Materials and Methods

1. Animal and retina preparation

Retinas were obtained from New Zealand White rabbits aged from postnatal day 20 to 22 (p20~p22). Control animals were kept in the normal light/dark cycle. For dark rearing, pregnant rabbits were placed in a completely dark room 1-3 days prior to the estimated date of delivery. After the baby rabbits were born, they were kept in the dark room continuously until the proper age for experiment. All of the daily maintenances were conducted under the dim red illumination by animal keepers.

Animals were deeply anesthetized by intramuscular injection of a mixture of ketamine (150 mg/kg) and xylazine (30 mg/kg). The local anesthetic (Alcaine; Alcon-Couvreur, Belgium) were applied in both eyes before dissection. Eye balls were then removed form the orbit and hemisected along the limbus by a scissor. The vitreous was removed from the posterior eye cup, and the retina were carefully isolated from the sclera with a glass rod in the oxygenated Ames' medium (Sigma, St. Louis, MO; Ames and Nesbett, 1981) or modified Ames' medium (120 mM NaCl, 3.1 mM KCl, 0.5 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.15 mM $CaCl_2$, 6.0 mM D-glucose). In order to stain the cell nuclei, isolated retinas were incubated in 30 ml of 5.7 μM 4,6-diamidino-2-phenylinodole (DAPI; Sigma, D-9542) containing the oxygenated AMES medium in a light-tight bottle for one hour. After rinsed by the fresh AMES medium, the DAPI-labeled retina was made a few cuts to flatten on a black Millipore filter paper (Millipore, Bedford, MA; HABP02500) with the ganglion cell side up and placed in the AMES medium (Sigma, A1420) for gene gun shooting.

2. Gene gun labeling

A small amount (40 ~ 50 mg) of tungsten particles (~1.7 μm in diameter; Bio-Rad, Hercules, CA; 1652269) were spread out on a glass slide. The dye solution was made by dissolving 4 mg of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ('DiI'; $DiIC_{18}(3)$, Molecular Probe, Eugene, OR; D-282) in 200 μl of methylene chloride. The solution was quickly applied on the tungsten particles on the glass slide. After methylene chloride evaporated, the dye-coated tungsten particles were scraped off with a blade, sonicated in 3 ml distilled water for 20 minutes, and vortexed to mix particles evenly with water. The tubing in the Bio-Rad tubing prep station was pretreated with polyvinyl pyrrolidone (PVP, Sigma; P5288) in isopropanol (10 mg/ml). The particle solution was quickly sucked into the PVP-pretreated tubing. The tubing was rotated 90 degrees every 10 seconds for 8 times to settle down the beads onto the inside surface of the tubing. The suspended beads and water were slowly drawn out and the rest of beads were dried onto the tubing. The beads–coated tubing was cut into around 40 small segments as bullets for gene gun shooting. The Helios Gene Gun system (Bio-Rad) was used to deliver dye-coated particles onto the retina. The whole set up for shooting is illustrated in Appendix Figure 1A. There were two kinds of nylon mesh used to block large tungsten clumps. A small piece of nylon mesh with pore size 105μm (Small Parts, Inc.; CMN-0105-D) was locked with the diffusion screen (Bio-Rad; 165-2475) in the barrel liner. A 35-mm plastic dish was drilled a hole (15 mm in diameter) on the dish cover and the bottom. The dish bottom was inverted and capped with the dish cover, so that another nylon mesh with pore size 10μm (Small Parts, Inc.; F010N-12-C) can be placed in between. The flattened retina was placed under the inverted dish and the excess of medium was removed. Each retina was shot with 1 or 2 bullets. The Helium pressure for shooting was 100-110 psi. After shooting, the retinas were immediately fixed for 25

minutes with 4% para-formaldehyde (Electronic Microscopy Science Co.; 15710) in 0.1M phosphate buffer (PB). The fixed retinas were rinsed 3 times with 0.1 M PB for 10 minutes, and then mounted with 0.1M PB on the glass slides. In order to maintain the thickness of the retina, the sample region was painted a wall by nail polish on the glass slide before mounting.

3. Image acquisition and measurements of morphological parameters

All images were taken by a cool CCD camera (AxioCam HRm, Zeiss, Germany) under the fluorescent microscope (Axioskop 2 mot Plus, Zeiss, Germany). Two sets of images were taken for each cell. DiI-labeled ganglion cells were recorded from the axons to the distal dendrites by using a long-pass filter for rhodamine (excitation, 546nm band pass; beam splitter, 580nm; emission, 590nm long pass; filter set 15, Zeiss). Afterward, DAPI-stained nuclei of the ganglion cell layer (GCL) and of the inner nuclear layer (INL) closest to the inner plexiform layer (IPL) were imaged respectively to estimate the thickness of the IPL. The GCL and the INL were set as 100% and 0% of the IPL, respectively. The ganglion cell stratification was calculated from the Z-axis reading of the labeled cell dendrites. The soma size was estimated by a polygon connecting the rim of the cell soma. For quantifing the dendritic field size of the labeled cell, a polygon connecting the tips of the dendrites was drawn for each cell (Appendix Fig. 1B). Both soma areas and dendritic areas were calculated by using the AxioVision LE 4.2 (Zeiss). The nucleus density was calculated by manual counting the numbers of nuclei in the GCL from the DAPI image (Appendix Fig. 1C).

4. Data analysis

Besides the effect of light deprivation, the regional difference in the retina has to be considered since the dendritic field sizes of RGCs are known to vary with the retinal eccentricity (Dann et al., 1987; Wong, 1990; Sernagor et al., 2001). We chose the nucleus density as an alternative eccentricity parameter to include RGCs from both dorsal and ventral retinas. To determine the influences of nucleus density and light deprivation simultaneously, we used the general linear model (GLM) to analyze our data (Der and Everitt, 2002; Walker, 2002). The linear model of this study can be expressed as the following equation:

$Y = b_0 + b_1X_1 + b_2X_2$,

where Y is the logarithmic dendritic field area for each RGC, X_1 is the logarithmic nucleus density, and X_2 is the rearing condition (giving 1 for cells in the control group and 0 for those in the dark-reared group). This linear model is assumed that there is no interaction between the nucleus density and the rearing condition. Analyses were conducted using the SAS (version 8.01; SAS Institute Inc., Cary, NC). Two types of sums of squares (SS) are given as results, and they are calculated as follows:

Term	**Type I SS**	**Type III SS**
X_1 (nucleus density) X_2 (rearing condition)	$SS(X_1)=R(1)-R(X_1)$ $SS(X_2\|X_1)=R(X_1)-R(X_1,X_2)$	$SS(X_1\|X_2)=R(X_2)-R(X_1,X_2)$ $SS(X_2\|X_1)=R(X_1)-R(X_1,X_2)$
H_0	$Y = b_0$ $Y = b_0 + b_1X_1$	$Y = b_0 + b_1X_1$ $Y = b_0 + b_2X_2$
H_1	$Y = b_0 + b_1X_1$ $Y = b_0 + b_1X_1 + b_2X_2$	$Y = b_0 + b_1X_1 + b_2X_2$ $Y = b_0 + b_1X_1 + b_2X_2$

Type I sum of squares (SS) reflects that the parameter effect is considered in the order of entering the model. Type III SS, which is sample-size independent, shows that the effect

of each parameter is evaluated after all other factors have been accounted for. In our analyses, we should take the nucleus density into concern before considering the effect of the rearing condition (i.e., the type I SS). However, our sampling numbers in the control and the dark-reared groups are not equal for all cell types, therefore the type III SS should be considered. We included analyses of both type I and III SS in this study and examined whether they show consistent results among all cell types.