ON-Type Retinal Ganglion Cells are Preferentially Affected in STZ-Induced Diabetic Mice

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PURPOSE. We investigate morphologic and physiologic alterations of ganglion cells (GCs) in a streptozocin (STZ)-induced diabetic mouse model.

METHODS. Experiments were conducted in flat-mount retinas of mice 3 months after the induction of diabetes. Changes in morphology of four subtypes of GCs (ON-type RGA2 [ON-RGA2], OFF-type RGA2 [OFF-RGA2], ON-type RGC1 [ON-RGC1], and ON-OFF type RGD2 [ON-OFF RGD2]) were characterized in Thy1-YFP transgenic mice. Using whole-cell patch-clamp recording, passive membrane properties and action potential (AP) firing properties were further investigated in transient ON- and OFF-RGA2 cells.

RESULTS. Morphologic parameters were significantly altered in the dendrites branching in the ON sublamina of the inner plexiform layer (IPL) for ON-RGA2 cells and ON-OFF RGD2 cells. MUCH less significant changes, if any, were seen in those arborizing in the OFF sublamina of the IPL for OFF-RGA2 and ON-OFF RGD2 cells. No detectable changes in morphology were seen in RGC1 cells. Electrophysiologically, increased resting membrane potentials and decreased membrane capacitance were found in transient ON-RGA2 cells, but not in transient OFF-RGA2 cells. Similar alterations in AP firing properties, such as an increase in AP width and reduction in maximum spiking rate, were shared by these two subtypes. Furthermore, in response to depolarizing current injections, both cells generated more APs suggesting an enhanced excitability of these cells in diabetic conditions.

CONCLUSIONS. These differential changes in morphology and electrophysiology in subtypes of GCs may be responsible for reduced contrast sensitivity known to occur during the early stage of diabetic retinopathy.

Keywords: diabetic retinopathy, ganglion cells, dendrites, intrinsic membrane property, K+ currents

Diabetic retinopathy (DR), the most common complication of diabetes, is a leading cause of blindness in adults.1–3 Although DR has long been recognized as solely a vascular disease,4–6 increasing evidence shows that this complication also may be considered a neurodegenerative disorder, since damage to retinal neurons and visual function deficits, such as reduced contrast sensitivity, could be observed before the onset of vascular symptoms.4,5,7–22

It is known that retinal ganglion cells (GCs), output neurons in the retina, are some of the severely damaged retinal neurons in diabetes.3 Loss of GCs has been reported in diabetic patients and animal models of diabetes.5,7–17,19–21,23–25 Consistent with this, the amplitudes of the pattern electroretinogram (ERG) and the scotopic threshold response (STR), which are likely related to GC and amacrine cell activities,56–28 were reduced in such circumstances.20,29–32 However, there are little data concerning changes in morphologic and electrophysiologic properties of GCs at individual cell levels.33–36 Moreover, GCs could be categorized into several subgroups according to morphologic and physiologic criteria. Morphologically, GCs could be classified into RGA, RGB, RGC, and RGD subgroups based on their soma sizes and dendritic characteristics using the nomenclature of He’s group.37 Based on their light response properties, GCs are functionally classified into ON, OFF, and ON-OFF subtypes.38 It was reported previously in type-I diabetic mice that the spontaneous spiking activity of ON-GCs, but not OFF-GCs was significantly increased compared to that in normal retinas.56 However, whether morphology and physiology of these GC subtypes could be affected differentially in diabetes remains an open question.

We investigated how four subtypes of GCs (ON-RGA2, OFF-RGA2, ON-RGC1, and ON-OFF RGD2) labeled by yellow fluorescent protein (YFP)39 were changed in morphologic features at the early stage of hyperglycemia in a streptozocin (STZ)-induced diabetic Thy1-YFP-H mouse (maintained under the C57BL/6 background) model of human type-I-like diabetes. Using whole-cell patch clamp recording techniques, we further explored changes in passive and active membrane properties of ON- and OFF-RGA2 cells in this mouse model. Our data clearly shows that GCs/GC dendrites mediating ON- and OFF-signaling are affected differentially in early experimental diabetes.
Changes of Retinal Ganglion Cells in Diabetic Mice

MATERIALS AND METHODS

Animals and Induction of Diabetes

All animal protocols were made in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee of Fudan University. All experimental mice were housed in a 12:12-hour light/dark cycle. Thy1-YFP-H transgenic mice (003782), male and female (6–7 weeks old), purchased from the Jackson Laboratory (Bar Harbor, ME, USA)39 and maintained under the C57BL/6J background, were used for morphologic analysis. This mouse line carries a YFP reporter gene under direction of the mouse Thy1 gene promoter (Tg[Thy1-YFP]23Jrs) and Thy1-YFP is expressed throughout the entire neuronal structure in small numbers of all morphologic subtypes of GCs.40 Male C57BL/6J mice, purchased from SLAC Laboratory Animal Company (Shanghai, China) were used for electrophysiologic experiments.

The mice were assigned randomly to diabetic or control groups. Hyperglycemia was induced following the procedures described previously36 with minor modifications. After being fasted for 4 hours, mice in the diabetic group received an intraperitoneal injection of 85 mg/kg STZ (Sigma-Aldrich Corp., St. Louis, MO, USA), freshly dissolved in sodium citrate buffer (1%, pH 4.2), for 3 consecutive days. Control animals received injections of an equal volume of citrate buffer. Blood glucose concentrations were measured using a glucometer (Accu-Chek Advantage, Roche, Germany). Oxygenated bathing solutions at 32°C were continuously perfused into the recording chamber at a flow rate of 1.5 to 2 ml/min with a peristaltic pump.

Immunohistochemistry

Retinas, isolated from Thy1-YFP mice deeply anesthetized, were fixed in 4% paraformaldehyde in PBS and then blocked with 6% donkey serum, 1% bovine serum albumin, and 0.3% Triton X-100 in 0.1 M PBS for 2 hours at room temperature. The retinas were incubated with the following primary antibodies diluted in blocking solution: chicken anti-GFP (1:1000 dilution; Aves Labs, Inc., Tigard, OR, USA) and goat anti-choline acetyltransferase (ChAT; 1:1000 dilution; Millipore, Billerica, MA, USA) for 3 days at 4°C. Immunoreactivity was detected with the following secondary antibodies: Alexa Fluor 488-conjugated donkey anti-chicken IgG (1:200 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for labeling GFP; Alexa Fluor 555-conjugated donkey anti-goat IgG (1:200 dilution; Invitrogen, Carlsbad, CA, USA) for ChAT, which can reveal the boundaries of the inner plexiform layer (IPL) by labeling somata of cholinergic amacrine cells. All retinas were flat-mounted and coverslipped, ganglion cell side up.

Image Capture and Analysis

All GCs were viewed and scanned with an Olympus FV1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) using a ×40 objective lens. Image stacks were collected at intervals of 1 μm from the optical nerve layer to the inner part of the INL, with a high resolution of 1024×1024 pixels.

GCs were classified using the nomenclature of He’s group.57 Complete soma-dendritic profile reconstruction of individual GCs was obtained by manually tracing using NeuroLucida software (MicroBrightFields, Inc., Colchester, VT, USA), and analyses were performed with NeuroLucida Explorer software (MicroBrightFields, Inc.). The following morphologic parameters were measured to evaluate alterations in morphology during the early stage of hyperglycemia: (1) Soma size was calculated by drawing contour lines around each cell body. (2) Dendritic field size was calculated by measuring the area enclosed by the outermost segments of the distal dendritic branches. (3) Total dendritic length was the sum of the length of all dendritic branches of individual GCs. (4) Dendritic branch number was the total number of all the branches of individual GCs. Sholl analysis was used to quantify dendritic complexity as a function of distance from the soma. That is, a set of concentric circles were drawn centering on the soma at 10-μm intervals, and the number of dendrites intersecting each circle was counted.

Electrophysiologic Recordings

Retinas were prepared following the procedures described previously56 with minor modifications. In brief, before each experiment, animals were dark adapted for 2 to 4 hours. All experiments were performed under dim red light or infrared illumination. Retinas were dissected immediately and placed in extracellular solution gassed with 95% O₂ and 5% CO₂. The extracellular solution contained (in mM): 125 NaCl, 2.5 KCl, 1 MgSO₄, 2 CaCl₂, 1.25 NaH₂PO₄, 20 glucose, and 25 NaHCO₃. The flat-mount retina was placed in a recording chamber with ganglion cell side up, which was positioned on the stage of an upright microscope (Axioskop 2 FS Mot; Carl Zeiss, Jena, Germany). Oxygenated bathing solutions at 32°C were continuously perfused into the recording chamber at a flow rate of 1.5 to 2 ml/min with a peristaltic pump.

Cells and pipettes were viewed on a video monitor coupled to a CCD camera (C11440; Hamamatsu, Hamamatsu City, Shizuoka Prefecture, Japan) mounted on the microscope. The pipette was advanced to a RGA2 cell in the ganglion cell layer (see Results for details) using visual control under infrared illumination. Spike activities in response to a 1-second green light stimulus were recorded extracellularly with patch electrodes that were made using a micropipette puller (P-97, Sutter Instrument Co., Novato, CA, USA) and of 4–7 MΩ tip resistance when filled with the extracellular solution in the cell-attach configuration. Whole-cell patch clamp recordings were made by patch electrodes filled with an intracellular solution containing the following (in mM): 120 K-gluconate; 5 NaCl; 5 KCl; 5 HEPES; 1 MgCl₂, 0.1 guanosine 5-triphosphate, and 0.1% Lucifer yellow, adjusted to pH 7.4 with KOH. The pipettes controlled by a motor-driven micromanipulator (MP-285, Sutter) were connected to a patch-clamp amplifier (EPC-9; Heka Elektronik, Lambrecht/Pfalz, Germany). Fast capacitance was fully canceled and cell capacitance was canceled by the circuits of the amplifier as much as possible. Data were acquired at a sampling rate of 20 kHz and then stored for further analysis. To further identify types of the recorded cells, Lucifer yellow in the pipette was dialyzed into neurons to reveal cell morphology. Three-dimensional cell morphology was visualized in flat-mount retinas using Lucifer yellow fluorescence with the Olympus confocal laser scanning microscope.

Light Stimulation

A green-light light-emitting diode (LED; λ = 525 nm) was used to deliver a full-filed light stimulation to the retina through the microscope condenser. The LED was controlled by Pulse software and the light intensity was adjusted by varying the output voltage from the software. The stimulus intensity varied from 3.36 × 10⁸ to 3.36 × 10¹¹ photons/cm²/s.
changes in morphologic features in diabetes. RGA2 cells, which are equivalent to z-GCs, exhibit a large receptive field center, short response latency, and fast conducting axon. RG2 cells are bistratified GCs and they respond transiently to the onset and termination of a light stimulus and show a strong directional selectivity to a moving rectangle. RG1 cells are ON direction-selective cells and respond to a stationary flashing spot with sustained spiking during light onset.

Changes in Morphology of RGA2 Cells in Diabetic Retinas. Changes in morphology were first examined in RGA2 cells. In the normal retina, these cells are characterized by a large soma, large dendritic field area, and four to seven primary dendrites that branch repeatedly proximal to the soma. They could be subdivided into two groups with dendrites stratifying either in the ON (ON-RGA2) or OFF sublamina of the IPL (OFF-RGA2). Figures 2A and B show the stacked confocal fluorescence images of two typical ON-RGA2 cells (green) respectively from an age-matched control mouse and a diabetic one with hyperglycemia for 3 months.
Changes of Retinal Ganglion Cells in Diabetic Mice

Table 1. Measurements of Morphologic Parameters of Various GC Subtypes in Control and Diabetic Retinas (Mean ± SEM)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Group</th>
<th>ON-RGA2</th>
<th>OFF-RGA2</th>
<th>RGC1</th>
<th>ON-Sublamina</th>
<th>OFF-Sublamina</th>
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<tr>
<td>Soma size, μm²</td>
<td>Control</td>
<td>329.90 ± 9.51</td>
<td>337.20 ± 10.50</td>
<td>299.80 ± 10.16</td>
<td>194.87 ± 7.44</td>
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<tr>
<td></td>
<td>Diabetic</td>
<td>335.75 ± 9.75</td>
<td>341.85 ± 12.46</td>
<td>306.45 ± 9.53</td>
<td>195.40 ± 7.17</td>
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<tr>
<td>Total dendritic</td>
<td>Control</td>
<td>4692.81 ± 97.81</td>
<td>4917.15 ± 206.57</td>
<td>3915.80 ± 211.02</td>
<td>1899.96 ± 131.57</td>
<td>2704.60 ± 95.37</td>
</tr>
<tr>
<td>length, μm</td>
<td>Diabetic</td>
<td>4554.04 ± 118.30</td>
<td>4902.03 ± 207.54</td>
<td>3748.85 ± 187.01</td>
<td>1634.37 ± 152.63</td>
<td>2736.41 ± 125.46</td>
</tr>
<tr>
<td>Dendritic filed</td>
<td>Control</td>
<td>95,472.47 ± 3687.06</td>
<td>75,673.01 ± 4476.81</td>
<td>61,810.46 ± 6203.43</td>
<td>30,129.08 ± 2621.89</td>
<td>31,843.85 ± 1141.74</td>
</tr>
<tr>
<td>size, μm²</td>
<td>Diabetic</td>
<td>79,674.37 ± 3631.88†</td>
<td>70,959.84 ± 6570.61</td>
<td>56,599.56 ± 5252.71</td>
<td>20,324.28 ± 1722.44†</td>
<td>30,020.97 ± 1407.17</td>
</tr>
<tr>
<td>Number of</td>
<td>Control</td>
<td>42.92 ± 1.79</td>
<td>48.13 ± 2.56</td>
<td>59.07 ± 3.46</td>
<td>70.76 ± 3.01</td>
<td>99.88 ± 6.58</td>
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<tr>
<td>dendritic branches</td>
<td>Diabetic</td>
<td>50.90 ± 2.25†</td>
<td>62.36 ± 3.50†</td>
<td>58.75 ± 4.03</td>
<td>67.82 ± 6.13</td>
<td>110.91 ± 10.54</td>
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<tr>
<td>Number of cells/mice</td>
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<td>39/9</td>
<td>16/9</td>
<td>15/9</td>
<td>17/8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>30/14</td>
<td>14/7</td>
<td>14/6</td>
<td>15/8</td>
<td></td>
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</table>

* P < 0.05 vs. control, Student’s t-test.
† P < 0.01 vs. control, Student’s t-test.

These images are projections from a series of section scans across the entire dendritic fields. The dendritic stratification patterns of these two cells are shown below as side view images (Figs. 2C, 2D), in which the dendrites occupy the lower half of the ON sublamina of the IPL, somewhat below (vitreal to) the ON ChAT-labeled band (red). Soma areas of ON-RGA2 cells were unchanged (335.75 ± 9.75 μm² [n = 30, from 14 mice] vs. 329.90 ± 9.51 μm² [n = 39, from 9 mice]) in control, P = 0.58; Table 1). Total dendritic lengths of ON-RGA2 cells in diabetic mice also were not significantly different from those obtained in control mice (4554.04 ± 118.30 μm in diabetic vs. 4692.81 ± 97.81 μm in control, P = 0.57; Table 1). The numbers of dendritic intersections were not significantly different from those obtained in control mice (4902.03 ± 207.54 μm² in diabetic vs. 4292.00 ± 97.81 μm² in control, P = 0.91; Table 1). In contrast, two other parameters, dendritic field areas and total numbers of dendritic branches, were changed in diabetic mice. Compared to those obtained in control mice, the dendritic field areas were markedly reduced (79,674.37 ± 3631.88 μm² in diabetic vs. 95,472.47 ± 3678.06 μm² in control; P < 0.01), whereas the total numbers of dendritic branches were considerably increased (50.90 ± 2.25 in diabetic vs. 42.92 ± 1.79 in control, P < 0.01; Table 1). Sholl analysis revealed that in control retinas the numbers of dendritic intersections were increased with the distance from the soma when it was <80 μm, but decreased when it was >90 μm (Fig. 2G), showing a bell-shaped curve. In diabetic retinas, compared to those obtained in control mice, the numbers of dendritic intersections were increased when the distance from the soma was approximately 30 to 80 μm, but decreased when it was approximately >150 μm, thus shifting the whole curve to the left. Such a shift was induced mostly because some long dendrites were shortened, thus resulting in an increased shorter dendrite number.

In diabetic OFF-RGA2 cells, like ON-RGA2 cells, soma sizes and total dendritic lengths were unchanged (soma area, 341.85 ± 12.46 μm² in diabetic [n = 14, from seven mice] vs. 337.20 ± 10.50 μm² in control [n = 16, from nine mice], P = 0.79; dendritic length, 4902.03 ± 207.54 μm in diabetic vs. 4917.15 ± 206.57 μm in control, P = 0.96; Figs. 2E-H, Table 1). However, unlike ON-RGA2 cells, the dendritic field area was comparable to control (70,959.84 ± 6570.61 μm² in diabetic vs. 75,673.01 ± 4476.81 μm² in control; P = 0.47) and the “bell” curve yielded by Sholl analysis coincided with that obtained in the control group (Fig. 2I). It was of interest that the overall dendritic branch number was significantly increased in diabetic retinas (62.36 ± 3.30 in diabetic vs. 48.15 ± 2.56 in control, P < 0.01; Table 1).

Laminar-Specific Dendritic Alterations in RGD2 Cells in Diabetic Retina. RGD2 cells are bistratified ON-OFF direction-selective GCs. A, RGD2 cell is shown in Figure 3A, which had extremely thin and recursive dendrites that arborized in ON and OFF sublaminae of the IPL. Diabetic RGD2 cells did not show changes in soma size (195.40 ± 7.17 μm² in diabetic n = 15, from eight mice) vs. 194.87 ± 7.44 μm² in control [n = 17, from eight mice], P = 0.91; Figs. 3A-D, Table 1). By processing three other morphologic parameters for dendrites in ON and OFF sublaminae of the IPL separately, there were no significant differences in total dendritic lengths and branch numbers for either ON or OFF sublamina between diabetic and control retinas (P > 0.21 in the two cases; Table 1), but a statistically significant decrease in dendritic field areas in the ON sublamina (20,324.28 ± 1722.44 μm² in diabetic vs. 30,129.08 ± 2621.89 μm² in control; P < 0.05), not for dendrites in the OFF sublamina (30,020.97 ± 1407.17 μm² in diabetic vs. 31,843.83 ± 1141.74 μm² in control; P = 0.33). Additionally, Sholl analysis revealed that in the ON sublamina the numbers of dendritic intersections were not significantly different except for the location 40 μm far from the soma (Fig. 3E). No such changes occurred for the dendrites in the OFF sublamina (Fig. 3F).

RGD2 Cells were Unchanged in Morphology in Diabetic Retinas. RGC1 cells exhibit medium dendritic fields and smooth, recursive dendrites extending from large primary ones and branching in the innermost tier of the IPL. They represent the ON direction-selective cells. A, as shown in Table 1, for all morphologic parameters examined, no differences were found between diabetic and control retinas, including soma area, total dendritic length, dendritic field area, dendritic branch number, and number of dendritic intersections (P > 0.55 in all cases; n = 14, from six diabetic mice; n = 15, from nine control mice).

Electrophysiology

Changes in Passive Membrane Properties of RGA2 Cells. Use of the transgenic mouse line for electrophysiological experiments that require a large number of GCs for each special subtype GC to accumulate data is poorly economic, since only a small population of GCs are YFP-labeled in this line. Instead, C57BL/6J mice, with a background on which the transgenic mouse line was originally created, were used. We first determined the identity of a cell being recorded according to its light response feature and then reconfirmed it
FIGURE 2. Dendritic alterations of ON- and OFF-RGA2 cells in diabetic retinas of Thy-1 YFP mice. (A, B) Maximum intensity projection confocal fluorescent images of two typical ON-RGA2 cells respectively in whole-mounted control (A) and diabetic (B; green for YFP) retinas. Arrows, axons. (C, D) Side views of the same cells in (A, B), showing the dendritic stratification patterns, in which the boundaries of the IPL are shown by ChAT-labeled somata of cholinergic amacrine cells (red). The areas in dotted rectangle are enlarged in the bottom panels. (E, F) Maximum intensity projection confocal fluorescent images of typical OFF-RGA2 cells from control (E) and diabetic (F) retinas. (G, H) Side views of the same cell in (E, F), showing the dendritic stratification depth of the dendritic arbor within the off sublamina. (I) Sholl analysis revealing the increased branches of proximal dendrites (30–80 μm away from somata) and reduced branches of distal dendrites (120 and 140–210 μm away from somata) of ON-RGA2 cells in diabetic retinas. (J) Sholl analysis showing no changes in the number of dendritic intersections along dendrites of OFF-RGA2 cells in diabetic retinas. *P < 0.05 and **P < 0.01 vs. control. OFF, OFF sublaminae of the IPL; ON, ON sublaminae of the IPL. Scale bar: 25 μm.
morphologically by filling the cell with Lucifer yellow. GC subtypes were identified according to well-established morphologic and physiologic criteria.\textsuperscript{46,52,53} RGA2 cells/α-GCs, which are characterized by large (~20 µm) somata, could be distinguished easily from other GCs and displaced amacrine cells.\textsuperscript{36,37,54} Based on their light response properties, α-GCs could be further classified into four subtypes: transient ON (with a brief burst at the start of the bright phase), sustained ON (with maintained firing during the bright phase), transient OFF (with a burst of spikes at the start of the dark phase followed by rapid decay to little or no firing), and sustained OFF (with maintained firing during the dark phase and little or no firing during the bright phase).\textsuperscript{46} All four physiologic subtypes share similar morphology.\textsuperscript{46}

Transient ON and OFF cells were chosen for physiologic analysis because these two cell subtypes were rather commonly encountered and stable recordings from these cells often were made under our experimental conditions. Figures 4A and 4B show the maximum intensity projection confocal fluorescent images of a transient ON-RGA2 cell and a transient OFF-RGA2 cell filled with Lucifer yellow (green), respectively, and their dendritic stratification patterns are shown below as side view images. These two cells generated a burst of spikes respectively at light (525-nm full field stimulus) onset (right in Fig. 4A) and offset (right in Fig. 4B) under dark-adapted conditions with cell-attached recording.

Under current-clamp mode, a range of passive membrane parameters were examined (Fig. 4C, for details see Materials and Methods). For transient ON-RGA2 cells, the mean $V_m$ obtained in diabetic mice was significantly less hyperpolarized than that in normal animals ($-61.64 \pm 0.83$ mV [$n = 21$] in diabetic vs. $-64.42 \pm 0.42$ mV [$n = 22$] in control; $P < 0.01$; Fig. 4D, Table 2), whereas the mean $C_m$ was decreased (55.78 \pm 3.56 pf in diabetic vs. 76.28 \pm 6.68 pf in control; $P < 0.01$; Fig. 4E). No changes in $\tau_m$ (Fig. 4F) and $R_m$ (Fig. 4G) of these cells were observed in diabetic retinas ($P > 0.13$ in the two cases).

No differences in all passive membrane properties examined in transient OFF-RGA2 cells were detected between control and diabetic retinas ($P > 0.08$ in all cases; Figs. 4H–K).

Alterations in Active Membrane Properties of RGA2 Cells. Whether AP properties were changed in diabetic retinas
was further examined. Under current clamp mode, we measured and compared the spike width, threshold, and amplitude of transient RGA2 cells in normal and diabetic mice. Figures 5A and 5B show the spike waveforms of transient ON-RGA2 cells in control and diabetic retinas, respectively. AP widths were measured as the full width at half height (dashed lines in Figs. 5A, 5B). Statistical analysis showed that the mean AP width of transient ON-RGA2 cells was larger in diabetic retinas (0.35 ± 0.01 ms, n = 21) than in control ones (0.27 ± 0.01 ms, n = 22; P < 0.01; Fig. 5C, Table 2). However, no changes in thresholds and amplitudes of APs were seen for transient ON-RGA2 cells (threshold, −56.20 ± 0.84 mV; amplitude, 81.51 ± 1.57 mV) in diabetic vs. 84.89 ± 1.74 mV in controls, P > 0.16 in the two cases; Figs. 5D, 5E, Table 2).

Transient OFF-RGA2 cells displayed similar changes in AP properties, such as an increase in AP widths (0.30 ± 0.01 ms [n = 18] in diabetic vs. 0.26 ± 0.01 ms [n = 20] in control, P < 0.01; Figs. 5F–I), no changes in thresholds (Fig. 5I) and amplitudes (Fig. 5J, Table 2).

AP firing patterns of GCs in response to depolarization steps, which determine the signal that is sent from the retina, were also tested. Since the V_m of a cell is related to the firing rates of the cell in response to a certain current step, the pre-stimulus V_m of each GC was adjusted to the same level (approximately −65 mV) by using an appropriate current injection \(^{55}\) to facilitate comparisons among the data obtained in different GCs. To elicit AP firing, GCs were injected with 500 ms depolarizing current steps (+200, 300, and 400 pA). Figure 6B shows the firing rate, which was much higher than that of another cell recorded in normal retina (Fig. 6A), of a transient ON-RGA2 cell in response to 400 pA current injection in diabetic retina. The bar chart in Figure 6C shows a comparison of the mean numbers of spikes in response to current steps of three magnitudes recorded in normal and diabetic retinas. In response to all current steps, transient ON-RGA2 cells (n = 19) in diabetic retinas fired more APs than those (n = 22) in normal retinas (P < 0.05 for 500 pA, P < 0.01 for 200 pA and 400 pA), suggesting an increased excitability of these cells in diabetic mice.

The changes in firing rates of transient OFF-RGA2 cells in response to depolarizing current steps of increasing amplitudes in diabetic mice followed the same pattern of significant changes as those seen in ON-RGA2 (P < 0.01 for all cases, n = 19 for controls, and n = 14 for diabetics; Figs. 6E–G).

We also examined the maximum firing rate of GCs, which reflects the dynamic range of signal being processed by these cells. Maximum firing rates of transient ON- and OFF-RGA2 cells in diabetic mice were significantly lower than those in control mice (ON-RGA2, 428.55 ± 11.29 Hz [n = 21] in diabetic vs. 353.20 ± 13.90 Hz, P < 0.01; OFF-RGA2 cells, 387.30 ± 14.29 Hz [n = 18] in diabetic vs. 458.01 ± 10.87 Hz [n = 19] in control, P < 0.001; Figs. 6D, 6H).

**Effects of Hypoglycemia on Voltage-Activated K^+ Currents of RGA2 Cells.** K^+ channels are a powerful regulator of dendritic excitability.\(^{56–57}\) It has been shown that blockade of voltage-dependent, outward K^+ currents with 4-AP or TEA increases AP widths in mouse retinal GCs\(^{58}\) and rat hippocampal CA3 neurons.\(^{59}\) Therefore we examined whether K^+ currents of transient RGA2 cells were affected under hyperglycemia. Outward K^+ currents of GCs were evoked by a series of 300-ms depolarizing voltage pulses from a holding potential of −80 mV to +40 mV in increments of 20 mV in the presence of 0.5 μM of TTX, which eliminated the inward Na^+ current. Delayed rectifier outward K^+ currents (I_{Kr}) and A-type transient outward K^+ currents (I_{to}), which are two major components of outward K^+ currents of mouse GCs,\(^{58}\) were respectively derived from the currents recorded in the presence of 4-AP (5 mM)/TEA (20 mM) according to the procedures described previously.\(^{60}\) Figures 7A and 7C show the isolated I_{to} of two representative ON-RGA2 cells from control and diabetic retinas, respectively, whereas the data for isolated I_{Kr}, respectively, are shown in Figures 7B and 7D. Amplitude of I_{to} was measured as the plateau current at the end of a 300 ms voltage pulse. When pooled current density (current amplitude/C_m) versus voltage relationships were

<table>
<thead>
<tr>
<th>Measurements</th>
<th>ON-RGA2</th>
<th>OFF-RGA2</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
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<tr>
<td>V_m, mV</td>
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<tr>
<td>R_m, MQ</td>
<td>106.24 ± 6.53</td>
<td>121.51 ± 7.44</td>
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<td>C_m, pF</td>
<td>76.28 ± 6.68</td>
<td>53.78 ± 5.56 ‡</td>
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<td>t_m, ms</td>
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<td>6.59 ± 0.40</td>
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<td>Number of cells/mice</td>
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**TABLE 2. Measurements of Membrane Properties of Two GC Subtypes in Control and Diabetic Retinas (Mean ± SEM)**

- † P < 0.01 vs. control, Student's t-test.
- ‡ P < 0.01 vs. control, Student's t-test.
- † P < 0.001 vs. control, Student's t-test.
constructed for isolated Ik, no significant difference was found between the two curves obtained in control and diabetic ON-RGA2 cells (two-way RM ANOVA followed by Sidak’s multiple comparisons test, F[1, 15] = 0.55, P = 0.47; Fig. 7E). In contrast, a significant difference was seen for the two curves of IA densities obtained in control and diabetic ON-RGA2 cells (two-way RM ANOVA followed by Sidak’s multiple comparisons test, F[1, 15] = 5.96, P < 0.05; Fig. 7F). It is noteworthy that Ik and IA densities of transient OFF-RGA2 cells were significantly decreased in diabetic retinas compared to controls (two-way RM ANOVA followed by Sidak’s multiple comparisons test: F[1, 22] = 7.06, P < 0.05 for Ik; Figs. 8A, 8C, 8E; F[1, 22] = 14.28, P < 0.05 for IA, Figs. 8B, 8D, 8F). These results suggest that the increased AP width observed in ON- and OFF-RGA2 cells could be caused by a reduction of Ik (for ON- and OFF-type) and Ik (for OFF-type) induced by hyperglycemia, respectively.

**DISCUSSION**

Visual deficits, especially ones at the early stages of diabetes, may be induced by effects of hyperglycemia on inner retinal neurons, as demonstrated by morphologic and physiologic studies. Since a variety of GCs are responsible for diverse visual functions, a detailed analysis of the hyperglycemia-induced morphologic and physiologic changes of different subtypes of GCs is extremely necessary, since it could provide information useful for developing new treatment options targeting these cellular alterations, thus ultimately preserving vision.

**MORPHOLOGY AND PASSIVE MEMBRANE PROPERTIES OF GCs/GC DENDRITES MEDIATING ON LIGHT SIGNALING ARE PREFERENTIALLY AFFECTED AT THE EARLY STAGE OF HYPERGLYCEMIA**

Experiments with ON- and OFF-RGA2 cells clearly demonstrated that hyperglycemia preferentially affected the dendrites arborizing in the ON sublamina in the IPL over those arborizing in the OFF sublamina. ON-RGA2 cells exhibited decreased dendritic field areas, whereas OFF-RGA2 cells showed no changes in dendritic field areas. It is of interest that in ON-OFF RGD2 cells that send dendrites arborizing in ON and OFF sublaminae, hyperglycemia caused a reduction in ON-stratified
dendritic field areas, but not in the OFF-stratified ones. All these results strongly suggested that GCs/GC dendrites mediating ON light signaling are preferentially affected at the early stage of hyperglycemia.

A possible explanation for the decreased dendritic field size of ON-RGA2 cells (Table 1) may be that longer dendrites were somehow shortened in diabetes. The occurrence of such shortening during the early stages of diabetes may lead to an

![Figure 5](image-url)

**FIGURE 5.** Changes in active membrane properties of RGA2 cells. (A, B) Representative AP waveforms of transient ON-RGA2 cells in control (A) and diabetic (B) retinas. Dashed lines show the AP widths that are measured as the full width at half height. (C–E) Quantification of various parameters of APs examined for transient ON-RGA2 cells in control and diabetic retinas. (F, G) Representative AP waveforms of transient OFF-RGA2 cells in control (F) and diabetic (G) retinas. (H–J) Averaged values of AP width (H), threshold (I) and amplitude (J) were differentially affected in diabetic OFF-RGA2 cells. Cell numbers are noted in the bars (C, H). **P < 0.01 vs. control.

![Figure 6](image-url)

**FIGURE 6.** Altered spiking patterns of RGA2 cells in diabetic mice. (A, B) Example traces from typical control (A) and diabetic transient ON-RGA2 (B) cells, showing the voltage responses to a depolarizing current injection (+400 pA, 500-ms duration). (C) Mean number of APs elicited in transient ON-RGA2 cells by depolarizing current steps of varying amplitudes. Cells (n = 19) from diabetic mice fired significantly more APs than control neurons (n = 22). (D) Averaged maximum firing rate was decreased in diabetic ON-RGA2 cells compared to control ones. (E, F) Example traces from typical control (E) and diabetic transient OFF-RGA2 cells (F), showing the voltage responses to a depolarizing current injection (+400 pA, 500-ms duration). (G) OFF-RGA2 cells (n = 14) from diabetic mice fired significantly more APs than control neurons (n = 19) in response to depolarizing current steps of varying amplitude. (H) Averaged maximum firing rate was decreased in diabetic OFF-RGA2 cells compared to control ones. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.
Increase in the number of short dendrites and a decrease in the number of long dendrites. As a result, the number of intersections at the distance from the soma less than 80 μm was increased, but it decreased when it was over approximately 130 μm, thus leading to the bell curve, yielded by Sholl analysis, to be shifted left. In contrast, no significant changes in dendritic field sizes and bell curve shapes were found in diabetic OFF-RGA2 cells compared to control ones.

An increase in total number of dendritic branches was seen in ON- and OFF-RGA2 cells. This result is different from that obtained in InsAkita mice, in which such an increase was detected only in large ON-GCs (corresponding to ON-RGA2 cells), but not in large OFF-GCs. The increase in dendritic branch numbers could be in part due to a compensatory response to a potential loss of inhibitory presynaptic inputs, as suggested by some studies conducted in diabetic animal models. RG1 cells were quite unique in that these cells were hardly changed in morphology in diabetic mice even though they represent ON-direction selective cells. Much evidence is available showing that these cells are highly resistant to various pathologic conditions. In an experimental glaucoma mouse model, neither somata nor dendrites were affected at the early stage of elevated IOP. RG1 cells also did not show any detectable morphologic changes in a mouse model of retinal degeneration.

The effect of hyperglycemia on passive membrane properties of GCs also was subtype-dependent. Hyperglycemia made transient ON-RGA2 cells less hyperpolarized and Cm decreased, but it changed none of the passive membrane properties for transient OFF-RGA2 cells. While different from the differential effects of hyperglycemia on morphology and passive membrane properties of ON- and OFF-RGA2 cells, hyperglycemia exerted actions on active membrane properties of the two cell subtypes in a similar way: hyperglycemia increased the AP width, enhanced the excitability and reduced the maximum firing rate without changing the AP threshold and amplitude in both cell subtypes.

A plausible explanation for this inconsistency may be that the morphologic parameters used in the present work were for detecting changes in dendritic regions rather than soma regions, which are mainly responsible for generating active membrane properties. If the changes in morphology are largely restricted to the dendritic regions of these cells, but it did not affect the soma region at the early stage of hyperglycemia, it would be understandable why no changes in active membrane properties were detected while the changes in morphology were detectable.

**Functional Implication of Morphologic and Physiologic Alterations in Diabetic Retinas**

A major change in GC morphology in diabetic retinas was a reduction in dendritic field size of ON-RGA2 cells. Reduced dendritic field sizes in GCs also were seen in postmortem human retinas with diabetes. It has been reported that the dendritic field size is closely related to the receptive field center dimension. Indeed, Xiao et al. have demonstrated that the receptive field size of ON-RGA cells in db/db mice is significantly reduced from 12 weeks after birth. However, whether the receptive field size of ON-RGA2 cells in STZ-induced diabetic mice is reduced should be further explored. Moreover, it should be noted that in contrast to the our results
changes in dendritic field sizes of ON-GCs. 

With the AP threshold being unaltered, the fact that the resting membrane potential of ON-RGA2 cells became less hyperpolarized in diabetic retinas suggests that hyperglycemia could make the membrane potential of these cells closer to the AP threshold. This means that these cells would generate more APs to a certain depolarizing current step in diabetic retinas implying an enhanced excitability of these cells. Less hyperpolarizing membrane potentials of ON-RGA2 cells may be due to a reduction of presynaptic inhibition of GCs, which has been suggested by several studies in various animal models of diabetes.22,36,63–65,75 It is shown, for instance, that diabetes could disrupt the rod bipolar-AI-A17 amacrine cell microcircuit in rats which results in a decrease in GABA release from A17 amacrine cells causing a disinhibition of the negative feedback from A17 amacrine cells to rod bipolar cells and an increase in glutamate release from the rod bipolar cells.64

It was shown that the reduced presynaptic inhibition led to an increase in spontaneous spiking activity of ON-GCs in STZ-induced diabetic mice.36,38,40,60,61 Spontaneous spiking activity of a neuron could be regarded as a background noise. The increased background noise could result in a decreased signal-to-noise ratio, meaning that it is more difficult for the animal to detect a luminance increment on a background (i.e., an elevation in luminance threshold). Indeed, an elevated luminance threshold in ON-GCs was reported in db/db mice.35 Moreover, the reduced maximum firing rates of ON- and OFF-RGA2 cells in diabetic mice (Figs. 6D, 6H) suggest a reduced dynamic range of visual signals that can be processed by these cells.

In summary, the effects of early hyperglycemia on GC morphology and passive membrane properties were cell subtype-dependent with GCs/GC dendrites mediating light ON signaling being preferentially affected. These differential changes in morphology and electrophysiologic function in subtypes of GCs may in part account for visual function defects in early diabetes.

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