerged in DCF (cat# D6883; Sigma-Aldrich Corp., St. Louis, MO, USA) at 10  $\mu\text{M}$ , and incubated at 37°C for 60 minutes. Sections then were washed 3 times 5 minutes, then cover-slipped using ProLong Gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI; cat# P36935; Invitrogen, Carlsbad, CA, USA), and photographed by fluorescence microscopy.

### Immunoblotting Technique

Retinas were isolated, sonicated, and centrifuged, and the supernatants were used for Western blots. Samples (usually approximately 30  $\mu\text{g}$ ) were fractionated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and membranes then blocked in Tris-buffered saline containing 0.02% Tween 20 and 5% nonfat milk. Antibodies for ICAM-1 (1:2000; Proteintech Group, Inc., Chicago, IL, USA) and iNOS (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-IkB and IkB (1:200 and 1:1000 dilutions, respectively; Santa Cruz Biotechnology) were applied, followed by secondary antibody for 1 hour. After washing, nitrocellulose membranes were visualized for enhanced chemiluminescence. Protein levels were quantified relative to  $\beta$ -actin loading controls (1:5000 dilution, Abcam, Inc., Cambridge, MA, USA) in the same samples.

### Visual Function

Spatial frequency threshold (a marker of visual acuity in rodents) was measured at 3 and 8 months of diabetes (5 and 10 months of age) with the Virtual Optokinetic system.<sup>19–21</sup> The maximum spatial frequency capable of driving head tracking was determined as the spatial frequency threshold. The experimenter was masked as to the identity of the experimental animals.

### Electroretinography (ERG)

ERGs were measured at 8 months of diabetes (10 months of age). After overnight dark adaptation, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg), the cornea was anesthetized with 1% proparacaine hydrochloride, and the pupils were dilated with 1% tropicamide, 2.5% phenylephrine hydrochloride, and 1% cyclopentolate. Mice were placed on a temperature-regulated heating pad throughout each recording session, which has been described.<sup>22</sup> In brief, responses of the outer retina were recorded with a contact lens electrode

TABLE. Glycemia in WT and *Gnat1*<sup>-/-</sup> Mice Over 8 Months of Study

Genotype	Final bw, g	Blood Glucose,* Non-Fasting; mg/dL	HbA1c,* %
N mice			
WT	42 ± 7	156 ± 18	3.1 ± 0.1
<i>Gnat1</i> <sup>-/-</sup>	41 ± 8	156 ± 10	3.1 ± 0.1
D mice			
WT	28 ± 3	489 ± 48	9.7 ± 0.9
<i>Gnat1</i> <sup>-/-</sup>	29 ± 4	514 ± 53	10.1 ± 0.4

\* Glucose levels and HbA1c were measured as described in the Methods section.

referenced to a needle electrode placed in the cheek in response to strobe-flash stimuli presented in the dark or superimposed on a steady 20 candela (cd)/m<sup>2</sup> rod-desensitizing adapting field. The amplitude of the a-wave was measured 6 ms after flash onset from the pre-stimulus baseline. The amplitude of the b-wave was measured from the a-wave amplitude at 6 ms to the peak of the b-wave.

### Leukocyte-Mediated Cytotoxicity Toward Endothelial Cells<sup>23–25</sup>

Transformed retinal endothelial cells<sup>26</sup> were grown in control medium (Dulbecco's modified Eagle medium [DMEM] with 5 mM glucose) containing 10% serum. The serum concentration was reduced to 2% just before cells were placed either in 5 mM glucose or high glucose (30 mM). Media was changed every other day for 3 days. When cells reached 80% confluency level (~300,000 cells), freshly isolated leukocytes from blood (100,000 cells) were added and incubated for 6 additional hours, after which cells were collected and washed with PBS. Cells were stained with an antibody against CD144 (1:50 dilution; BD Biosciences Pharmingen, San Diego, CA, USA) to identify endothelial cells, and the viability of the endothelial cells was identified by flow cytometry based on 7-AAD staining. Endothelial cell death was expressed as the percentage of endothelial cells that stained positively with dye. Approximately 10,000 cells were counted in each sample. Experiments were repeated twice with similar results each time.

### Leukostasis

At 2 to 3 months of diabetes, blood was removed from the vasculature of anesthetized animals (100 mg/mL ketaset; 100 mg/mL xylazine = 5:1) by complete perfusion with PBS via a heart catheter. Animals then were perfused with fluorescein-coupled Concanavalin A lectin (20 µg/mL in PBS; Vector Laboratories, Burlingame, CA, USA) as described previously.<sup>16</sup> Flat-mounted retinas were imaged via fluorescence microscopy, and the number of leukocytes adherent to the vascular wall was counted.

### Statistical Analyses

Data are expressed as mean ± SD. All statistical analyses were performed with ANOVA followed by Fischer's test.  $P < 0.05$  was considered statistically significant.

## RESULTS

There was no significant difference with respect to bw or glycemia between N members of the strains studied. Glycemia was elevated in all animals assigned to D groups, and the

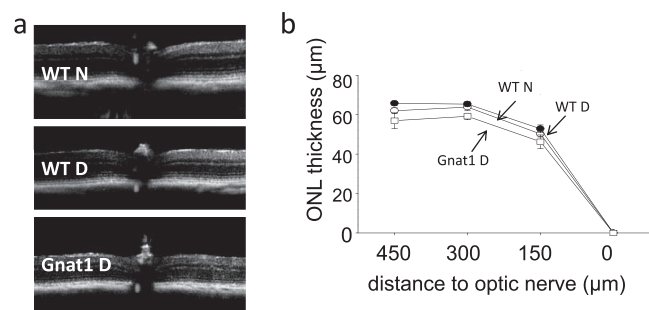


FIGURE 1. OCT images (a) and quantitation of data from those images (b) show that diabetes for 8 months (10 months of age) resulted in essentially no loss of retinal photoreceptors (ONL thickness) in WT C57Bl/6J mice compared to age-matched N controls. *Gnat1*<sup>-/-</sup> mice diabetic for the same duration likewise show little loss of retinal photoreceptors ( $n = 4-7$  per group).

severity of diabetes was not different among the D groups. HbA1c and blood glucose were measured multiple times over the entire duration of the 8-month experiment, and these values were significantly greater than normal in all D groups ( $P < 0.01$ ). Data are summarized in the Table.

### Effect of Diabetes and Deletion of *Gnat1* on Photoreceptor Survival

OCT analysis indicated that diabetes of 8 months duration did not cause a significant increase or decrease in retinal thickness in wildtype (WT) animals ( $213 \pm 5$  µm compared to  $212 \pm 5$  for N and D groups 450 µm from the optic nerve, respectively). *Gnat1*<sup>-/-</sup> mice that had been diabetic for a comparable duration showed only a slight (but significant,  $P < 0.05$ ) reduction in thickness ( $196 \pm 6$  µm) compared to age-matched WT mice. The thickness of the ONL was not significantly different from normal in D WT and in *Gnat1*<sup>-/-</sup> mice (Fig. 1).

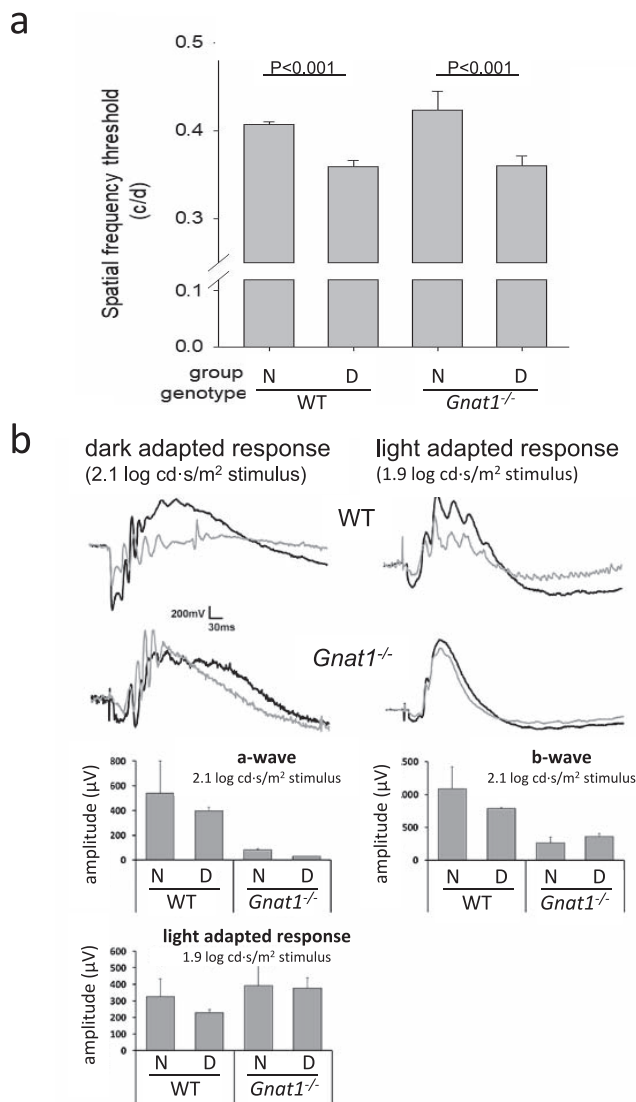
### Visual Function

Spatial frequency threshold is a psychophysical measure that assesses the function of retinal and central visual pathways in cone cells. At 5 months of age, N mice from the *Gnat1*<sup>-/-</sup> mice showed spatial frequency thresholds that were equivalent to those observed in WT N mice (Fig. 2a). The fact that this model did not have a major effect on vision is not surprising, since the optokinetic technique measures predominantly cone function, and not the rod cells. Diabetes for 3 months' duration (5 months old) caused a significant decrease in spatial frequency threshold in WT and *Gnat1*-deficient mice (Fig. 2a), and this decrease was maintained also at 8 months of diabetes ( $0.397 \pm 0.003$  c/d;  $0.371 \pm 0.008$ , and  $0.350 \pm 0.006$  for WT N and D, *Gnat1*<sup>-/-</sup> D groups, respectively; all  $P < 0.001$  compared to WT N).

We next examined ERGs as a confirmatory test (Fig. 2b). WT mice diabetic for 8 months (10 months of age) exhibited reductions in a- and b-wave amplitudes as well as the light-adapted response. N *Gnat1*-deficient mice exhibited small dark-adapted responses, and low a- and b-wave. Light-adapted responses were of normal amplitude in the mutants, and the results seemed not affected by diabetes.

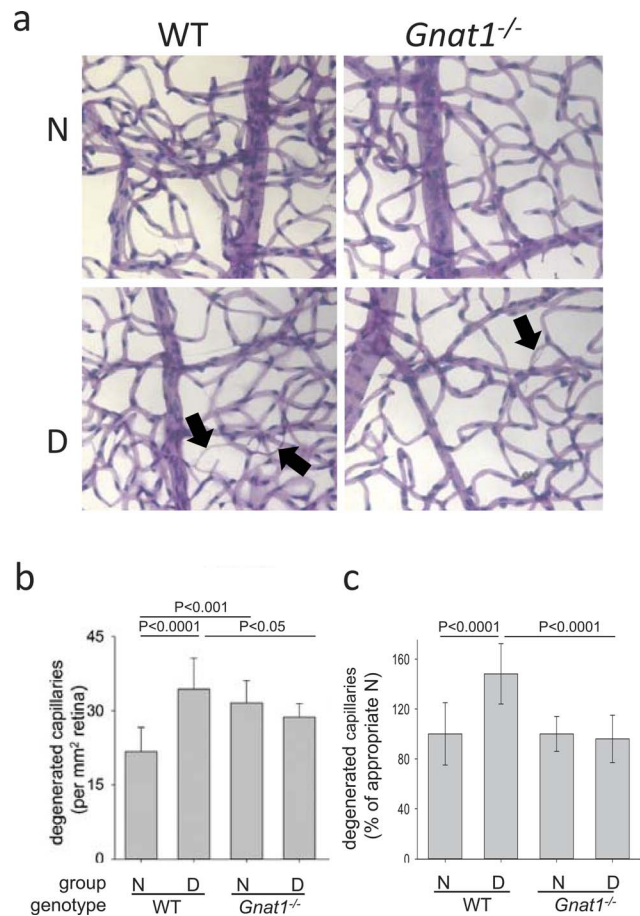
### Retinal Vascular Histopathology

N WT C57Bl/6J mice had few degenerate capillaries at 10 months of age (Fig. 3). *Gnat1*-deficient N mice showed a significant increase in retinal capillary degeneration compared to N WT C57Bl/6J mice (Fig. 3b;  $P < 0.0001$ ).



**FIGURE 2.** Visual function in N and D C57Bl/6J mice and *Gnat1*<sup>-/-</sup> mice. **(a)** Spatial frequency threshold was not impaired in N mice of either of the strains at 5 months of age, and diabetes reduced it significantly and to a similar degree in C57Bl/6J mice and *Gnat1*<sup>-/-</sup> mice ( $n = 4$  per group). **(b)** Averaged strobe-flash ERG responses were recorded at 10 months of age (8 months diabetes) at increasing light intensities for each group. Representative waveforms generated for each group in response to a 2.1 log cd·s/m<sup>2</sup> stimulus flash presented after overnight dark adaptation are shown. Light-adapted responses in response to a 1.9 log cd·s/m<sup>2</sup> stimulus flash on a steady adapting field also are shown. N, black lines; D, gray lines. Amplitude of the a-wave was measured at 6 ms and amplitude of the b-wave was measured at the peak. ERG responses were generated for illustrative purposes (only  $n = 2$  animals per group), and so were not analyzed statistically.

As expected, diabetes 8 months in duration (age 10 months) significantly increased the number of degenerated capillaries in WT C57Bl/6J mice compared to their N C57Bl/6J controls (Fig. 3). Since even the N *Gnat1*-deficient mice showed an increase in retinal capillary degeneration, we evaluated the diabetes-induced increase in capillary degeneration as the number of degenerate capillaries per unit retinal area (Fig. 3b), and as a ratio compared to the number of degenerate capillaries in nondiabetic members of the same mutant strain (Fig. 3c). *Gnat1*-deficient D mice showed fewer degenerated retinal capillaries compared to WT D controls, and showed no diabetes-induced increase compared to their N *Gnat1*-deficient



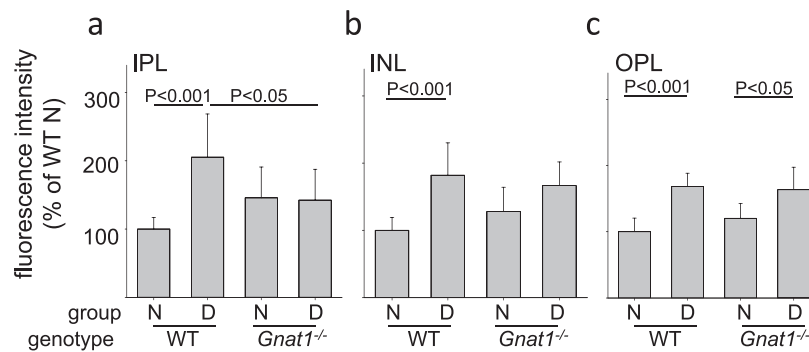
**FIGURE 3.** Effect of diabetes and rod cell mutations on the retinal vasculature. **(a)** Representative photomicrographs demonstrating the retinal vasculature in N (top row) and D (lower row) WT and *Gnat1*<sup>-/-</sup> mice. Examples of degenerated capillaries are indicated by arrows. Samples were collected and prepared at 8 months of diabetes (10 months of age). Data are graphed as degenerate capillaries per unit area of retina **(b)** and as a ratio compared to that in N controls of the same genetic strain **(c)**. Vascular histopathology was quantitated microscopically following isolation of the vasculature by the elastase digestion method. Horizontal lines indicate statistically significant differences between groups ( $n = 5-9$  for all groups). Mean  $\pm$  SD were calculated.

controls. Thus, the D mice lacking phototransduction in their rod cells were protected from the diabetes-induced capillary degeneration, and this protection occurred without any reduction in glycemia.

Diabetes of 8 months' duration significantly increased the amount of leakage of FITC-BSA into each multiple layer of the neural retina containing a vascular plexus (IPL, IN, OPL) in WT animals (Fig. 4). The diabetes-induced increase in permeability was inhibited in D *Gnat1*<sup>-/-</sup> mice in the IPL, but there was no significant inhibition of the diabetes-induced increase in albumin leakage in the OPL or INL layers at this time point.

### Retinal Oxidative Stress

Diabetes of 3 months' duration (5 months of age) appreciably increased the generation of superoxide or reactive oxygen species in the photoreceptors of WT D mice (Fig. 5). Our studies as well as others have reported previously that this increase is present in vivo and maintained for at least an additional 6 months of diabetes,<sup>18</sup> demonstrating that this oxidative stress is not an artifact due to ex vivo processing or a



**FIGURE 4.** Diabetes of 8 months' duration significantly increased accumulation of FITC-BSA in the (a) IPL, (b) INL, and (c) OPL of retinas from WT mice. FITC-BSA was injected intravenously, allowed to circulate for 20 minutes, and then fluorescence in areas of the appropriate retinal layer was quantitated from retinal cross-sections. *Gnat1*<sup>-/-</sup> diabetic mice were significantly protected from the diabetes-induced increase in permeability only in the IPL, whereas leakage remained significantly elevated compared to N controls in the OPL and INL layers ( $n = 3-8$  per group).

transient abnormality. Generation of reactive oxygen species also was evaluated by measuring DCF staining in freshly isolated retinal slices. Under these conditions, no DCF fluorescence was observed in slices obtained from N control mice (Fig. 5b), while it was consistently detected in photoreceptor cells of D WT mice. The DCF fluorescence was detectable predominantly in the rod outer segments. We found that the deletion of transducin1 (and, thus, phototransduction) in *Gnat1*<sup>-/-</sup> mice did not inhibit that retinal oxidative stress in diabetes, and in fact, induced it in photoreceptor cells even in N mice.

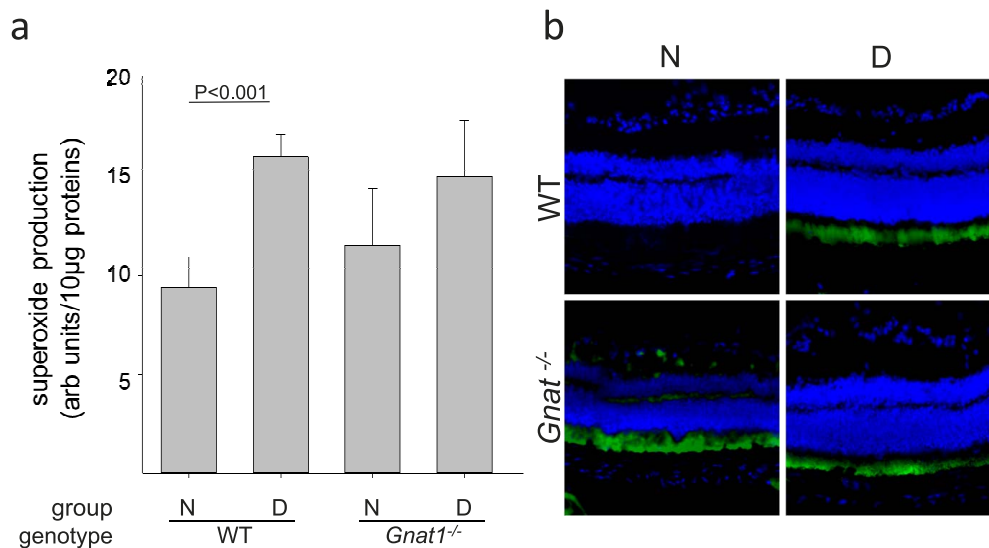
To further investigate the effects of eliminating phototransduction on retinal superoxide generation, we maintained other N and D WT animals in total darkness for 2 months to mimic the absence of phototransduction that would occur in rods of the *Gnat1*-deficient D mice. Compared to N mice under a standard day/night cycle, superoxide generation in the retinas of D mice with the same diurnal light cycle was 316%  $\pm$  155% above that in the N mice, whereas superoxide generation by retinas from the dark-maintained N and D

animals was 259%  $\pm$  137% and 389%  $\pm$  244% above that in WT N mice maintained under a diurnal cycle, respectively ( $n = 5$  in all groups).

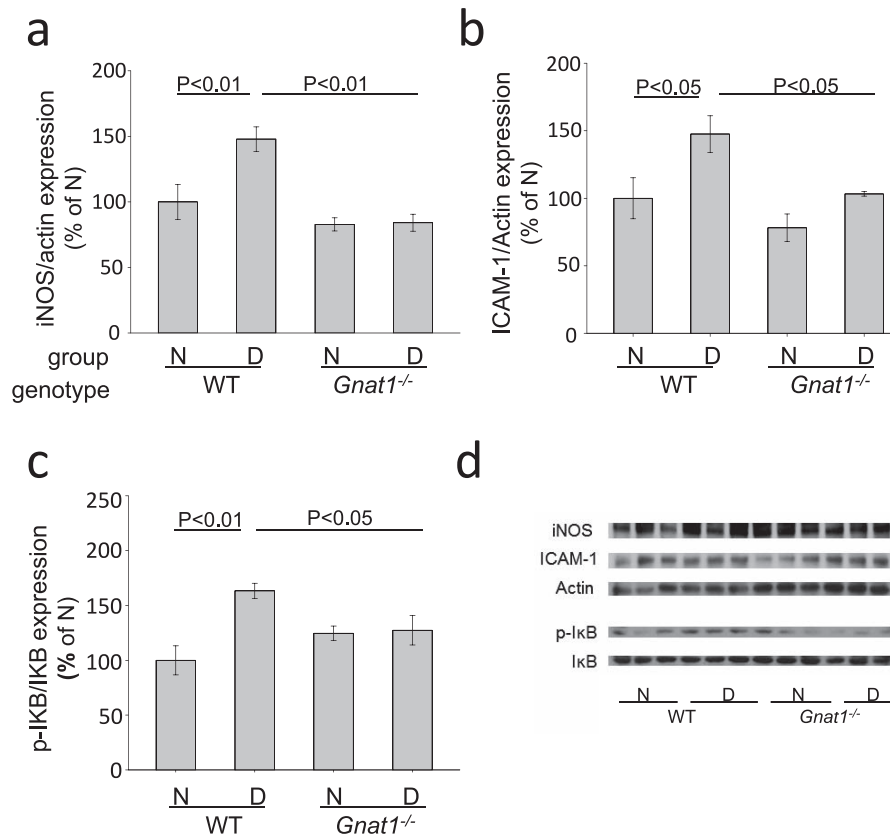
### Markers of Inflammation

Since increased expression of proinflammatory proteins and cytokines has been implicated in the pathogenesis of the retinopathy,<sup>5,18,27</sup> we measured the effect of diabetes and transducin1 deficiency on expression of iNOS, ICAM-1, and other proinflammatory proteins in the retina. Diabetes of 2 to 3 months in duration resulted in a significant increase in expression of retinal iNOS, ICAM-1, and in the ratio of p-I $\kappa$ B/total I $\kappa$ B in WT animals (expression of iNOS and ICAM-1 is regulated by NF- $\kappa$ B, and I $\kappa$ B is an important regulator of NF- $\kappa$ B activation; Fig. 6). The expected diabetes-induced increase was significantly inhibited for all of these proinflammatory proteins in retinas from D *Gnat1*<sup>-/-</sup> mice (Figs. 6a-c).

Leukocytes are intimately involved in inflammatory processes, and we previously implicated leukocytes as contribut-



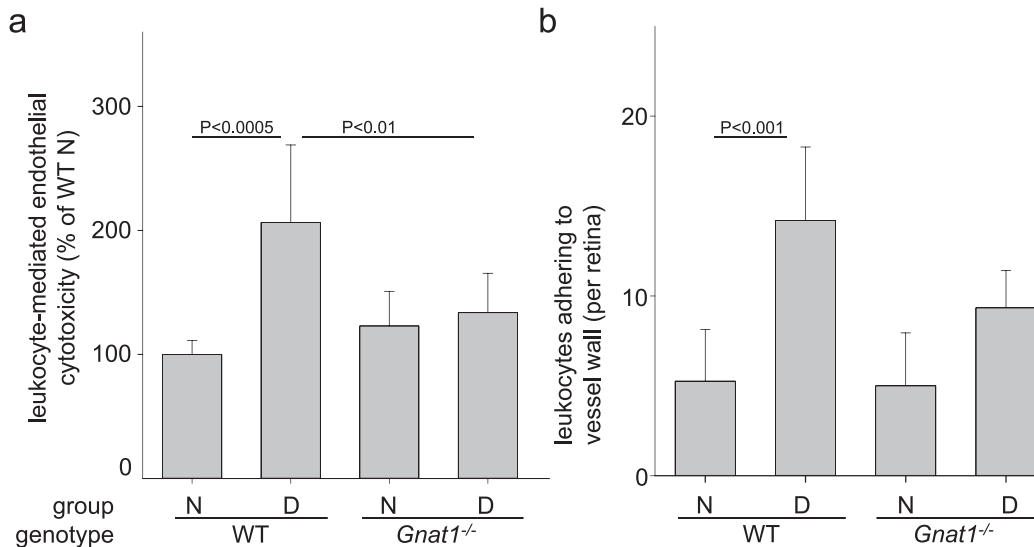
**FIGURE 5.** Effect of diabetes and loss of transducin1 on retinal generation of superoxide in mice at 5 months of age (3 months of diabetes). (a) Superoxide generation by freshly isolated retinal tissue using the lucigenin method, and (b) demonstration of oxidative stress in unfixed cryosections using the DCF method. Horizontal lines indicate statistically significant differences between groups ( $n = 6$  for all groups); there was no statistically significant difference between N mice of the WT and *Gnat1*-deficient groups. In the micrographs, DCF staining for reactive oxygen species is shown in green, and DAPI staining of nuclei is shown in blue. Reactive oxygen species were not measured at longer durations of diabetes due to use of the limited retinal tissue for the measurements of histopathology and permeability ( $n = 3-4$  in all groups for the DCF method). Inner retina is shown on the top of the micrographs, and outer retina is on the bottom.



**FIGURE 6.** Effect of diabetes and deletion of *Gnat1* on retinal expression of proinflammatory proteins in mice. (a–c) summarize expression of iNOS, ICAM, and the ratio of phosphorylated IκBα/total IκBα at 4 months of age (2 months of diabetes). (d) Representative Immunoblots of inflammatory proteins quantified, retinal homogenates, and expressed relative to actin in the same sample (*n* = 4–5 in all groups).

ing to the development of DR.<sup>23–25,28</sup> Consistent with prior studies, we found that diabetes significantly increased the leukocyte-mediated cytotoxicity against retinal endothelial cells in WT D mice, and increased also leukostasis within retinal capillaries (Fig. 7). Even though transducin1 is

expressed only in photoreceptor cells, the data indicate that deletion of the protein (and, thus, phototransduction) from rod cells significantly inhibited or tended to inhibit leukocyte-mediated killing of retinal endothelial cells and leukostasis, respectively.



**FIGURE 7.** In WT animals, (a) leukocyte-mediated cytotoxicity toward retinal endothelial cells and (b) leukostasis are significantly increased by diabetes. In contrast, the leukocyte-mediated cytotoxicity is significantly inhibited in *Gnat1*-deficient mice (*Gnat1*<sup>-/-</sup>), and leukostasis tends to be inhibited (but these results did not achieve statistical significance in this limited sample). Total duration of diabetes was 12 weeks. Horizontal lines above the figure indicate significant differences (*n* = 4 per group for the cytotoxicity assay; and *n* = 3–5 per group for leukostasis).

## DISCUSSION

Compared to other tissues, the retinal vasculature has been recognized to be uniquely sensitive to adverse effects of hyperglycemia,<sup>29</sup> but the cause of that susceptibility remains unclear. One unique function of the retina is phototransduction, where external light is absorbed in photoreceptor cells and converted into an electrical signal that results in vision. Our study provided evidence that phototransduction itself has a previously unrecognized role in the pathogenesis of retinal vascular lesions of early DR.

Transducin1 is a subunit of the heterotrimeric G-protein encoded by *Gnat1*.<sup>30</sup> It is found on the disk membrane of rod photoreceptor cells in the retina, and is a key component of the vertebrate phototransduction pathway. Therefore, the absence of transducin1 prevents phototransduction in rods, causing the cGMP-gated ion channels on the rod cells to remain continually open. Interestingly, in contrast to the original study describing *Gnat1*-deficient mice that reported that these mice had mild photoreceptor degeneration, we did not find such degeneration. This might be due to the fact that their animals were on a BALBc background, whereas our animals had been crossbred (>10 generations) onto a C57Bl/6J background. In addition, possible differences in ambient light in animal rooms might be a factor, because *Gnat1*<sup>-/-</sup> mice are sensitive to light-induced photoreceptor degeneration.<sup>31,32</sup>

Consistent with prior studies, diabetes in WT animals significantly increased capillary degeneration and albumin leakage into the neural retina in our study. Deletion of *Gnat1* increased retinal capillary degeneration in N animals. We reported previously that mutation or deletion of another critical gene in photoreceptor cells (opsin) results in a large increase in degeneration of the retinal vasculature,<sup>2</sup> and deletion of *Gnat1* apparently represents a less severe example of that relationship. Interestingly, the capillary degeneration caused by diabetes apparently occurs by a mechanism that differs from that caused by the gene deletion, because total elimination of *Gnat1* significantly inhibited the diabetes-induced increase in capillary degeneration (independent of any reduction in overall glycemia). These findings provided evidence that phototransduction in rod cells contributes to the observed susceptibility of the retinal vasculature to diabetes. Since phototransduction still persists in cone cells of *Gnat1*<sup>-/-</sup> mice, the continued phototransduction in cone cells of D mice apparently has no obvious adverse effect on the vasculature.

The effect of the transducin1 deletion on the diabetes-induced increase in permeability, however, was less impressive. We found that the permeability defect at 8 months of diabetes was significantly inhibited in the IPL, but not in the OPL or INL. The reasoning behind as to why the influence of phototransduction on vascular permeability would be most apparent in the retinal layer that is furthest from the photoreceptors is not yet clear. Nevertheless, the results suggested that capillary degeneration (a permanent pathologic change) and increased vascular permeability (a potentially reversible functional change) might be caused in part by different mechanisms, with phototransduction having a stronger effect on capillary degeneration than on the permeability defect.

Some data by others have suggested that diabetes might impair phototransduction. The light-sensitive, guanosine triphosphate-dependent functions and levels of transducin have been reported to be subnormal in diabetes,<sup>33,34</sup> but it is not clear whether these changes adversely affect vision *in vivo*. Evidence that rod cell ion channels, which are normally closed secondary to light-induced phototransduction (and open in the dark), are paradoxically closed in dark-adapted D mice<sup>35</sup>

provide evidence of a functional defect. This partial inhibition of phototransduction by diabetes clearly does not inhibit the microvascular degeneration to the extent that total elimination of phototransduction in rod cells did in our study of *Gnat1*<sup>-/-</sup> mice.

In an effort to understand how deletion of *Gnat1* (and thereby inhibiting phototransduction) inhibited capillary degeneration in diabetes, we investigated the *Gnat1*<sup>-/-</sup> animals with regard to metabolic abnormalities that have previously been implicated in the development of the retinopathy.

Inhibition of retinal oxidative stress in diabetes by antioxidants or overexpression of anti-oxidant enzymes previously has been reported to preserve the retinal vasculature despite persistent hyperglycemia.<sup>36-38</sup> Two methods to assess the oxidative stress in the present studies showed that diabetes or transducin1-deficiency both increased oxidative stress in photoreceptor cells. Our data suggested that phototransduction in rod cells is not a major contributor to the diabetes-induced increase in retinal oxidative stress, because elimination of the phototransduction in *Gnat1*<sup>-/-</sup> mice did not inhibit the oxidative stress. Animals dark-adapted overnight<sup>6</sup> or for two months (present study) likewise lack phototransduction, and the increase in retinal oxidative stress in those animals further confirms that retinal oxidative stress is increased by the absence of phototransduction. The evidence that the *Gnat1*-deficient D mice were protected from the diabetes-induced capillary degeneration in the *Gnat1*-deficient D mice, however, dissociates the oxidative stress from the capillary degeneration in these D animals.

Diabetes has been recognized to induce a local inflammatory state in the retina, and induction of those inflammatory proteins in the retina also has been linked to the development of DR.<sup>24,27,39-42</sup> Our study demonstrated that the diabetes-induced increase in the expression of proinflammatory proteins, leukostasis, and leukocyte-mediated cytotoxicity to endothelial cells are (or tended to be) inhibited in the *Gnat1*-deficient D mice, thus indicating that phototransduction has an important role in the development of this proinflammatory state, and presumably also the subsequent development of retinopathy. It was interesting to find that the leukocyte-mediated cytotoxicity to retinal endothelial cells in diabetes was entirely suppressed by the deletion of a protein that is expressed only in photoreceptor cells, suggesting that photoreceptors somehow are communicating with leukocytes. We previously reported that photoreceptors release proinflammatory cytokines that can stimulate leukocytes to affect retinal endothelial cells in diabetes.<sup>4,43</sup>

In contrast to the present data indicating that inhibition of light-induced phototransduction inhibited lesions of DR, others have postulated that dark caused lesions of the retinopathy by exacerbating ischemia in retinas of D patients.<sup>44-46</sup> Accordingly, they postulated that continuous light could inhibit dark-adaptation by rods cells, thus diminishing photoreceptor oxygen consumption and retinal levels of VEGF. They reported evidence that continuous illumination of the eyes of diabetic patients throughout the night had beneficial effects, resulting in reversal of diabetic macular edema.<sup>44-46</sup> Animal evidence that the diabetes-induced increase in retinal VEGF was inhibited in rodents whose photoreceptors had degenerated was consistent with this hypothesis,<sup>47</sup> but a recent two-year clinical test<sup>48</sup> as well as other data<sup>38,49</sup> did not support the hypothesis. Thus, the postulated role of darkness on DR remains controversial.

Cone cells are the other photoreceptor cell-type, and their role in DR remains unknown. The light-adapted ERG test showed no effect of diabetes on cone function, whereas a psychophysical optokinetic test that is the summation of information from cones all the way to the brain did show a



defect. Thus, diabetes does impair aspects of vision, but we have no data that the cause of this defect includes cones. Moreover, the present studies provide no evidence that cone cells contribute to diabetes-induced retinal vascular injury.

Our findings suggested that light-induced phototransduction in rod cells and the molecular processes regulated by it contributing to the diabetes-induced degeneration of the retinal vasculature. Whether pharmacologic inhibition of phototransduction is a meaningful therapeutic target to inhibit retinopathy will depend on whether a partial reduction in phototransduction that does not compromise normal daytime vision can inhibit the microangiopathy. As an alternate approach, however, pharmacologic slowing of visual cycle activity (which is activated by phototransduction) by retinylamine already has been shown to significantly inhibit the development of the vascular lesions of the retinopathy.<sup>5</sup>

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### References

- Kern TS. Do photoreceptor cells cause the development of retinal vascular disease? *Vision Res* 2017;139:65-71.
- Liu H, Tang J, Du Y, et al. Photoreceptor cells influence retinal vascular degeneration in mouse models of retinal degeneration and diabetes. *Invest Ophthalmol Vis Sci*. 2016; 57:4272-4281.
- Kern TS, Berkowitz BA. Photoreceptors in diabetic retinopathy. *J Diabetes Investig*. 2015;6:371-380.
- Tonade D, Liu H, Palczewski K, Kern TS. Photoreceptor cells produce inflammatory products that contribute to retinal vascular permeability in a mouse model of diabetes. *Diabetologia*. 2017;60:2111-2120.
- Liu H, Tang J, Du Y, et al. Retinylamine benefits early diabetic retinopathy in mice. *J Biol Chem*. 2015;290:21568-21579.
- Du Y, Veenstra A, Palczewski K, Kern TS. Photoreceptor cells are major contributors to diabetes-induced oxidative stress and local inflammation in the retina. *Proc Natl Acad Sci U S A*. 2013;110:16586-16591.
- Xu HZ, Song Z, Fu S, Zhu M, Le YZ. RPE barrier breakdown in diabetic retinopathy: seeing is believing. *J Ocul Biol Dis Infor*. 2011;4:83-92.
- Palczewski K. G protein-coupled receptor rhodopsin. *Annu Rev Biochem*. 2006;75:743-767.
- Arshavsky VY, Lamb TD, Pugh EN Jr. G proteins and phototransduction. *Annu Rev Physiol*. 2002;64:153-187.
- Luo DG, Xue T, Yau KW. How vision begins: an odyssey. *Proc Natl Acad Sci U S A*. 2008;105:9855-9862.
- Fu Y, Yau KW. Phototransduction in mouse rods and cones. *Pflugers Arch*. 2007;454:805-819.
- Golczak M, Maeda A, Bereta G, et al. Metabolic basis of visual cycle inhibition by retinoid and nonretinoid compounds in the vertebrate retina. *J Biol Chem*. 2008;283:9543-9554.
- Golczak M, Kuksa V, Maeda T, Moise AR, Palczewski K. Positively charged retinoids are potent and selective inhibitors of the trans-cis isomerization in the retinoid (visual) cycle. *Proc Natl Acad Sci U S A*. 2005;102:8162-8167.
- Cheng Y, Du Y, Liu H, Tang J, Veenstra A, Kern TS. Photobiodulation inhibits long-term structural and functional lesions of diabetic retinopathy. *Diabetes*. 2018;67:291-298.
- Berkowitz BA, Kern TS, Bissig D, et al. Systemic retinaldehyde treatment corrects retinal oxidative stress, rod dysfunction, and impaired visual performance in diabetic mice. *Invest Ophthalmol Vis Sci*. 2015;56:6294-6303.
- Veenstra A, Liu H, Lee CA, Du Y, Tang J, Kern TS. diabetic retinopathy: retina-specific methods for maintenance of diabetic rodents and evaluation of vascular histopathology and molecular abnormalities. *Curr Protoc Mouse Biol*. 2015; 5:247-270.
- Antonetti DA, Barber AJ, Khin S, Lieth E, Tarbell JM, Gardner TW. Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Penn State Retina Research Group. *Diabetes*. 1998;47:1953-1959.
- Du Y, Cramer M, Lee CA, et al. Adrenergic and serotonin receptors affect retinal superoxide generation in diabetic mice: relationship to capillary degeneration and permeability. *FASEB J*. 2015;29:2194-2204.
- Prusky GT, Alam NM, Beekman S, Douglas RM. Rapid quantification of adult and developing mouse spatial vision using a virtual optomotor system. *Invest Ophthalmol Vis Sci*. 2004;45:4611-4616.
- Lee CA, Li G, Patel MD, et al. Diabetes-induced impairment in visual function in mice: contributions of p38 MAPK, RAGE, leukocytes, and aldose reductase. *Invest Ophthalmol Vis Sci*. 2013;93:135-143.
- Liu H, Tang J, Lee CA, Kern TS. Metax and early stages of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2015;56: 647-653.
- Samuels IS, Sturgill GM, Grossman GH, Rayborn ME, Hollyfield JG, Peachey NS. Light-evoked responses of the retinal pigment epithelium: changes accompanying photoreceptor loss in the mouse. *J Neurophysiol*. 2010;104:391-402.
- Veenstra AA, Tang J, Kern TS. Antagonism of CD11b with Neutrophil Inhibitory Factor (NIF) inhibits vascular lesions in diabetic retinopathy. *PLoS One*. 2013;8:e78405.
- Talahalli R, Zarini S, Tang J, et al. Leukocytes regulate retinal capillary degeneration in the diabetic mouse via generation of leukotrienes. *J Leukoc Biol*. 2013;93:135-143.
- Li G, Veenstra AA, Talahalli RR, et al. Marrow-derived cells regulate the development of early diabetic retinopathy and tactile allodynia in mice. *Diabetes*. 2012;61:3294-3303.
- Su X, Sorenson CM, Sheibani N. Isolation and characterization of murine retinal endothelial cells. *Mol Vis*. 2003;9:171-178.
- Tang J, Kern TS. Inflammation in diabetic retinopathy. *Prog Retin Eye Res*. 2011;30:343-358.
- Tian P, Ge H, Liu H, et al. Leukocytes from diabetic patients kill retinal endothelial cells: effects of berberine. *Mol Vision*. 2013;19:2092-2105.

29. Kern TS, Engerman RL. Capillary lesions develop in retina rather than cerebral cortex in diabetes and experimental galactosemia. *Arch Ophthalmol*. 1996;114:306-310.
30. Hurley JB. Molecular properties of the cGMP cascade of vertebrate photoreceptors. *Annu Rev Physiol*. 1987;49:793-812.
31. Hao W, Wenzel A, Obin MS, et al. Evidence for two apoptotic pathways in light-induced retinal degeneration. *Nat Genet*. 2002;32:254-260.
32. Krishnan J, Chen J, Shin KJ, et al. Gene expression profiling of light-induced retinal degeneration in phototransduction gene knockout mice. *Exp Mol Med*. 2008;40:495-504.
33. Kowluru A, Kowluru RA, Yamazaki A. Functional alterations of G-proteins in diabetic rat retina: a possible explanation for the early visual abnormalities in diabetes mellitus. *Diabetologia*. 1992;35:624-631.
34. Kim YH, Kim YS, Noh HS, et al. Changes in rhodopsin kinase and transducin in the rat retina in early-stage diabetes. *Exp Eye Res*. 2005;80:753-760.
35. Berkowitz BA, Bissig D, Patel P, Bhatia A, Roberts R. Acute systemic 11-cis-retinal intervention improves abnormal outer retinal ion channel closure in diabetic mice. *Mol Vis*. 2012;18:372-376.
36. Kowluru RA, Tang J, Kern TS. Abnormalities of retinal metabolism in diabetes and experimental galactosemia. VII. Effect of long-term administration of antioxidants on the development of retinopathy. *Diabetes*. 2001;50:1938-1942.
37. Kanwar M, Chan PS, Kern TS, Kowluru RA. Oxidative damage in the retinal mitochondria of diabetic mice: possible protection by superoxide dismutase. *Invest Ophthalmol Vis Sci*. 2007;48:3805-3811.
38. Berkowitz BA, Gadianu M, Bissig D, Kern TS, Roberts R. Retinal ion regulation in a mouse model of diabetic retinopathy: natural history and the effect of Cu/Zn superoxide dismutase overexpression. *Invest Ophthalmol Vis Sci*. 2009;50:2351-2358.
39. Jousseaume AM, Poulaki V, Le ML, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J*. 2004;18:1450-1452.
40. Adamis AP, Berman AJ. Immunological mechanisms in the pathogenesis of diabetic retinopathy. *Semin Immunopathol*. 2008;30:65-84.
41. Kaul K, Hodgkinson A, Tarr J, Kohner EM, Chibber R. Is Inflammation a Common Retinal-Renal-Nerve Pathogenic Link in Diabetes? *Curr Diabetes Rev*. 2010;6:294-303.
42. Tang J, Lee CA, Du Y, et al. MyD88-dependent pathways in leukocytes affect the retina in diabetes. *PLoS One*. 2013;8:e68871.
43. Tonade D, Liu H, Kern TS. Photoreceptor cells produce inflammatory mediators that contribute to endothelial cell death in diabetes. *Invest Ophthalmol Vis Sci*. 2016;57:4264-4271.
44. Ramsey DJ, Arden GB. Hypoxia and dark adaptation in diabetic retinopathy: interactions, consequences, and therapy. *Curr Diab Rep*. 2015;15:118.
45. Arden GB, Jyothi S, Hogg CH, Lee YF, Sivaprasad S. Regression of early diabetic macular oedema is associated with prevention of dark adaptation. *Eye (Lond)*. 2011;25:1546-1554.
46. Arden GB, Gunduz MK, Kurtenbach A, et al. A preliminary trial to determine whether prevention of dark adaptation affects the course of early diabetic retinopathy. *Eye (Lond)*. 2010;24:1149-1155.
47. de Gooyer TE, Stevenson KA, Humphries P, et al. Rod photoreceptor loss in Rho<sup>-/-</sup> mice reduces retinal hypoxia and hypoxia-regulated gene expression. *Invest Ophthalmol Vis Sci*. 2006;47:5553-5560.
48. Sivaprasad S, Vasconcelos JC, Prevost AT, et al. Clinical efficacy and safety of a light mask for prevention of dark adaptation in treating and preventing progression of early diabetic macular oedema at 24 months (CLEOPATRA): a multicentre, phase 3, randomised controlled trial. *Lancet Diabetes Endocrinol*. 2018;6:382-391.
49. Kur J, Burian MA, Newman EA. Light adaptation does not prevent early retinal abnormalities in diabetic rats. *Sci Rep*. 2016;6:21075.