2. MATERIALS AND METHODS

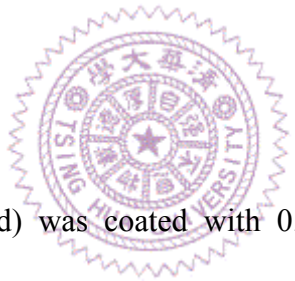
Tissue preparation

The normal-reared rabbits (albino New Zealand White) of various postnatal stages were purchased from the local breeder (AHRI, Zhunan). For dark-reared rabbits, we raised the pregnant rabbit and its pups in a complete dark room at Laboratory Animal Center (National Tsing Hua University, Hsinchu). All animals were anesthetized by 1:1 mixture of Ketamine (75-100 mg/kg) and Xylazine (15-200 mg/kg). After enucleation and hemisection, the vitreous was removed. The retina was carefully detached from the RPE, and fixed in 4% paraformaldehyde in 0.1M PB for 20 minutes. After washing for 3 times, a small piece of retina (8 mm x 5 mm) was embedded in 2% low melting point agarose (Sigma, St. Louis) at 37°C. To ensure sampling similar retinal areas at different postnatal stages, the retinal location 2 mm away from the visual streak in the ventral side was chosen. The retinal piece was then cut to 200-300 um vertical slices via Vibratome.for dye injection or DiOlistic gene gun labeling.

Dye injection

The retina slice was prepared as described above without fixing. The sample was put in a perfusing chamber with oxygenated Ames medium on the stage of the

fluorescence microscope (Axioskop 2 FS, Zeiss, Germany). The aluminosilicate glass pipette (prepared from the P97 puller, Shutter Instrument) was filled with 4% Lucifer Yellow (in 0.1M Tris buffer) for microinjection. Cell somata located in the upper two-third of the Inner Nuclear Layer (INL) were targeted for dye injection. To ensure better injection, the cell somata 50 um deep in the retinal slice were selected. The dye injection was delivered by the AM1600 intracellular amplifier (A-M Systems, Inc.) with negative current of 0.3-3 nA for 10-60 seconds. The injected cells were selected randomly in the INL based on the Random Number Generator program.



DiOlistic gene gun labeling

The Tefzel tube (Bio-Rad) was coated with 0.1mg/ml polyvinyl pyrrolidone (PVP) solution in pure ethanol and dried completely in the Tubing Prep Station (Bio-Rad). A total of 4 mg DiI (Molecular Probes) was dissolved in 200 µl methylene chloride (0.02 M), and mixed with 50 mg tungsten particles (diameter 1.7 µm). After spreaded out on a clean glass slide, tungsten particles were scraped with a razor blade onto a weighting paper, and transferred into the PVP-precoated tubing. Fine particle dispersion was achieved by holding the tubing into an ultrasonic bath for several minutes.

DiI particles were delivered by a Bio-Rad Helious gene gun device, and the helium pressure was set at 75-85 psi. A piece of nylon filter (Small Part, Inc.) with 5 um pore size and another piece of same nylon filter with 100 μm pore size were interposed between the gene gun and the specimen. The gene gun was positioned perpendicularly to these nylon filters and 2 cm above the retinal surface. After the shooting, the specimen was incubated in Ames medium for 10 minutes and mounted for confocal microscopy.

Confocal microscopy and data analysis

The images of gene gun labeled cells were collected using a Laser Scanning Confocal Microscope (LSM 5 Pascal, Zeiss, Germany). Either the 40X objective lens (Plan-Neofluar, 0.75 NA, Zeiss) or the 63X objective lens (C-Apochromat, 1.2 NA, Zeiss) were used, depending on the sizes of cell processes.

We calculated the distinguishable types of bipolar cells appeared following the age between dark-reared and normal-reared rabbit. The “undefined bipolar cells” were the cells that we could ensure being bipolar cells. The “B/M” cells were unrecognizable either bipolar cells or Muller cells. Soma aspect ratio and location of cell soma in the INL could be used as estimates of bipolar cell development. The soma aspect ratio was defined as the length of long-axis divided by the length of short

axis (see Appendix 2), and the location of cell soma was assigned to N1, N2 and N3, where N1 is the soma located toward the outer segment (see Appendix 3).