2. Materials and Methods

2.1 Retina preparation

New Zealand White rabbits raised under normal light-dark conditions were either bred in the animal facility of National Tsing Hua University, or purchased from a local breeder. For obtaining dark-rearing neonates, pregnant rabbits were transferred to a complete dark room before parturition and pups were kept with mothers in the darkness until experimentation. Daily monitoring and routine maintenance of dark-reared rabbits were carefully handled using a dim red light. The day of birth of the pups is termed P0, and the stage groups used in our study are P8-9, P10-14, P15-21, and P22-adult.

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Animals were dark adapted for at least one hour before dissection. A 1:1 mixture of Ketamine (150 mg/kg) and Xylazine (30 mg/kg) were injected intramuscularly to anesthetize rabbits, and topical anesthesia of 0.5% proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon-Couvreur, Belgium) was applied before enucleation under dim red light. After hemisection, lenses and vitreous humors were removed immediately. The posterior eyecups were everted over the round head of a Teflon rod and immersed 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). Retinas were

carefully detached from the retinal pigment epithelium. Rabbits were then euthanized with an overdose of anesthetic. All procedures met the Institutional Animal Care and Use Committee of National Tsing Hua University. To label the nuclei of the neurons, retinas were incubated in 5 μM 4', 6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO) in oxygenated Ames' medium for one hour. The whole-mount retina was adhered photoreceptor-side down on a coverslip coated with tissue adhesive (Cell-Tak; BD Biosciences, Bedford, MA). This preparation was transferred to a recording chamber mounted on the stage of a Zeiss Axioskop 2 FS Plus fluorescence microscope, and superfused with oxygenated Ames' medium (1.5-2 ml/min) at 34-37 °C.

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2.2 Light stimuli

Visual stimuli generated by the VisionWorks (Vision Research Graphics, Durham, NH) were displayed on a CRT monitor (refresh rate 100 Hz; SyncMater 757NF; Samsung, Korea) and reflected upward by a mirror positioned beneath the microscope stage. A 20X microscope objective (A-plan, NA 0.45, Zeiss) replaced the condenser was used to focus the stimulus onto the photoreceptor layer of the retina. The DSGCs were identified initially by direction selective responses to a bar of light maneuvered manually. A flashing square 180x180 μm^2 was then used to map the overall receptive

field. For characterizing the detailed receptive field properties of the DSGCs (well-studied in adult rabbit retina) throughout development, six different visual stimulus paradigms were used in this study. First, the preferred direction of the DSGCs was determined by a moving bar (540x180 μm^2, ~900 μm/sec = ~5.14 deg/sec) swept across the receptive field in twelve directions equally spanning 360° at 30° intervals (Fig. 2A). Second, velocity tuning was examined by a moving bar (540x180 μm^2) swept in the preferred direction with various speeds (1 mm/sec = 5.71 deg/sec; Fig. 3A). Third, center-surround interaction was studied by flashing (167 ms) white circles of different diameters (Fig. 4A). Forth, surround inhibition induced by preferred direction motion was determined by various sizes of moving rectangles (~900 μm/sec = ~5.14 deg/sec) extending perpendicularly to the preferred-null axis swept in the preferred direction (Fig. 5A). Fifth, we offered a square wave grating (1 cycle per receptive field center) moving in the preferred direction (~900 μm/sec = ~5.14 deg/sec) in the receptive field center and a windmill pattern (16 vanes) rotating clockwise in the surround annulus to study moving surround inhibition (Fig. 6A; (Werblin, 1972; Chiao and Masland, 2003). Lastly, similar to the fifth stimulus paradigm, but the surround annulus was replaced by a square wave grating (same spatial and temporal frequency as the center grating) moving in the preferred direction (~900 μm/sec = ~5.14 deg/sec; Fig. 7A; (Chiao and Masland, 2003). We varied the

phase of annulus surround grating, so that center and surround could either in-phase or out-phase to each other. The size of the center grating patch in last two stimulus paradigms closely matched the excitatory region of the receptive field, and the width of the surround annulus was equal to the diameter of the center grating. The surround annulus was immediately adjacent to the center grating, with no gap. The presence of the surround stimulus alone caused no response from the recorded cell.

2.3 Extracellular recording

RGCs labeled with DAPI were visualized under brief fluorescence illumination (365 nm excitation) using a 40X water immersion objective (Achroplan, NA 0.8, Zeiss), and the DSGCs were targeted with the aid of soma features described previously (Vaney, 1994; Yang and Masland, 1994; Chiao and Masland, 2002). The activity of single ganglion cells was recorded with the tungsten-in-glass electrode (Levick, 1972). A LabVIEW based data acquisition system (National Instrument, Austin, TX) was used to identify action potentials; their time of occurrence relative to the stimulus generation was recorded by computer for later offline analysis.

2.4 Intracellular dye injection

After recording, the tungsten-in-glass electrode was withdrawn and replaced by a

micropipette pulled from the thick-wall borosilicate glass capillaries with filament (o.d. = 1.0 mm, i.d. = 0.5 mm; Sutter Instrument, Novato, CA) using a programmable Flaming-Brown P97 puller (Sutter Instrument) for intracellular dye injection. The micropipette was back-filled with 2% Lucifer Yellow (Sigma) and 4% Neurobiotin (Vector Laboratories, Burlingame, CA) in 0.1M Tris buffer. An intracellular amplifier (Neuroprobe Amplifier 1600, A-M Systems, Carlsborg, WA) was used to perform the iontophoresis with biphasic current (1-2 nA) for 1-2 minutes. To allow diffusion of Neurobiotin across potentially gap junction connected ganglion and amacrine cells, the tracer-filled cell was left in the oxygenated Ames' medium for at least 30 minutes, prior to fixation in 4% paraformaldehyde (in 0.1 M PB) for 40 minutes. The injected cells were then visualized by incubating the retina with a FITC-conjugated streptavidin (diluted 1:50 in 0.1M PB with 0.1% Triton X-100; Vector Laboratories) at room temperature overnight. The retina was flat-mounted in the mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) for confocal imaging.

2.5 Image acquisition

Images of the injected cells were acquired using a laser scanning confocal microscope (LSM 5 Pascal, Zeiss) with a 20X objective lens (Plan-NEOFLUAR, NA 0.5, Zeiss) or a 40X objective lens (Plan-NEOFLUAR, NA 0.75, Zeiss), depending on

the dendritic field size. A series of z-stack images was taken from the focal plane of axon terminals to the inner nuclear layer to reveal both ON/OFF dendritic arbors of the DSGCs and their tracer coupling patterns. LSM 5 image examiner (v3.1.0.99, Zeiss) was used to improve the image intensity and contrast.

2.6 Data analysis

Offline data analysis of recorded extracellular spike trains was carried out using MATLAB (The MathWorks Inc, Natick, MA). To calculate the strength of the directional tuning, we used the direction selective index (DSI) described in Taylor and Vaney (2002). Action potentials were recorded in each of twelve directions, and DSI was defined for the recorded spikes as:

$$DSI = \left| \frac{\sum \vec{v}_i}{\sum \vec{r}_i} \right| ,$$

where v_i are vectors pointing in the direction of the moving stimulus and having length, r_i, equal to the averaged number of spikes recorded during that stimulus direction (Fig. 1B). The vector sum of twelve directions points to the preferred direction of the DSGC. The DSI can range from 0 (when the responses are equal in all twelve directions), to 1 (when the response is obtained only from a single direction).

Thus, the DSI values close to 1 indicate sharp directional tuning.

Statistical analyses were performed using SAS (SAS Institute Inc, Cary, NC). We evaluated the difference between rearing conditions using student *t*-test (*P* value < 0.05 was considered statistically significant). One-way and two-way analysis of variance (ANOVA; *P* value < 0.05 was considered statistically significant) were used to examine the difference amongst different age groups with *post hoc* (Bonferroni/Dunn) test (*P* value < 0.0333 was considered statistically significant).