Morphology and Immunoreactivity of Retrogradely Double-Labeled Ganglion Cells in the Mouse Retina

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PURPOSE. To examine the specificity and reliability of a retrograde double-labeling technique that was recently established for identification of retinal ganglion cells (GCs) and to characterize the morphology of displaced (d)GCs (dGs).

METHODS. A mixture of the gap-junction-impermeable dye Lucifer yellow (LY) and the permeable dye neurobiotin (NB) was applied to the optic nerve stump for retrograde labeling of GCs and the cells coupled with them. A confocal microscope was adopted for morphologic observation.

RESULTS. GCs were identified by LY labeling, and they were all clearly labeled by NB. Cells coupled to GCs contained a weak NB signal but no LY. LY and NB revealed axon bundles, somas and dendrites of GCs. The retrogradely identified GCs numbered approximately 50,000 per retina, and they constituted 44% of the total neurons in the ganglion cell layer (GCL). Somas of retrogradely identified dGs were usually negative for glycine, ChAT (choline acetyltransferase), bNOS (brain-type nitric oxidase), GAD (glutamate decarboxylase), and glial markers, and occasionally, they were weakly GABA-positive. dGs averaged 760 per retina and composed 1.7% of total GCs. Sixteen morphologic subtypes of dGs were encountered, three of which were distinct from known GCs. dGs sent dendrites to either sublaminae of the IPL, mostly sublamina a.

CONCLUSIONS. The retrograde labeling is reliable for identification of GCs. dGs participate in ON and OFF light pathways but favor the OFF pathway. ChAT, bNOS, glycine, and GAD remain reliable AC markers in the GCL. GCs may couple to GABAergic ACs, and the gap junctions likely pass NB and GABA. (*Invest Ophthalmol Vis Sci.* 2011;52:4886–4896) DOI:10.1167/iov.10-5921

In the vertebrate retina, ganglion cells (GCs) are axon-bearing output neurons. Their axons form the optic nerve to bring visual signals from the eye into the brain. Amacrine cells (ACs), on the other hand, are interneurons; their function is to modulate signal transmission inside the inner retina. The morphology of GCs in the GCL and ACs in the inner nuclear layer (INL) has been extensively studied in the vertebrate retina,^{1–4} and nearly 20 morphologic types of GCs and ACs have been described; yet, the corresponding information on displaced

(d)GCs (dGs)^{5,6} and displaced ACs is still missing. Because of the small population of dGCs and a lack of highly specific GC markers, the morphology and function of dGCs in the mammalian retina are still unclear.

Individual GCs are not easily distinguishable from ACs because of their similar morphology, electrophysiology, and soma location. Observation of soma size is helpful in identifying GCs, as somas of GCs are generally thought to be larger than those of ACs.² This rule is often violated in the central retina, since GCs have small somas there and some subtypes of ACs can have larger ones.^{4,7} GCs may also be identified by observation of their axons, although axons are also found in a minority of ACs.^{8,9} GCs and ACs also share qualitatively similar patterns of light responses,^{10,11} and both may fire action potentials.^{9,12} Antibodies for Thy1,^{13–15} neurofilaments,^{16,17} and parvalbumin^{18,19} have been used to label GCs. However, their immunoreactivity in the GCL is not fully restricted to GCs, and antibodies may be specific only for certain subpopulations of GCs rather than the whole population^{16,18–20} Although ACs can be distinguished from GCs by expression of glycine or GABA, as well as ChAT and bNOS, it is still uncertain how AC–GC coupling^{2,21,22} may alter the immunoreactivity of GCs.

The simplest yet most important difference between GCs and ACs is that GC axons form the optic nerve, and ACs do not. Because of this anatomic characteristic, the GC population may be studied by counting axons in the optic nerve or by retrograde labeling of GC somas. Among a variety of mouse strains, the GC population measured by counting axons in the optic nerve was estimated at 44,860 to 59,897 per retina.^{23,24} A slightly higher density of GCs (50,920–70,000 per retina) was obtained by counting retrogradely identified somas^{6,25} in the GCL. Axonal terminals of GCs are able to take up fluorescent dyes and transport them into their somas, and retrograde labeling^{26,27} is based on this mechanism. Using retrograde tracers to identify mouse retinal GCs was pioneered by Dräger and Olsen.⁶ Later on, in a few reports, NB²⁸ and LY^{13,29} were used as retrograde dyes to label retinal GCs. LY is a fluorescent dye. It is known to be impermeable for gap junctions³⁰ and harmless for neuronal function.²¹ It is widely used in investigation of individual cell morphology in living and fixed retinal preparations.^{20,21} NB is usually adopted for studying gap junctions in the central nervous system, including the retina.^{31–33} By applying retrograde dyes to the cut end of the optic nerve, instead of applying them to brain areas, researchers in a few studies have been able to reduce the complexity of the technique and shorten the labeling time from several days^{13,25,34} to several hours.^{28,29} However, axonal counting gives no information about GC morphology, and retrograde labeling currently is still challenged by a prolonged labeling time, the complexity of the procedure, and uncertainty regarding labeling efficiency and how gap junctions between GCs and ACs affect the labeling.^{6,13,28,29}

Recently, we have described a novel method for retrograde double-labeling of mouse retinal GCs in vitro using LY and NB.³⁵ This procedure requires a much shorter labeling time

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(see the Methods section) than other techniques and is able to reveal GC somas, axons, and dendrites. In this study, we performed a systematic evaluation of the efficiency and specificity of the retrograde labeling method for labeling retinal GCs, and on the basis of the results, we further studied the distribution and morphology of retrogradely identified dGCs.

MATERIALS AND METHODS

Animals

The animals used in this study were C57BL/6J purchased from Jackson Laboratory (Bar Harbor, ME). The mice were 2- to 6-month-old males and females. All procedures used in this study complied with the NIH guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the relevant requirements of the Baylor College of Medicine Animal Care and Use Committee. All mice were dark adapted for 1 to 2 hours before the experiment. The animals were anesthetized with an intraperitoneal injection of ketamine (200 mg/kg) and xylazine (10 mg/kg). The eyes were enucleated after the animals were deeply anesthetized. The animals were euthanized thereafter by an overdose of the anesthetic.

Antibodies and Specificity

The primary antibodies used in this study have been used in previous reports. They included polyclonal guinea pig anti-GABA (1:1K, AB175; Chemicon, Temecula, CA),²⁹ polyclonal goat anti-CHAT (choline acetyltransferase, 1:100, AB144P; Chemicon),³⁶ rat anti-glycine antiserum (1:1000, a generous gift from David Pow, University of Queensland, Brisbane, QLD, Australia),^{37–39} polyclonal rabbit anti-GAD65 (glutamate decarboxylase, 1:1000, Chemicon),⁴⁰ monoclonal rat anti-GFAP (glial fibrillary acidic protein, 1:1000, Zymed Laboratory, Inc., South San Francisco, CA),⁴¹ monoclonal mouse anti MHC II (major histocompatibility complex Class II molecules, 1:500; Chemicon),^{42,43} monoclonal mouse anti-GS (glutamine synthetase, 1:1000; BD Transduction Laboratories, Palo Alto, CA),³⁹ and a mouse monoclonal antibody against Go α (1:1000, clone 2A; Chemicon).⁴⁴ The specificity of these primary antibodies has been demonstrated in previous studies and their staining patterns in our results were similar to the previous published data. Rabbit anti-5-HT antiserum was obtained from Immunostar Inc. (1:500, Hudson, WI). In the retinas that were not preincubated with 5-HT, 5-HT-immunoreactivity (IR) was absent in the neuronal somas in the GCL and the inner nuclei layer (INL), confirming the specificity of the 5-HT antibody. Cy3-conjugated and Cy5 conjugated secondary antibodies were used at 1:200 dilution (Jackson ImmunoResearch, West Grove, PA). A fluorescent nuclear dye,⁴⁵ TO-PRO-3 (1:3000; Molecular Probe, Eugene, OR), was used to visualize the nuclei in the retinas, together with secondary antibodies.

We also used IgG fraction of rabbit antiserum against bNOS (brain-type nitric oxidase, 1:4000, N7155; Sigma-Aldrich, St. Louis, MO) to label NOS. We had examined the specificity of this antibody³⁵ in bNOS knockout mice (*Nos^{1tm1Pl}/–*),⁴⁶ and the study indicated that bNOS labeling in ACs was specific.

Retrograde Labeling of GCs and Immunohistologic Staining

The technique has been previously described.³⁵ Briefly, a mixture of neurobiotin (NB), a gap-junction-permeable dye (MW 322.85; Vector Laboratories, Burlingame, CA), and Lucifer yellow (LY), a less permeable dye (MW 457.24; Sigma-Aldrich),^{31–33} were used for the labeling. The cells double-labeled by LY and NB were recognized as GCs; and those that contained only NB but no LY were identified as being coupled with GCs. Eyeballs with an attached optic nerve stump were chosen for retrograde labeling. First, the nerve stump was dipped into a small drop of a cocktail that contained 3% LY and 8% NB in the internal solution³⁵ for 20 minutes. Afterward, the eyeball was thoroughly rinsed with oxygenated Ames medium (Sigma-Aldrich) to re-

move the extra dye. The eyeball was then dissected under infrared illumination. The eye cup with intact retina and sclera tissue was transferred into fresh oxygenated Ames medium and kept at room temperature for 40 minutes under a 10-minute dark/10-minute light cycle. The medium in which the retinas were incubated was replaced every few minutes during the labeling. After the light cycle, the whole retinas were rinsed and fixed in darkness in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Hatfield, PA) and 0.5% glutaraldehyde (Sigma-Aldrich) in phosphate buffer (D-PBS, pH 7.4; Invitrogen, Carlsbad, CA), for 30 to 45 minutes in room temperature. The retinas were blocked with 10% donkey serum (Jackson ImmunoResearch) in TBS (D-PBS with 0.5% Triton X-100 (Sigma-Aldrich) and 0.1% NaN₃ (Sigma-Aldrich) for 2 hours at room temperature or at 4°C overnight to reduce nonspecific labeling. Afterward, the retrogradely filled whole retinas were incubated in Cy3-conjugated streptavidin (1:200; Jackson ImmunoResearch) in 3% normal donkey serum-TBS for 1 day at 4°C.

The retinas were subsequently cut into 40- μ m-thick vertical sections with a microtome (Vibratome; Leica Microsystems, Bannockburn, IL). The whole-mounted retinas or free-floating sections were incubated in primary antibodies in the presence of 3% donkey serum-TBS for 3 to 5 days in 4°C. Controls lacking primary antibodies were also processed. After several rinses, the slices and whole retinas were then transferred into Cy3- and/or Cy5-conjugated secondary antibodies (1:200; Jackson ImmunoResearch) and/or Alexa Fluor 488-conjugated secondary antibodies (1:200; Molecular Probes), in 3% normal donkey serum-TBS solution in 4°C overnight. After extensive rinsing, the slices and whole retinas were coverslipped. Two small pieces of filter paper (180- μ m-thick, MF membrane filters; Millipore, Billerica, MA) were mounted beside whole retinas to prevent them from being overly flattened.

The preparations were observed with a laser scanning confocal microscope (LSM 510; Carl Zeiss Meditec, Oberkochen, Germany). Images were further processed with image-management software (Photoshop ver. 9.0.2; Adobe Systems, San Jose, CA). For better clarity, some images were presented in black and white, in which fluorescent signals were in white against a dark background (see Figs. 8, 9) or in black against a bright background (see Figs. 3a, 3b, 4); and some color images (see Figs. 5, 6) were inverted as well, in which fluorescent signals were presented against a bright background.

Data Analysis

All data are presented as the mean $\pm$ SEM. The differences between paired data were analyzed by two-tail Student's *t*-test. Histograms of GC soma size were plotted and further analyzed (SigmaPlot ver. 8; Statist, San Jose, CA). The data were fitted by the normal Gaussian distribution function. The statistical significance of the data was determined by *t*-test and variance analysis.

RESULTS

Before investigation of the GC morphology, we examined how thoroughly and specifically GCs were labeled by our retrograde labeling method. The data are presented in the following three sections.

Under our experimental conditions, GCs were labeled evenly throughout the entire retina. The retrograde dye LY was restricted to GCs, and NB was primarily found in GCs as well, consistent with our previous observations.³⁵ The GCs were identified by LY labeling, and they were all clearly labeled by NB (Fig. 1). A small population of retrogradely labeled somas contained only NB but not LY, and these cells were identified as tracer-coupled ACs. NB signals were usually a few to 10 times weaker in the ACs than in the GCs; but they were still distinguishable from background noise. Prolonged labeling time may cause higher noise on the surface and the edge of the retina; thus the labeling time was restrictively followed. The proximal margin of the inner plexiform layer (IPL; nearest to

FIGURE 1. Retrograde labeling of the mouse retina. (a, b) Confocal micrographs of vertical retinal sections. (a) Low-power micrograph double-labeled by NB (red) and Goα (green). Goα antibody labels the IPL and OPL. NB is largely restricted in GCs. A brightly labeled optic nerve stump is outstanding. (b) Triple-labeling by LY (blue), NB (red), and the nuclear dye TO-PRO-3 (green). In the GCL, GC (purple) and AC (green) somas were usually arranged in a single layer and they were clearly distinguishable because of the labeling. Scale bar: (a) 100 μm; (b) 20 μm.

the GCL) was defined as 100% of the IPL depth, and this definition was used to describe the location of neurites in the IPL.

Dye-Coupling between GCs and Glial Cells Was Not Observed in the Mouse Retina

Glial cells have been extensively studied in the central nervous system. So far, three major types of glial cells have been reported in the mammalian retina: astrocytes, Müller cells, and microglia. To exclude glial cells from the neuron count in the GCL, we studied the morphology of glial cells in triple-labeled retinal preparations (Fig. 2).

We observed that in the mouse retina neurons and glial cells were morphologically distinguishable. In addition to other characteristics, neurons, including GCs and ACs, usually showed spherical somas and cablelike processes that became progressively thinner when leaving the soma. However, glial cells in the retina usually possessed flat and much wider processes, less extensive branching, and irregular somas.

Astrocytes in the central nervous system expressed GFAP. In the retina, our data showed that GFAP-IR was primarily located in astrocytes and was not present in displaced ACs or GCs, consistent with our previous observations.³⁵ These GFAP-IR astrocytes resembled snowflakes, with a flat body and short processes (Fig. 2).

In retinal slices and confocal optical sections of flat-mounted retinas, we observed that the GCL was covered proximally by a three-layer “quilt”; and the layers, from the proximal surface of the retina to the GC somas, were astrocytes, blood vessels, and GC axons (nerve fiber layer; NFL). Confocal micrographs focusing on the GC soma plane, which were used for counting the total nuclei of the neurons, rarely included somas of astrocytes (Fig. 2). GFAP-IR sometimes could be found to invade the IPL with blood vessels, but those were mostly processes of astrocytes and they were negative for TO-PRO-3.

Similarly, Müller cells³⁹ and microglia⁴² can be immunologically identified. In our results, GS-IR Müller cells were usually distributed vertically inside the retina and each cell covered only a rather narrow region. Their somas were spherical or oval, but were located in the middle of the INL instead of the GCL. Their end feet, although reaching the GCL, were much thicker and irregular than neuronal processes; and they were negative for TO-PRO-3. The location and look of the Müller cells were distinguishable from that of retinal GCs.

Microglia cells have been reported in most retinal layers, except the outer nuclear layer (ONL). When these cells have their somas locating in the GCL or INL and branches ramifying into the IPL, they may bear some similarity to retinal GCs.

However, our data showed that microglia cells revealed by MHC II-IR had far fewer branches than did the GCs, including the highest-level branches. The somas of these cells were small and usually quite irregular, like a twisted pie, triangle, or a polygon, and so were their nuclei. Their processes were much thicker than the neuronal processes, and they showed large spines and irregular varicosities, especially at branching points. Higher order branches were as thick as primary branches. Their TO-PRO-3-labeled nuclei were stained 2 to 4 times brighter than those of the neurons in the GCL. The cell mor-

FIGURE 2. Glial cells and GCs in the mouse retina. (a–c) Labeling with NB (red), LY (blue), and glial cell markers (green). (d₁, d₂) Triple labeling with NB (red), TO-PRO-3 (blue, or white in the insets), and MHC II (green). (a₁) Confocal micrograph from a flat-mounted mouse retina, showing the morphology of astrocytes (GFAP-IR). In the same area, astrocyte somas (double arrows in a₁) are nearly invisible in the focal plane of GC somas (a₂). (b) Stacked confocal micrograph from a vertical retinal slice. Astrocytes were mostly located in a layer separated from the GCL (a, b), except those processes of astrocytes wrapping the blood vessels (*). (c) A confocal micrograph from a vertical retinal slice shows that Müller cell somas (GS-IR) are located in the INL and are not to be confused with somas in the GCL. (d₁, d₂) Micrographs of a flat-mounted retina focused on the GCL (d₁) and IPL (d₂). TO-PRO-3 labeled nuclei (d₂, inset; d₁, arrow) of microglia cells (MHC II-IR) are more intense than that in the GCs (d₁, open arrow). NB and LY do not clearly reveal astrocytes, microglia, and Müller cells. GFAP-, GS- and MHC II-IR were not found in retrogradely labeled GCs. IPL, the inner plexiform layer; INL, the inner nuclear layer. Scale bar, 20 μm.

FIGURE 3. Observation of retrogradely identified GCs and total neurons in the GCL. Stacked confocal micrographs were taken from the same area over the GCL, which had been stained with TO-PRO-3 (a, black; c, green) and retrograde dye NB (b, black; c red). GC somas were nearly absent in a blood vessel (b, *), where quite a few nuclei (12.5%, 88/705 nuclei) were revealed by TO-PRO-3 (a, c). These nuclei were either spindle shaped, as is typical of endothelial nuclei, or oval. They were not counted as neurons. Scale bar, 20 μ m.

phology that we observed was similar to that in previously published data.⁴³

In our experimental conditions, retrograde dyes LY and NB did not clearly label cells with the morphology of astrocytes, Müller cells or microglia and GFAP-, GS-, and MHC II-IR did not present in retrogradely identified GCs. The data suggested that there was no clear dye-coupling between these glial cells and GCs in the mouse retina. Subsequently, retrograde identification of GCs was not significantly affected by communication between these cells and GCs, if any.

Because of their location, nuclei of Müller cells were not included in total neuron counts, and neither were the nuclei of astrocytes. Furthermore, to exclude nuclei of microglia, we did not count nuclei with an irregular shape and very intense TO-PRO-3 signal.

Retrogradely Identified GCs and Total Neurons in the GCL

We investigated the number of retrogradely double-labeled GCs and total neurons in the GCL. Total neurons were visualized by the nuclear dye TO-PRO-3 (Fig. 1) and counted in the GCL, with an exclusion of endothelial nuclei and extensively stained irregular nuclei that belonged to microglia cells. Endothelial nuclei in blood vessels were recognized by their typical spindle shape or by their location in GC-absent blood vessel territories (Fig. 3). The shapes of endothelial nuclei were largely dependent on their location relative to the viewing angle. With a thin pie shape, endothelial nuclei on sidewalls of blood vessels usually appeared to be spindle-like, and those on the top and bottom walls of blood vessels were mostly oval shaped. In flat-mounted retinas, most of the endothelial nuclei were observed in a nearly oval shape. Their size was similar to those of GCs and displaced ACs.

The total number of GCs was obtained either by counting cells in whole retinas ($n = 2$ retinas/animals) or by sampling an equal number of areas in the central and peripheral retina ($n = 12$ retinas/animal). The entire sampling area was approximately 10% of the total retinal area, to assure an accurate sampling.⁴⁷ A whole-retina image was composed routinely for all retinas used in the GC counting and from which the retinal area was measured. We first took confocal micrographs over an entire flat-mounted retina. Images were overlapped slightly at the edges to ensure complete coverage. They were then stitched together carefully (Photoshop; Adobe Systems) to form a complete retinal image, and overlapped parts were eliminated to assure accurate counting. We counted cells on such a whole retinal image by putting dots on a new layer of the image at positions of labeled somas to keep track of which

cells had been counted. Displaced cells were counted in whole retinas, either directly from the confocal microscope or from both images and the microscope. The two methods yielded similar results. From nine triple-labeled retinas/animals, a total of $113,222 \pm 4,340$ neurons were found in the GCL. GCs accounted for 44% ($49,823.12 \pm 1,792$ cells) of the total neurons in the GCL, and the remaining neurons were displaced ACs. GCs with somas in the INL averaged 760 ± 27 per retina and amounted to 1.7% of the total GCs. Our data were similar to previous observations^{6,23,24} and suggested that GCs were revealed thoroughly in the retina.

In the whole retinas, the average GC density was 3817 ± 160 cells/mm². The density was progressively decreased toward the peripheral retina (Fig. 4). In randomly chosen confocal micrographs, with a step of 0.01 μ m, we measured the soma size of GCs in the GCL. For better accuracy, the measurement was performed on 400 $\times$ confocal micrographs with an image browser (LSM; Carl Zeiss Meditec). An advantage of using a confocal microscope instead of a regular light microscope in this observation is that the microstructure of a cell can

FIGURE 4. Soma size of GCs in the GCL. (a) Low-power confocal micrograph of a flat-mounted retina retrogradely labeled with LY and NB (black), focusing on the GCL. (a) A progressive decrease of GC density toward the peripheral retina is obvious. The average GC soma size (major axis) in the central retina was significantly smaller than that in the peripheral retina (b). The soma size is well fit by a normal Gaussian function (line).

FIGURE 7. Topographic distribution of dGCs in the INL. A flat-mounted whole retina labeled by LY (black). Stacked confocal micrographs were taken from the INL and recombined. dGCs were counted, and their soma locations were depicted with dots. dGCs were preferentially distributed in the temporal and peripheral retina.

regions where GCs in the GCL were very brightly labeled. However, some dGCs, as we presented here, were labeled more brightly than regular GCs. By choosing appropriate exposure times, we were able to take images of these cells with a relatively low background. Not all the labeled retinas were useful for this purpose. We deliberately selected retinas with distinguishable dGCs for this study.

Currently, four parameters are often used for morphologic classification of conventional GCs in the GCL: soma size and ramification level, stratification pattern, and the coverage of their dendritic arbors in the IPL. We examined the morphology of retrogradely identified dGCs in both whole-mounted retinas and retinal slices. To obtain precise morphologic features of GCs, for each targeted cell we routinely scanned images in the x - y plane at successive levels along its depth (z -axis) and then reconstructed a three-dimensional image (x - y - z stack) of the cell. Whether a retrogradely labeled dendrite belonged to a GC was verified by rotating the three-dimensional image of the cell to find a connection between the dendrite and the soma. For dGCs in flat-mounted retinas, the location of dendrites in the IPL was examined in x - z and y - z views of their stacked images. However, we felt that this approach was less accurate in determination of ramification levels of the dendrites than was direct observation of dendrites in slice preparations (Fig. 8). The reason was that dendrites of the GCs in retinal slices had much better resolution than those in x - z or y - z views of stacked GC images from flat-mounted retinas. In fixed retinal slices, the diameter of most dendritic processes of GCs were less than 2 μ m and the thickness of the IPL was near 40 μ m; but in x - z and y - z views of stacked GC images, dendrites appear to be thicker relative to the IPL depth. To clearly present the morphology of dGCs, we also manually traced axons and dendrites of GCs in stacked confocal images (Fig. 8). We used the somas of GCs and ACs as boundaries for the IPL. This IPL definition was very helpful for us to accurately describe the location of processes of GCs in the IPL.

To take advantage of the accuracy of the slice preparation in localization of dendrites in the IPL and the accuracy of flat-mounted retinas in measurement of the size and shape of dendritic arbors, we took three steps to classify dGCs. First, we

characterized more than 10 morphologic subtypes of dGCs in slice preparations using soma size, the size of dendritic fields, and the ramification pattern of dendritic arbors in the IPL, and with similar parameters, we also characterized more than 10 subtypes of dGCs in flat-mounted retinas. Second, we compared the morphology of the two sets of cells. Third, we paired up similar cells, reinvestigated cells with doubts and reclassified dGCs. In this way, we were able to classify dGCs that we encountered into 16 morphologic types (Figs. 9–12). Most of the dGCs bore morphology similar to that of their counterparts that were previously recognized in the GCL. However, three types of dGCs, dG5, -7, and -14 were not reported before. For easier comparison, dGCs and their counterparts are listed in Figure 12.

The soma size of the dGCs fell into three groups: small (<13 μ m), medium (13–17.5 μ m), and large (>17.5 μ m). The size of the dendritic field fell into four groups: small (<100 μ m), medium-small (100–250 μ m), medium-large ($\leq$ 400 μ m and >250 μ m), and large (>400 μ m). Most dGCs possessed a medium-size soma and a medium-size and monostratified dendritic arbor. Dendrites of dGCs could stratify in either or both sublamina a and b. Dendritic arbors that ramified in two or three levels of the IPL and had at least 20% of the IPL thickness separating any two adjacent levels were described as bi- or tristratified, respectively (Fig. 12). Although most of the dendrites of bi- and tri-stratified dGCs were located in separate levels in the IPL, some of them also crossed from one level to another and were termed interlevel dendrites. Dendritic arbors were described as monostratified if occupying $\leq$ 50% of the total IPL thickness, and they were termed diffuse if occupying >50% of the total IPL thickness. Of 16 types of dGCs, 2 (dG5 and -6) showed diffuse dendrites in the IPL, 9 monostratified (dG1, -2, -4, -7, -9, -10, -11, -15 and -16), 4 bistratified (dG3, -8, -12, and -13), and 1 tristratified (dG14).

FIGURE 8. Comparison of retrogradely labeled dGCs in whole-mounted retinas and retinal slices. (a) Image scanning with a confocal microscope. A set of consecutive optical sections in x - y plane (ai and aii) is obtained first and is reconstructed into a single three-dimensional image (aiii). (b, c, e) Retinas are double labeled by LY and NB (white). (b) An x - y view of a stacked image of a dGC from a whole-mounted retina; an x - z view (x : y : z = 1:1:2) of the cell is depicted in (c). (e) A stacked image of a dGC in a retinal slice, whose soma size, ramification level of dendrites, and dendritic field are similar to those of the cell in (c). The morphology of the cell in (b) and the cell in (e) are manually traced and presented in (d) and (f), respectively; but they are scaled down to fit the space. (c, e, g, arrow) axon. Scale bar, 20 μ m.

FIGURE 9. Morphology of dGCs in vertical sections. Stacked confocal micrographs of the 16 subtypes of dGC in retinal slices double-labeled by LY and NB (*wbite*). The images optimize the visibility of the GC dendrites, thus axon bundles and some GC somas, especially in thicker stacks of images, are saturated. Dendritic fields of some cells are not fully visible because of image trimming. dGCs have somas in the IPL (7) and INL. Their axons are visible (*arrow*). (5, *) A diffuse dGC. Scale bar, 20 μm .

dG5 cells had medium-size somas and narrow dendritic fields, with a few primary dendrites emanating from the soma and branching sparsely throughout 10% to 90% of the IPL depth. dG5 cells sometimes formed small clusters of two to five cells (Fig. 9). The narrow receptive field was not a result of sectioning, as cells with similar morphology were frequently observed (Fig. 9). dG7 was a unique type of GC with the soma displaced in the IPL instead of the INL. These GCs had the smallest somas (8–12 μm) of various shapes, and their dendrites were thin and located at 30% to 40% of IPL depth. dG14 cells were tristratified, with medium somas and medium-large dendritic fields. Their dendrites were thin and wavy, ramifying at 0%, 40%, and 80% of the IPL depth (Fig. 11).

Most of the dGCs showed nearly symmetric dendritic trees, except for dG8, -9, -10 and -15 (Fig. 10). The dendrites of the dG8 cells were mostly located to one side of the soma, and they were characterized by dendritic spines and some almost parallel branches. dG9 and dG10 usually had two to three primary dendrites, and their dendritic trees formed two wings at opposite sides of their soma. dG15 had a sparse dendritic tree with a few thin and very long dendrites. Subtypes dG1 to -4, -11, and -12 had similar shapes and branching pattern of their dendritic trees and a nearly even dendritic density from the center to the periphery of dendritic fields. Yet, the dendritic density was not uniform among these cells, and it was slightly higher for dG1, -3 and -4 but lower for dG2, -11, and -12. dG1, -3, and -11 also showed slightly asymmetric dendritic density, which was lower on one side of the dendritic field and higher at the other side, with a difference of ~ 1 dendrite/ $20 \mu\text{m}^2$. In contrast to these cells, dG6, -13, and -14 had the highest dendritic density near their somas, which progressively decreased toward the periphery of the dendritic fields. They also bore fewer higher order branches. dG5, -6, and -16 had small dendritic fields and sparse dendritic trees. dG16, as well as dG10, had some branches with a branching angle greater than 90° . For the remaining dGCs, the angle was often less than 60° . Further morphologic details are included in Figure 12.

DISCUSSION

Retrograde LY-NB Double-Labeling Is Reliable for Identification of Mouse Retinal GCs

LY was impermeable and NB was permeable for gap junctions among retinal neurons. Thus, in this study LY was used to label GCs, and NB was used to label GCs and ACs that were coupled to GCs. The result, consistent with our previous publication,³⁵ supported that retrograde dyes primarily accumulated in GCs. In other previous studies, the total number of neurons in the GCL has been reported to be $\sim 110,000^{6,23}$ in the mouse retina. GC axons counted in the optic nerve are 44,860 to 59,897 per retina. GCs make up nearly 41% to 44% of the total neurons in the GCL,^{23,24} and dGCs account for nearly 2% of all GCs.⁶ Our data were in agreement with these findings on the GC population. Comparing GCs that we labeled (50,000 per retina) to those in other reports, it seems unlikely that we have significantly overestimated or underestimated the GC population. Although there is a possibility that we slightly underestimated the GC population because GCs at the edge of the retina may have been cut off by imperfect dissection and some GCs may have died before fixation, we believe that this possibility was minimized by a shorter labeling time and careful dissection. This idea is also supported by total neuron counts (see below). There was also a potential for us to slightly overestimate the GC population because of GC-AC coupling, yet this chance was largely reduced by co-application of both gap-junction-permeable and -impermeable dyes. In general, the data did not indicate a significant undercounting of the GC population or incomplete labeling of the GCs.

The total number of neurons in the GCL that we revealed (113,222 per retina) was close to that in the previous reports (110,000 per retina^{6,23}), supporting that dissection-related cell loss was effectively minimized. Thus, it is most likely that neurons in the GCL, whether GCs or ACs, were largely preserved during our retrograde labeling process.

FIGURE 10. Morphology of dGCs in flat-mounted retinas. The 16 subtypes of dGCs retrogradely labeled by LY and NB are included: dG1 to dG16. The morphology of the cells was manually traced from stacked confocal micrographs. Their axons are clear in *x-z* views of the stacked images (Fig. 8) but not clear in *x-y* views. Scale bar, 20 μ m.

Because of their specific soma location, Müller cells were excluded from total neuron counts. Meanwhile, we routinely mounted flat retinas with a piece of filter paper nearly 180- μ m-thick, to prevent the retinas from artificially flattening, so that in our flat-mounted preparations, the distance between the GCL and the astrocytes was better preserved. We counted total nuclei of neurons in the GC soma plane; thus, the nuclei of astrocytes were largely avoided from the counting. Although sometimes GFAP-IR was observed in the IPL and GS-IR near the GCL, the labeled was mostly processes of the glial cells, and they were negative for TO-PRO-3.

Microglia cells have a density of 7×10^5 cells μ m² in the P28 mouse retina,⁴³ and a mouse retina was averaged approximately 13.5 mm² in our results. With such a cell density and retinal area, we estimated a total of approximately 935 microglial cells per retina. Since the GCL, IPL, INL, and outer plexiform layer share these microglia cells, the maximum overestimation of the total neurons in our results because of microglia, if any, is most likely below 0.2% to 0.9%.

Furthermore, our data showed that retrogradely identified GCs were seldom positive for the AC markers glycine, CHAT, bNOS, GAD, and GABA. They were negative for glial markers GFAP, GS, and MHC II, and they were rarely present in blood vessel territories. These results, together with our previous observations,³⁵ confirmed that these cells are not ACs, glial cells, or endothelial cells. Therefore, we believe that the retrograde double-labeling technique is specific and reliable for visualization of the entire GC population in the mouse retina. It is likely that the whole GC population in the mouse retina was faithfully revealed by our retrograde labeling methods.

The retrograde labeling method, compared to *in vivo* retrograde labeling and other GC staining methods, has several advantages: (1) It requires much shorter labeling time; (2) it specifically reveals GCs and tracer-coupled ACs; (3) it can clearly label GC axons and somas simultaneously throughout the entire retina; (4) LY signal is visible in living retina preparations; and (5) LY and NB signals are stable and can be maintained after further sectioning and labeling. Because of its smaller molecular mass, NB usually labeled GCs more brightly than LY did, and although NB also revealed coupled ACs, its signal in ACs was very weak. Thus, NB, just like LY, may be used alone to identify GCs in the mouse retina.

Glycine, CHAT, bNOS, and GAD are Reliable AC Markers in the GCL, Whereas 5-HT Labels Both GCs and ACs

GABA-IR cells and 5-HT-accumulating cells have been reported in both the GCL and INL in mammalian retinas.^{48–50}; yet, whether they are GCs is still uncertain. In the present study, our data showed that bNOS-, glycine-, GAD- and CHAT-IR were rarely present in GCs. This result confirms that bNOS, ChAT, GAD, and glycine are reliable AC markers in the GCL. We also observed that bNOS-IR was mostly colocalized with GABA-IR, which is in line with previous studies.^{51–53} In contrast, GCs and ACs were both found to accumulate 5-HT, and these cells resided in either the GCL or INL. Thus, GCs and ACs cannot be distinguished by observation of 5-HT accumulation.

Tracer-Coupling between GCs and ACs

GABA has a molecular weight of 103.12, slightly larger than that of glycine (75.07). It is still unknown whether GABA is gap-junction-permeable in neurons. However, besides the observation of weak GABA-IR in GCs, we also found weak NB (MW 322.85) in ACs. Combined with previous studies,^{2,21,22} our data suggest that some GCs may couple to ACs and receive GABA via gap junctions between them.

We have previously shown that the retrograde dye NB could label ACs coupled to GCs in the mouse retina.³⁵ NB signal in coupled ACs was usually weak. We identified dGCs by bright LY and NB signals, thus dye-coupling between GCs and ACs caused no problems for identification of dGCs.

Recently, a systematic survey was made on the tracer coupling pattern of GC subtypes in the mouse retina.² Several morphologic subtypes of retinal GCs have been revealed to be

FIGURE 11. Morphology of bistratified (dG13) and tristratified (dG14) dGCs in flat-mounted retinas. The cells were manually traced from *x-y* views of stacked confocal micrographs labeled by LY and NB. For dG13 (a), the first level of dendrites is near 25% of the IPL depth (red) and the second (blue) is near 70% of the IPL depth. For dG14 (b), the first (red), second (blue), and third (green) levels of dendrites are near 10%, 40%, and 80% of the IPL depth, respectively. Scale bar, 20 μ m.

FIGURE 12. Summary of 16 morphologic types of dGCs in the mouse retina. dGCs are named dG1 to -16. Their dendritic arbors are arranged in an artificial 10-layer IPL. Dendritic arbors in IPL are presented in strata with 100% as the proximal margin (nearest the GCL) of the IPL. Most GC dendrites are stratified in the IPL, in three levels. Monostratified cells have only one level (the first level) of dendrites. The soma size illustrated in the artificial IPL is relevant to the original size, but the dendritic fields are not in some cells. Soma size: large (L), >17.5 μ m; medium (M), 13 to 17.5 μ m; and small (S), <13 μ m. Dendritic field size: large (L), >400 μ m; medium-large (M_L), $\leq$ 400 μ m and > 250 μ m; medium-small (M_S), 100 to 250 μ m; and small (S), <100 μ m; -, not applicable; Y, observed; N, rarely found; arrows axons.

coupled with ACs, such as G₁, G₂, G₃, G₆, G₇, G₈, G₁₀, G₁₃, G₁₈, G₂₀, and G₂₁. In our results, most dGCs morphologically resembled their counterparts in the GCL. It is likely that they may couple to ACs in similar patterns.

5-HT-Accumulating GCs Comprise a Large Subpopulation of GCs in the Mouse Retina

5-HT-accumulating GCs have been revealed in cat and rabbit retina,^{48,49} but they have not been studied in the mouse retina. Our results, similar to findings in other mammals, showed that a large subpopulation of GCs in the mouse retina, especially dGCs, accumulated 5-HT. In mammals, retinal neurons lack enzymes for synthesizing 5-HT and are also absent of endogenous 5-HT, except in photoreceptors.^{54,55} 5-HT in the inner retina is believed to be released by axons of brain neurons terminating in the retina (termed centrifugal fibers or retino-petal fibers).⁵⁶⁻⁵⁸ 5-HT accumulated in GCs can be stored in vesicles and used as “borrowed neurotransmitters”⁵⁹; and retinal neurons in the mouse retina are found to express 5-HT receptors.⁵⁹ Yet, the significance of 5-HT in retinal physiology and pathology is still not fully understood.⁵⁹⁻⁶¹

Sixteen Morphologic Subtypes of dGCs Were Identified, and They Are Likely to Participate in More Activities in the Retinal OFF Light Pathway

In the mouse retina, we identified 16 morphologic subtypes of dGCs. These GCs were mostly medium-field cells with medium-size somas. Most of them morphologically resembled their counterparts previously revealed in the GCL. In the vertebrate retina, GCs receive excitatory inputs from bipolar cells, and they generally show three types of light responses, ON, OFF, and ON-OFF center light responses.⁶² Retinal neurons with neuronal processes (axonal terminals for bipolar cells and dendrites for GCs) ramified in sublamina a usually generate OFF-center light responses, cells with processes residing in

sublamina b usually generate ON-center light responses, and cells with bi-stratified processes in both sublaminae exhibit ON-OFF center-light responses.^{10,21,63} This general rule may not hold for all cells, especially those with diffuse or complex dendritic trees. However, since most dGCs that we identified sent dendrites to sublamina a of the IPL, it is likely that as a population, dGCs are functionally more important for retinal OFF light pathways.

We characterized the morphology of dGCs in both flat-mounted retina and retinal slices, to obtain a precise description of ramification levels of GC dendrites, as well as the dendritic field size. The data may be more reliable for cells with small to medium dendritic fields but less accurate for the cells with large dendritic fields, as some very long dendrites may be incompletely revealed. However, only few of the GCs in the mouse retina are currently known as large-field cells.^{1,2,64} Moreover, confocal microscopy allowed us to acquire stacks of images over entire dendritic arbors of targeted cells. For each GC, a three-dimensional image was routinely reconstructed to fully reveal all the dendrites. Based on these investigations and analysis, we believe that the uncertainty related to the dendritic field size in slice preparations and the inaccuracy related to the location of dendritic arbors in flat-mounted retinas were minimized.

To the best of our knowledge, three subtypes of dGCs, dG5, -7, and -14, have not been previously revealed in the mouse retina.^{1,2,64} Although a few types of diffuse GCs have been reported in the mouse retina, such as G₈, G₁₃,² and the mono-stratified GC clusters 4 and 5,⁶⁴ the dendritic arbors of these cells occupy the center region of the IPL, 45% to 68% of the IPL depth for G₈ and 39% to 65% of the IPL depth for G₁₃. Compared with these cells, our dG5 cells have more diffuse dendritic trees and narrower dendritic fields. G₅ cells from the human retina,⁴ however, possess a smaller and rather diffuse dendritic arbor (but >100 μ m) similar to that of our dG5. Our

dG14 cells bore some similarity to G₂₂ cells from the human retina,⁴ which are large-field cells (near 600 μ m) with dendritic processes that diffusely occupy the entire IPL.

In general, the retrograde labeling technique adopted here was a simple yet reliable way for identification of GCs in the mouse retina. We revealed 16 morphologic subtypes of dGCs in the mammalian retina for the first time. With regard to the morphology and immunoreactivity, dGCs generally resemble their counterparts in the GCL, but they probably favor the retinal OFF pathway.

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