Partial Rescue of Retinal Function in Chronically Hypoglycemic Mice

Yumiko Umino,^{1,2} Nicolas Cuenca,^{2,3} Drew Everhart,^{1,2} Laura Fernandez-Sanchez,³ Robert B. Barlow,^{1,4} and Eduardo Solessio¹

PURPOSE. Mice rendered hypoglycemic by a null mutation in the glucagon receptor gene *Gcgr* display late-onset retinal degeneration and loss of retinal sensitivity. Acute hyperglycemia induced by dextrose ingestion does not restore their retinal function, which is consistent with irreversible loss of vision. The goal of this study was to establish whether long-term administration of high dietary glucose rescues retinal function and circuit connectivity in aged *Gcgr*^{-/-} mice.

METHODS. *Gcgr*^{-/-} mice were administered a carbohydrate-rich diet starting at 12 months of age. After 1 month of treatment, retinal function and structure were evaluated using electroretinographic (ERG) recordings and immunohistochemistry.

RESULTS. Treatment with a carbohydrate-rich diet raised blood glucose levels and improved retinal function in *Gcgr*^{-/-} mice. Blood glucose increased from moderate hypoglycemia to euglycemic levels, whereas ERG b-wave sensitivity improved approximately 10-fold. Because the b-wave reflects the electrical activity of second-order cells, we examined for changes in rod-to-bipolar cell synapses. *Gcgr*^{-/-} retinas have 20% fewer synaptic pairings than *Gcgr*^{+/+} retinas. Remarkably, most of the lost synapses were located farthest from the bipolar cell body, near the distal boundary of the outer plexiform layer (OPL), suggesting that apical synapses are most vulnerable to chronic hypoglycemia. Although treatment with the carbohydrate-rich diet restored retinal function, it did not restore these synaptic contacts.

CONCLUSIONS. Prolonged exposure to diet-induced euglycemia improves retinal function but does not reestablish synaptic contacts lost by chronic hypoglycemia. These results suggest that retinal neurons have a homeostatic mechanism that integrates energetic status over prolonged periods of time and allows them to recover functionality despite synaptic loss.

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The retina is among the most metabolically active tissues in the body, requiring a constant supply of blood glucose to sustain glycolysis, oxidative metabolism, and retinal function.^{1–6} The sensitivity of the retina to glucose is underscored by the observations that acute hypoglycemia decreases rod⁷ and cone vision,^{8–10} blurs central vision, and produces temporary central scotomas.^{10,11} Dietary hyperglycemia can rapidly counteract these effects of acute hypoglycemia and restore retinal function.¹²

The consequence of sustained hypoglycemia on retinal function is less clear. Hypoglycemia is a common condition caused by poor nutrition,¹³ inborn errors of metabolism,¹⁴ and pancreatic tumors,¹⁵ and it is a frequent complication of diabetes medications.¹⁶ Our goal in this study was to understand the effects of sustained hypoglycemia on retinal and visual function. To this purpose we examined mice rendered chronically hypoglycemic by a null mutation in the glucagon receptor gene, *Gcgr*.¹⁷ *Gcgr*^{-/-} mice are moderately hypoglycemic and experience late-onset retinal degeneration with loss of vision.¹⁸ Their retinal function (as assessed by the electroretinographic [ERG] b-wave) begins to decline at 9 months of age and is severely limited by 12 to 14 months.¹⁸ Unlike the effects caused by acute hypoglycemia, the negative consequences of chronic hypoglycemia in the *Gcgr* mice model cannot be rescued by acute hyperglycemia, indicating that the loss of retinal function is not simply the result of decreased glucose availability. Lack of recovery after acute glucose ingestion suggests that chronic hypoglycemia causes irreversible neurodegeneration in the *Gcgr*^{-/-} retina. *Gcgr*^{-/-} retinas show modest histologic defects (7% loss of photoreceptors¹⁸ and thinning of the plexiform layers) compared with their 100-fold loss of retinal sensitivity. We therefore hypothesized that chronic hypoglycemia causes retinal cells to enter a “dormant,” possibly neuroprotective, state. If such a dormant state exists, then retinal neurons and retinal function might be partially rescued with sustained normal levels of glucose. If, on the other hand, irreversible neurodegeneration has occurred as a consequence of chronic hypoglycemia, no functional rescue would be observed after the recovery of normal blood glucose levels.

To test whether retinal function can be recovered after chronic hypoglycemia, we treated aged *Gcgr*^{-/-} mice with a carbohydrate-rich diet to raise their blood glucose levels and then assessed their retinal function. Surprisingly, we found that treatment with a carbohydrate-rich diet for 1 month induced euglycemia in *Gcgr*^{-/-} mice and partially rescued their retinal function as measured by ERG b-wave responses. We also performed a detailed histologic analysis of the synaptic connections between photoreceptors and bipolar cells. We found that chronic hypoglycemia caused a 20% reduction in the number of rod-to-bipolar cell synapses, with synapses in the distal

From the ¹Center for Vision Research and SUNY Eye Institute, Department of Ophthalmology, SUNY Upstate Medical University, Syracuse, New York; and the ²Department of Physiology, Genetics and Microbiology, University of Alicante, Alicante, Spain.

³These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Corresponding author: Eduardo Solessio, SUNY Upstate Medical University, 3258 Weiskotten Hall, 750 E. Adams Street, Syracuse, NY 13210; solessie@upstate.edu.

dendritic arbor of bipolar cells particularly vulnerable to hypoglycemic conditions. However, these synapses did not recover after treatment despite the recovery of the b-wave. This suggests that persistent synaptic loss is a consequence of chronic hypoglycemia and that retinal neurons may have a homeostatic mechanism that allows them to recover normal function despite synaptic loss. However, activation of this homeostatic mechanism depends on the sustained recovery of normal glucose levels.

MATERIALS AND METHODS

Animals

Mice with a null mutation of the glucagon receptor ($Gcgr^{-/-}$) were generated as described.¹⁷ We studied 12- to 13-month-old $Gcgr^{-/-}$ male and female mice and their littermate controls ($Gcgr^{+/+}$).¹⁸ All animals were bred and maintained at SUNY Upstate Medical University (Syracuse, NY). Mice were fed ad libitum a standard diet (58% carbohydrate content by weight; Formulab Diet #5008; Purina, St. Louis, MO) or, alternatively, a carbohydrate-rich diet (70% carbohydrate content; High Carbohydrate Purified Diet #49918; Purina) and were maintained on a 14-hour light/10-hour dark cycle. Blood glucose levels were measured from the tail vein with a glucose meter (One Touch Ultra; LifeScan, Milpitas, CA). All procedures were approved by the SUNY Upstate Medical University Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington, DC, 1996) and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Electroretinograms

Electroretinograms (ERGs) were recorded as detailed previously.¹⁸ In brief, dark-adapted mice were placed in a light-proof cage and anesthetized with 60 mg/kg pentobarbital (Nembutal; Lundbeck Inc., Deerfield, IL); pupils were dilated with tropicamide, corneas were kept moist with 0.3% glycerine/1.0% propylene glycol, and body temperatures were maintained at 37°C with a heating pad. ERGs were recorded with a Burian-Allen electrode (0.3–300 Hz; Hansen Ophthalmic Development Laboratory, Coralville, IA) in response to 10-ms LED flashes (520 nm) that delivered $55 \text{ cd} \cdot \text{s}/\text{m}^2$ ($0.9 \times 10^5 \text{ photons}/\mu\text{m}^2$) at the surface of the cornea at $\log I = 0$. The b-wave was measured from the a-wave trough to the peak of the corneal positive wave. We plotted the amplitudes of the b-waves as a function of light intensity and fit with a Hill function. Intensities required to evoke threshold 50- μV ERG b-waves were determined. Sensitivity was defined as the inverse of the threshold intensity.

Visuomotor Response

We measured the optomotor reflex behavior of mice in response to a vertically oriented sinusoidal pattern (100% contrast) rotating on a computer-controlled display,¹⁹ as described previously.¹⁸ During a trial, the stimulus rotated for 5-second periods at a speed of 12°/s under photopic luminance levels (70 cd/m^2). The task of the observer was to determine the direction of grating rotation based on animal behavior (two-alternative, forced-choice protocol). Spatial frequency of the grating was systematically varied using a staircase paradigm until thresholds (producing 70% correct responses) were determined.

Immunohistochemistry

Anesthetized animals were perfused by intracardiac injection with saline and then 4% paraformaldehyde in phosphate buffer. Eyes were enucleated and postfixed in 4% paraformaldehyde for 2 hours at 4°C. After removal of the cornea and lens, the tissue was cryoprotected sequentially in 15%, 20%, and 30% sucrose overnight. Next, the tissue was embedded in OCT compound and sectioned with a cryostat at 16- μm thickness. Sequential vertical sections cut through the center of

the eye were incubated in 10% normal goat or donkey serum for 1 hour (Jackson ImmunoResearch, West Grove, PA) to avoid nonspecific staining and were immunostained overnight at room temperature with primary antibodies diluted in phosphate-buffered saline (PBS) containing 0.5% Triton X-100. Subsequently, the sections were washed in PBS and exposed to the secondary antibodies. Sections were finally washed in PBS, mounted, and coverslipped for viewing by laser confocal microscopy (LSM510; Zeiss, Thornwood, NY). Immunohistochemical controls were performed by omission of either the primary or the secondary antibodies. Nuclear stain (TO-PRO-3 Iodide; Molecular Probes, Eugene, OR) diluted 1:1000 was used to label cell nuclei. Primary antibodies and their corresponding dilutions used for this study were mouse anti-bassoon 1:100 (Stressgen/Enzo, Plymouth Meeting, PA), rabbit anti-PKC α 1:300 and mouse anti-PKC α 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-recoverin 1:500 (Chemicon International, Temecula, CA).

Quantification of the numbers and distribution of rod-to-bipolar cell synapses were performed using a double-blind approach to minimize unintended bias in the measurements. Immunolabeled sections of retina were assigned an arbitrary identification number and handed to a naive observer for inspection and quantification. Information pertaining to mouse identity (genotype and treatment) was revealed only after tabulation of the measurements.

Statistical Analysis

For all experiments involving both $Gcgr^{-/-}$ and $Gcgr^{+/+}$ mice, one-way analysis of variance (the single factor was treatment with a carbohydrate-rich diet) with Holm-Sidak's procedure for pairwise multiple comparisons were performed to test the hypotheses that measurements obtained from $Gcgr^{-/-}$ mice were not different from those of $Gcgr^{+/+}$ mice, $Gcgr^{-/-}$ mice were not different from diet-treated $Gcgr^{-/-}$ mice, and diet-treated $Gcgr^{-/-}$ mice were not different from $Gcgr^{+/+}$ mice. Data analysis was performed with statistical software (SigmaStat; Systat Software, San Jose, CA).

RESULTS

Diet-Induced Elevation of Blood Glucose Levels and Rescue of Retinal Function

We first determined whether treatment with a high carbohydrate diet (see Materials and Methods for description of diet) could induce euglycemia in 12-month-old $Gcgr^{-/-}$ mice (Fig. 1A). Before treatment, $Gcgr^{-/-}$ mice were moderately hypoglycemic, and their blood glucose levels averaged $75 \pm 5 \text{ mg}/\text{dL}$. After 1 month of treatment with a carbohydrate-rich diet, blood glucose levels of $Gcgr^{-/-}$ mice rose significantly ($107 \pm 5 \text{ mg}/\text{dL}$), matching those of littermate $Gcgr^{+/+}$ control mice ($119 \pm 5 \text{ mg}/\text{dL}$). Prolonging the treatment period from 1 month to 2 months did not elevate their blood glucose levels further ($n = 4$ mice), indicating that the glucose-raising benefits of ingesting a carbohydrate-rich diet are acquired within 1 month after starting treatment.

Next we analyzed the effects of diet on retinal sensitivity using ERG recordings. Comparison of ERG responses from $Gcgr^{-/-}$ mice recorded before and after treatment shows that diet significantly improved the overall responses of the retina to brief flash presentations (Fig. 1B). Both b- and a-wave amplitudes increased significantly. To quantify the effectiveness of the treatment, we measured two indicators of retinal health: sensitivity of the ERG b-wave and its maximal response amplitude. Sensitivity reflects the ability of the retina to respond to light (arbitrarily defined as the inverse of the flash intensity necessary to elicit a 50- μV response), whereas the maximal amplitude of the b-wave is a measure of the electrical activity generated by second-order cells.²⁰ We found that on average, dietary treatment increased b-wave sensitivity by 10-fold (Fig. 1C) and doubled the size of its maximal amplitude (Fig. 1D).

FIGURE 1. Diet-induced euglycemia can rescue retinal function in hypoglycemic Gcgr^{-/-} mice. **(A)** Blood glucose levels of Gcgr^{-/-} mice before and after treatment with a carbohydrate-rich diet (Gcgr^{-/-} + diet) compared with results from sibling Gcgr^{+/+} mice. **(B)** ERGs recorded from a 12-month-old Gcgr^{-/-} mouse before (black traces) and after (red traces) treatment with a carbohydrate-rich diet. **(C, D)** ERG b-wave sensitivity and maximal amplitude, respectively. Data are mean $\pm$ SEM ($n = 10$ –15 mice per group). * $P < 0.05$, one-way ANOVA. **(E)** Diet-induced change in ERG b-wave sensitivity plotted as a function of sensitivity before treatment. The correlation of the variables is significant ($R^2 = 0.5$; $P = 0.02$). The regression line is shown in the graph. **(F)** Correlation between diet-induced change in maximal b-wave amplitude and maximal b-wave amplitude before treatment ($R^2 = 0.8$; $P < 0.001$).

However, the amount of rescue depended on the severity of the condition before diet treatment. Mice with poor initial sensitivities or small ERG amplitudes experienced the greatest benefit, exhibiting the largest improvements in their responses to light (Figs. 1E, 1F). Thus, treatment with a carbohydrate-rich diet increased blood glucose levels and partially rescued retinal function, as determined by the ERG. These results are surprising because acute hyperglycemia by dextrose ingestion does not improve retinal function in Gcgr^{-/-} mice.¹⁸ Together, these results suggest that rescue of function requires sustained exposure to euglycemic conditions.

We have previously demonstrated that retinal sensitivities of Gcgr^{-/-} and Gcgr^{+/+} mice increase in a near-linear relationship with blood glucose levels.¹⁸ Figure 2A shows that the sensitivity of diet-treated Gcgr^{-/-} mice follows a similar relationship. The sensitivities of Gcgr^{-/-} mice measured before treatment were scattered across the bottom-left quadrant of the graph, consistent with low sensitivity and low blood glucose (severely hypoglycemic animals with glucose levels <50 mg/dL were not included in the analysis). The sensitivities of control Gcgr^{+/+} mice populate the upper-right quarter of the graph, which is consistent with high sensitivity and high blood glucose levels. In contrast, the sensitivities of diet-treated Gcgr^{-/-} mice cluster about the center of the graph, matching the sensitivities of mildly hypoglycemic Gcgr^{+/+} mice (range, 100–115 mg/dL), proving not only that Gcgr^{-/-} mice can regain retinal function but that their sensitivity is as good as that of control mice with the same glucose levels. More important, the plot

shows that retinal sensitivity is strictly a function of blood glucose levels, where genotype and treatment dictate the range of operation. To quantify this relationship, we pooled all the data in the plot and applied linear regression analysis. The resultant regression coefficient reveals that sensitivity increases 0.025 log units per mg/dL increment of blood glucose ($R^2 = 0.3$; $P = 0.002$). A parallel analysis shows that the maximal response of the ERG (Fig. 2B) is also strongly dependent on blood glucose levels (regression coefficient of 6.5 μ V per mg/dL increment of blood glucose levels, with a coefficient of determination $R^2 = 0.42$, $P < 0.001$). We conclude that b-wave sensitivity and maximal amplitude in Gcgr^{-/-} mice improve dramatically after extended exposure to near euglycemic blood glucose levels.

It is intriguing that the b-wave sensitivity of diet-treated Gcgr^{-/-} mice recovers almost completely, whereas the maximal b-wave amplitude exhibits only a partial recovery (Figs. 1C, 1D). The explanation for this discrepancy may lie in the hyperbolic relationship that exists between b-wave sensitivity and maximal amplitude. As shown in Figure 2C, sensitivity falls precipitously when maximal amplitude is <300 μ V but remains relatively constant (given the variability of the measurements) when maximal amplitude is >300 μ V. Maximal amplitudes of treated Gcgr^{-/-} mice were in the order of 300 to 500 μ V, whereas the respective responses in Gcgr^{+/+} control mice reached 400 to 700 μ V. Thus, although the ERG amplitudes of treated Gcgr^{-/-} mice were subnormal, they clustered above the 300 μ V mark to yield approximately normal b-wave sensitivities. The basis for the incomplete recovery in ERG ampli-

tude after treatment is unclear but may be explained, at least in part, by an irreversible loss of rod-to-bipolar cell synapses as we describe here.

FIGURE 2. Effect of blood glucose levels on ERG b-wave sensitivity and maximal amplitude. (A) Relationship of b-wave sensitivity and blood glucose levels in Gcgr^{-/-} mice (circles), Gcgr^{-/-} mice fed the carbohydrate-rich diet (triangles), and Gcgr^{+/-} mice (squares). The regression line is $\log \text{Sensitivity} = -0.93 + (0.025 \times \text{Blood glucose})$. (B) Maximal amplitude of the ERG b-wave as a function of blood glucose. The regression line is $\text{Maximal amplitude} = -254.3 + (6.5 \times \text{Blood glucose})$. (C) The hyperbolic relationship between Sensitivity and maximal amplitude ($A_{\max}$) is well described ($R^2 = 0.8$) by an expression derived from the Hill equation:

Diet-Induced Rescue of the Visuomotor Response in Gcgr^{-/-} Mice

We have previously shown that the visuomotor response of Gcgr^{-/-} mice is significantly impaired at 12 months of age.¹⁸ Thus, we investigated whether treatment with a carbohydrate-rich diet can improve their visual performance. We found that the spatial resolution of the visuomotor response of Gcgr^{-/-} mice improved significantly after 1 month of treatment with the carbohydrate-rich diet (0.43 ± 0.01 vs. 0.51 ± 0.01 cyc/deg; $n = 13$), matching the resolution of their Gcgr^{+/-} littermates used for control (0.52 ± 0.01 cyc/deg). We concluded that treatment with the carbohydrate-rich diet improves both retinal and visual responses in Gcgr^{-/-} mice.

Age-Related Thinning of the Outer Plexiform Layer in Chronically Hypoglycemic Mice

To determine whether structure and function changed in parallel, we examined the morphology of the retinal layers in Gcgr^{-/-} and Gcgr^{+/-} mice. Figures 3A and 3B show vertical retinal sections of 12- to 13-month-old mice labeled with antibodies specific for recoverin, a marker for photoreceptors, and PKC- α , a marker for rod bipolar cells.²¹⁻²⁴ The side-by-side comparison suggests that the major retinal layers in Gcgr^{-/-} mice are thinner than in Gcgr^{+/-} mice. Systematic measurement of the various layers in sections labeled with the nuclear stain (TO-PRO-3 Iodide; Molecular Probes) (Figs. 3C, 3D) reveals significant reductions in the widths of the outer (21%) and inner (12%) nuclear layers and of the outer plexiform layers (OPLs) (38%) of Gcgr^{-/-} mice (Fig. 3E). The thickness of the inner plexiform layer did not change significantly. The reduction in the width of the outer nuclear layer was probably due to fewer photoreceptor cells.¹⁸ On the other hand, thinning of the OPL was consistent with the disruption and loss of connections between photoreceptor cells and second-order cells (bipolar and horizontal cells). Thus, we proceeded to carefully examine the numbers and distributions of these synapses in Gcgr^{-/-} and Gcgr^{+/-} mice.

Loss of Rod-to-Bipolar Cell Synaptic Contacts

We visualized rod-to-bipolar cell synapses using immunolabeling. Photoreceptor terminals were identified by labeling presynaptic ribbons with antibodies against bassoon. Staining of vertical sections showed two different immunoreactive structures in the OPL: a punctate staining with horseshoe morphology associated with the synaptic ribbons of rod spherules and a dislike presynaptic structure associated with cone pedicles^{25,26} (Fig. 4A, arrowheads). Rod bipolar cells were identified by their robust, specific immunoreactive labeling with PKC- α antibodies. Pairing of PKC- α (green)- and bassoon (red)-labeled processes indicates the site of a presumed rod-to-bipolar cell synapse (Fig. 4B, enlarged OPL region). Using this approach we detected clear differences in the distribution of synapses in the retinas of Gcgr^{+/-} and Gcgr^{-/-} mice at 12 to 13 months of age and in

$$\text{Sensitivity} = \left(\frac{A_{\max} - Y_T}{Y_T I_k^n} \right)^{1/n} \quad (1)$$

Here, $Y_T = 50 \mu\text{V}$ is the threshold response, $n = 0.5$ is the Hill coefficient, and $I_k = 0.54 \text{ cd} \cdot \text{s/m}^2$ is the intensity required to elicit half-maximal response. Parameter values adopted are from the study by Umino et al.¹⁸

FIGURE 3. Thinning of major retinal layers in $Gcgr^{-/-}$ mice. Retinas of 12- to 13-month-old (A) euglycemic $Gcgr^{+/-}$ and (B) hypoglycemic $Gcgr^{-/-}$ mice. Double labeling of specific cell classes using antibodies directed against recoverin (red) in photoreceptors and PKC- α in rod bipolar cells (green). (C, D) Nuclear stain highlights thinning of the OPL in $Gcgr^{-/-}$ compared with $Gcgr^{+/-}$. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 20 μ m (A, B); 40 μ m (C, D). (E) Thickness of retinal layers in $Gcgr^{-/-}$ and $Gcgr^{+/-}$ mice. Mean $\pm$ SEM ($n = 7$ mice per group). * $P < 0.05$ and ** $P < 0.001$, Student's t -test.

the retinas of $Gcgr^{-/-}$ mice receiving the carbohydrate-rich diet (Figs. 4A, 4C, 4E).

The retinas of $Gcgr^{-/-}$ mice had fewer synaptic contacts than the retinas of age-matched $Gcgr^{+/-}$ littermates used for control. Rod bipolar cells in $Gcgr^{+/-}$ retinas extended a primary dendrite into the OPL that branched profusely into fine dendritic processes that connected with rod spherules (Figs. 4A, 4B). Their synaptic contacts, usually present at the tip of the dendrites, were arranged in multiple rows along the distal

boundary of the OPL. In contrast, the OPLs of $Gcgr^{-/-}$ retinas were slightly disorganized. Rod bipolar cells extended into the OPL, but their dendritic arbors were thin and made few synaptic contacts (Figs. 4C, 4D). The lengths of the dendritic processes were generally shorter, and the scant synaptic contacts were arranged in as few as two or three rows. To quantitatively assess the structural differences between $Gcgr^{-/-}$ and $Gcgr^{+/-}$ retinas, we counted the number of synapses in each image. We found that on average the synaptic density

FIGURE 4. Synapses between rod photoreceptors and rod bipolar cells decrease in aged hypoglycemic $Gcgr^{-/-}$ mice. Synaptic contacts between rod photoreceptors and rod bipolar cells identified using antibodies against bassoon (red) and PKC- α (green) in retinas of $Gcgr^{+/-}$ (A), $Gcgr^{-/-}$ (C), and diet-treated $Gcgr^{-/-}$ (E) mice. Scale bars, 10 μm . B, D, and F are the enlarged regions indicated in A, C, and E, respectively. Arrowheads: disc-like structure characteristic of cone terminals. Nuclei stained with a nuclear marker (TO-PRO-3 Iodide, blue). Scale bar, 10 μm . (G) Density of rod-to-rod bipolar cell synapses in diet-treated and untreated $Gcgr^{-/-}$ mice and control $Gcgr^{+/-}$ mice. (H) Density of PKC-labeled rod bipolar cells. All measurements were taken from vertical sections of central retina (within 0.3 mm from the optic nerve head) to minimize potential biases arising from heterogeneities associated with retinal eccentricity. Synaptic density (or number of bipolar cells) for each animal was estimated as the average of 10 to 16 randomly chosen, nonoverlapping images, each spanning 100 μm in the longitudinal direction. Data represent mean $\pm$ SEM; $n = 3$ –6 mice per group. * $P < 0.05$, one-way ANOVA.

(defined as the number of synapses divided by the longitudinal distance) was approximately 20% lower in $Gcgr^{-/-}$ retinas than in $Gcgr^{+/-}$ retinas (Fig. 4G). Moreover, the number of rod bipolar cells was essentially the same in $Gcgr^{-/-}$ and $Gcgr^{+/-}$ retinas (Fig. 4H), consistent with the notion that chronic hypoglycemia leads to a reduction in the number of synaptic contacts made by each bipolar cell. Lost synapses (20%) outnumbered lost photoreceptors ($\sim 7\%$),¹⁸ suggesting that we could not detect a ribbon in approximately 13% of the photoreceptors in the $Gcgr^{-/-}$ retina. Treatment with the carbohydrate-rich diet did not reestablish these synaptic contacts. Organization of the OPL (Figs. 4E, 4F) and the numbers of synaptic contacts and bipolar cells (Figs. 4G, 4H) in treated mice appeared similar to those of untreated $Gcgr^{-/-}$ mice. Thus, rescue of retinal function was not accompanied by a corresponding rescue of retinal structure.

Is a thin OPL in aged hypoglycemic mice a reflection of lower synaptic density or a consequence of the retraction of bipolar cell processes? To distinguish between these alternatives, we measured the position of rod to rod bipolar cell synapses relative to the proximal border of the OPL (defined by the distal boundaries of the nuclei of the bipolar cells) (Fig. 5A). Next we computed histograms of the distance to each synapse (see Fig. 5 legend for details) for both $Gcgr^{-/-}$ and $Gcgr^{+/-}$ retinas. The corresponding histograms approximated

normal distributions, with a slight skew toward the distal (positive) direction (Fig. 5B). Direct comparison of the histograms indicated that, on average, there were fewer synapses in $Gcgr^{-/-}$ than in $Gcgr^{+/-}$ retinas at distances exceeding 12 μm from the proximal boundary of the OPL. On the other hand, $Gcgr^{-/-}$ retinas have more synapses than $Gcgr^{+/-}$ retinas at distances $< 12 \mu m$. Further analysis of the histograms provides important insights into the basis of the morphologic changes that occur in the OPL. First, the peak density of the histogram is approximately the same for both genotypes, thereby ruling out the possibility that synapses are lost uniformly across the OPL, as is illustrated in Figure 5C. Second, the position of the distribution peak has shifted toward the proximal border of the OPL by 3 or 4 μm , which is consistent with a slight retraction of the primary trunk of rod bipolar cells (Fig. 5D). In addition, the synapse distribution is approximately 20% to 25% tighter in $Gcgr^{-/-}$ than in $Gcgr^{+/-}$ retinas, which suggests a selective loss of synapses originally populating the most distal layers ($> 12 \mu m$) of the OPL (Fig. 5E). In sum, we conclude that rod bipolar cells in $Gcgr^{-/-}$ mice experience a slight retraction of their primary trunk (Fig. 5D) along with a selective loss of their apical synapses (Fig. 5E). Treatment with a carbohydrate-rich diet had no influence on the shape of the histogram, suggesting that the redistribution of rod-to-bipolar cell synapses caused by

FIGURE 5. Loss of distal synapses with shortening of the primary dendrite in rod bipolar cells. (A) Analysis of OPL thickness and density of synapses in $Gcgr^{+/-}$ and $Gcgr^{-/-}$ mice. We quantified the distribution of photoreceptor-bipolar synapses as follows: using antibodies to bassoon and PKC- α , we visualized synapses between photoreceptor terminals and bipolar cells (red dots) in 16- μm -thick sections imaged with a confocal microscope. Next we defined the proximal border of the OPL as the distal boundaries of bipolar cell nuclei across the width of the section (horizontal lines). Using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>), we measured the distance, d_n , of each bassoon/PKC- α punctum from the proximal border of the OPL and built a histogram of the number of synapses that occurred at each distance ($d_1 \dots d_n$). (B) Average histograms showing the distribution of synapses as a function of distance across the OPL in central retina for $Gcgr^{-/-}$, $Gcgr^{+/-}$, and diet-treated $Gcgr^{-/-}$ mice. Data represent the mean $\pm$ SEM; $n = 3$ –6 mice per group. (C–E) Alternative models (dashed lines) that may explain the changes observed in the histograms. ONL, outer nuclear layer; OPL, outer plexiform; INL, inner nuclear layer. Scale bar, 10 μm .

long-term metabolic stress is not reversed after 1 month of carbohydrate-rich diet treatment.

DISCUSSION

In this study we combined ERG recordings with measurements of visuomotor sensitivity and immunohistochemistry to determine how treatment with a carbohydrate-rich diet affects retinal function and structure in a mouse model of chronic hypoglycemia. We found that treatment with a carbohydrate-rich diet raised blood glucose levels and improved some aspects of retinal function, as measured with the ERG, but that it did not rescue synaptic connections in the OPL within the 1-month duration of our study. These results have important implications for our understanding of the connection between metabolism and retinal function and structure.

$Gcgr^{-/-}$ mice exhibit late-onset retinal degeneration and loss of retinal function.¹⁸ The extent of cell death is modest compared with the substantial losses in retinal function. In this study, we show that $Gcgr^{-/-}$ mice experience a disruption of the synaptic contacts between rods and second-order cells that parallel the early signs of reorganization observed in other forms of retinal degeneration. Such changes in connectivity,

also known as remodeling, are a common event in degenerations caused by defects in the sensory retina and is generally associated with photoreceptor death (see reviews by Marc et al.²⁷ and Jones and Marc²⁸). Cone death,^{29,30} rod death,^{22,24,31–35} and lack of rods during development³⁶ are associated with cellular remodeling and ectopic synaptogenesis. Cellular remodeling can also result from aging,^{37,38} light damage,³⁹ and detachment and reattachment of the RPE.⁴⁰

Maintenance of established synapses in the central nervous system depends both on the presence of neurotrophic factors and the level of synaptic activity.⁴¹ Our results suggest that in $Gcgr^{-/-}$ retinas, synapses located at the dendritic tips and farthest from the bipolar cell body are particularly vulnerable to metabolic stress. Although the cause of selective loss (as opposed to uniform loss) of synapses is unclear, it is possible that a hierarchy or spatial gradient exists, whereby synapses located in the periphery receive fewer metabolic resources than those positioned closer to the perinuclear housekeeping machinery. By this scenario, apical synapses may be at increased risk during the chronic hypoglycemia and metabolic deprivation characteristic of $Gcgr^{-/-}$ mice. Synaptic loss has also been linked to chronic stress^{42,43} and related pathologic conditions such as Cushing's disease.⁴⁴ Because $Gcgr^{-/-}$ mice

have altered stress responses,¹⁷ we cannot rule out that stress consequent to chronic hypoglycemia is detrimental to the long-term stability of apical rod-to-bipolar cell synapses.

The physical loss of synapses cannot account for the loss of ERG sensitivity in *Gcgr*^{-/-} mice. Indeed, the 20% reduction in the number of rod-to-bipolar cell contacts that we measured is probably insufficient to explain the substantial (~10- to 100-fold) loss in ERG b-wave sensitivity (assuming that every synapse contributes equally to the generation of the postsynaptic signal in rod bipolar cells⁴⁵). Further support for this notion follows from the observation that treatment with a carbohydrate-rich diet promotes a strong recovery of the ERGs without a corresponding recovery in the number of synaptic contacts. On the other hand, the permanent 20% reduction in the number of synapses between rods and bipolar cells may explain, to a good extent, the failure of the ERG b-wave to recover completely, which, as shown in Figures 1 and 2, is approximately 30% below normal.

A major result of this study was the demonstration that long-term treatment with a carbohydrate-rich diet can rescue retinal sensitivity in *Gcgr*^{-/-} mice. Because glucose is essential for retinal function,^{46–48} the expectation is that acute restoration of the amount of available glucose in *Gcgr*^{-/-} mice should lead to an immediate reestablishment of retinal function. However, when we previously tested this hypothesis, we found that acute increments in blood glucose levels by ingestion of dextrose did not improve retinal function in these mice, suggesting that metabolically challenged retinal cells lose their function irreversibly.¹⁸ In this study, we administered a carbohydrate-rich diet to elevate blood glucose levels for an extended period. Blood glucose levels recovered almost completely after 1 month of treatment. Surprisingly, the treatment also rescued retinal function. The sluggish reversal of the loss of retinal function suggests that retinal cells can sense their energy status, perhaps by way of the AMP-kinase, a regulator of metabolic energy balance,^{49–51} and can adjust the allocation of energy resources between metabolically demanding tasks involved in signaling the absorption of photons^{47,48,52,53} and processes that promote cell survival during metabolic stress conditions.

In summary, short-term reductions in glucose availability cause dramatic yet readily reversible alterations in retinal and visual function.^{4,10,11} However, chronic hypoglycemia also leads to loss of neurons¹⁸ and synaptic remodeling. In mice, the improvement of retinal function is possible after 1 month of treatment with a carbohydrate-rich diet; however, the same treatment did not rescue synaptic connectivity. Future studies should aim at determining end points for the recovery of retinal function.

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