2. MATERIALS AND METHODS

2.1 Tissue Preparation

Adult New Zealand White rabbits (*Oryctolagus cuniculus*) of either sex (1.0-2.0 kg) from local vender (The branch of drugs inspector, Animal health research institute, Council of agriculture, Executive Yuan) were used (Fig. 1). Use and handling of animals were strictly in accordance with the Animal Care Committee of National Tsing Hua University.

One day prior to each experiment, the animals were anesthetized by injecting the mixture of 75-100 mg/kg ketamine (Imalgene 1000; Merial, Lyon, France) and 15-20 mg/kg xylazine (Chanazine; Chanelle Pharmaceuticals Manufacturing Ltd., Loughrea, Ireland) intramuscularly, and 1-2 μl (1 μg / μl) of 4',6-diamidino-2-phenyl-indole (DAPI; Sigma, St. Louis, MO, all chemicals in the experiments were purchased from Sigma unless otherwise stated) was then injected intraocularly to pre-label DSGCs and SACs. On the experimental day, after 1-3 hours dark-adaptation (all surgery and electrophysiological experiments were accomplished under dim red illumination), the animals were given deep anesthesia through intramuscular (130-160 mg/kg ketamine and 26-32 mg/kg xylazine) and intravenous injection (50 mg/kg ketamine). The eyes were anesthetized locally with a few drops of 0.5% proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon-Couvreur, Belgium) before enucleated and hemisected.

The anterior segments of the eyeballs and the vitreous were removed (Fig. 2A, 2B). The eyecups

were immersed in oxygenated (95% O_2 and 5% CO_2) simplified 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, 23 mM $NaHCO_3$, pH 7.7) and the retinae were separated from the retinal pigment epithelium and the sclera carefully (Fig. 2C, 2D). The animals were then euthanized with an overdose of anesthetic.

A small piece of retina from the ventral side (7 × 7 mm) was adhered, photoreceptor-side down, to a coverslip coated with CellTak (BD Sciences, Bedford, MA) and transferred to a recording chamber mounted on the stage of a Zeiss Axioskop 2 FS Plus fluorescence microscope (Zeiss, Germany). This preparation was perfused at 2.5–3.5 ml/min with oxygenated simplified Ames' medium at 33-37°C. The remaining retinae were maintained by continually shaking and changed fresh Ames' medium every 1 hour at room temperature.

2.2 Visualization of the somata of DSGCs and SACs

The tissues were viewed through a long-working-distance water immersion objective lens (Zeiss, 40× ACHROPLAN, 0.80 NA). Under fluorescence illumination through an UV filter set (exciter = D360/40, emitter = E420lpv2, beamsplitrer = 400dclp; Chroma Technology, Brattleboro, VT), the nucleus of numerous retinal neurons can be revealed with DAPI staining (Fig. 3). In electrophysiology experiments, the neutral density filter with 6% light transmission (Zeiss) and luminous-field diaphragm were used in the optical path to minimize the photobleaching of visual pigments. Ganglion cells with medium-large somata and crescent-shaped nucleus were considered

to be the potential DSGCs, whereas SACs have small somata and round-shaped nucleus which were brighter-stained by DAPI (Fig. 3C, 3D).

2.3 Visual stimuli

A CRT monitor (SyncMaster 757NF; Samsung, Korea) was used to display visual stimuli (Fig. 4) with 100 Hz refresh rate driven by a Pentium 4 PC. Images were reflected by a mirror and focused via a sub-stage objective lens (Zeiss, 20× 0.45 NA) onto the photoreceptor layer of the retina. Illuminant values were calibrated using a spectrophotometer (USB-2000; Ocean Optics, Dunedin, FL), and typically fell between 0.1-12 cd/m^2.

Stimuli were generated by VisionWorks (Vision Research Graphics, Durham, NH). There were two kinds of stimulus in the experiment. A 180 × 180 μm flash light square was used to identify the receptive center of DSGCs. Then a moving bar of 540 ×180 μm (velocity ~900 μm s^{-1}) swept across the receptor field center of the DSGCs from 12 directions.

2.4 Electrophysiology

Intracellular recordings were performed to identify DSGCs and examine their prefer-null axis. The microelectrodes from borosilicate glass capillaries (o.d. = 1.0 mm, i.d. = 0.5 mm; Sutter Instruments, Novato, CA) were pulled from a programmable Flaming/Brown puller (P-97; Sutter

Instruments), and filled with 4 M potassium acetate (Mallinckrodt Baker Inc., Phillipsburg, NJ) using a 34 gauge microfill (World Precision Instruments, Sarasota, FL) in combination with a 0.22 μm filter. An electrode holder (World Precision Instruments) containing silver wire was used to connect the electrode to an amplifier (Neuroprobe Amplifier 1600; A-M Systems, Carlsborg, WA). The electrode holder and amplifier headstage were installed on a manual-controlled 3-D micromanipulator (MWS-31; Narishige, Japan) which was mounted close to the microscope (Fig. 4). Final resistance of these electrodes ranged from 90 to 120 MΩ. The offset potential was balanced and capacitance was compensated before impalement.

For intracellular recording, the targeted DSGCs were first recognized by a short DAPI illumination. The electrodes were then lowered in close proximity to the surface of inner limiting membrane and aimed at the target cells. The water hydraulic fine control of the manipulator (Narishige) was used to advance the electrodes toward the cell. When the electrode tip touched the membrane of cells, the “buzz” (generates oscillatory current and causes the electrode to vibrate) was utilized to facilitate the cell penetration. Usually, it took a few minutes to stabilize the membrane potential, and the resting potential was at least -50 mV in a successful impalement. A flash light square stimulus was projected onto the tissue for determining the ON-OFF response of DSGCs and the position of receptive field center. The stimulus was switched to a moving light bar to examine the direction selective response. Once the directionality was confirmed, a program written in LabVIEW (National Instruments, Austin, TX) was executed to record the acquired signal. Membrane potentials typically filtered and sampled at 10k Hz, and digitized with an analog-digital

converter (NI 6040E; National Instruments). The spike trains and the raw data were recorded and stored in a Pentium 4 PC. When the recording finished, the electrodes were retracted gently, and the position of the recorded cell was identified.

2.5 Microinjection

Different tracers were conducted for microinjection. In single cell morphology experiments, either 4% Lucifer Yellow (Sigma) or 4% *N*-(2-aminoethyl)-biotinamide hydrochloride (Neurobiotin; Vector Laboratories, Burlingame, CA) in distilled water were used (due to the achromatic appearance, Neurobiotin was always mix with 1% Lucifer Yellow, thus the micropipette can be revealed under fluorescence). Neurobiotin filled cells were visualized by the incubation of Cy3 (Amersham Pharmacia Biotech, Piscataway, NJ) or fluorescein (Vector Laboratories) conjugated streptavidin in the dendritic contacts and synaptic connections experiments, respectively. Occasionally, 5 mg/ ml DiI (Molecular Probes, Eugene, OR) dissolved in ethanol was also used for injection. In dendritic contact experiments, a SAC and a DSGC were individually filled by two tracers (usually Neurobiotin and Lucifer Yellow, but sometimes DiI were used to replace Neurobiotin), whereas only Neurobiotin were injected into both DSGCs and SACs in the synaptic connection experiments. When use Lucifer Yellow or Neurobiotin as the tracer, the tracer-filled micropipette can be visualized and tuned under fluorescence illumination through a GFP filter set (exciter = HQ450/50, emitter = HQ525/50, beamsplitrer = Q480lp; Chroma Technology), but

Rodamine filter set (exciter = BP546/12, emitter = BP575-640, beamsplitrer = FT560; Zeiss) for DiI injection.

The borosilicate micropipettes were first backfilled at their tips with the tracer, and then filled with 3M potassium chloride. Identical holder, headstage, amplifier and manipulator were used to perform the iontophoresis. The iontophoresis of Lucifer Yellow and DiI were both triggered by negative charge. Sometime a biphasic current (+2.0 nA and -0.5 nA at 0.33 Hz) for 1-3 minutes were used to injected Neurobiotin. However, the parameter for the current strength and inject time varied all the time because of the varied tip diameters and other factors. To ensure a good quality of dye injection, the dendritic morphology can be checked often during injection, and the injecting current was turned off when the terminal dendrites were completely filled.

2.6 Immunocytochemistry

Following microinjection experiments, the retinae were fixed immediately in a cold fixative of 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1 M phosphate buffer (PB) for 30-40 minutes at room temperature. The retinae were then detached from coverslip and transferred into 0.5 ml micro-centrifuge tubes. To obtain better immunoreaction, the whole processing was performed at 37°C, and 0.3% sodium azide (Sigma) was added to reagents to protect the retina from bacteria infection. The retinae were pre-incubated in 4% normal goat serum with 0.1% Triton X-100 in 0.1 M PB for 1 day to block nonspecific binding sites, and then

incubated in the primary antibody with 4% normal goat serum with 0.1% Triton X-100 in 0.1 M PB for 3-5 days. In this study, the primary antibody which is against α1 subunit of the $GABA_A$ receptor (1:50; Upstate Biotechnology, Lake Placid, NY) was used to localize inhibitory synapses on DSGCs. After rinsing the retinae for 3 hours (3 × 1 hour) in 0.1 M PB, secondary antibody which was raised in goats and conjugated to Alexa Fluor 555 (1:100; Molecular Probes) and streptavidin were applied together in 0.1 M PB (all manipulations from staining to image acquisition were conducted under dim red illumination). The retinae were rinsed 3 times in 0.1 M PB (at least 1 hour each time) and coverslipped in Vectashield (Vector Laboratories). Specificity of immunostaining was evaluated by omitting the incubation step with the primary antibody.

2.7 Image acquisition

Except the DAPI staining which were observed through an UV filter set (exciter = BP365/12, emitter = LP397, beamsplitrer = FT395; Zeiss) and snapped with a cooled CCD camera (AxioCam HRm; Zeiss) mounted on a Zeiss Axioskop 2 Mot Plus fluorescence microscope (Zeiss), other images were acquired using a confocal scanning module (LSM 5 PASCAL; Zeiss), installed on an identical model of microscope. The injected cells were first positioned in the field of view and focused using a low power objective lens (Zeiss, 20× Plan-NEOFLUAR, 0.5 NA) under fluorescence illumination. Sometimes the 40× objective lens (Zeiss, Plan-NEOFLUAR, 0.75 NA) was also used depending on the size of dendritic field. To minimize photobleaching, a neutral

density filter with 6% light transmission (Zeiss) was used in the optical path. The operation was then switched to confocal scanning mode. The SAC soma of the injected cell pair was placed on the center of scanning region, and the focus was set to reveal the dendrites. After properly adjusting detector gain and amplifier offset, a single plane or a stack of z-sectioning (only for single cell morphology experiments, in dendritic contacts and synaptic connection experiments, high magnification scanning was required. thus the less important low magnification stack was done at the end to minimize the photoblaching) images was scanned and saved (optic slice = 4 μm, resolution = 1024 × 1024, scan speed = 6, average = 2).

The co-localization of the dendrites and $GABA_A$ receptor puncta requires more optical magnification to be resolved, thereby high power water objective lens (Zeiss, 63× C-APOCHROMAT, 1.2 NA) was used for further detail. An advantage of this 63× water objective lens is that the lenses inside were optimized to correct vertical inaccuracy caused by different wavelength. The scanning region was zoom-in 1.6×, and the view region was determined according to the contents comparing with the corresponding region of the 20× image. As many as Z-sections containing the processes of the injected DSGCs and SACs were scanned and saved (optic slice = 0.6 μm, resolution = 1024 × 1024, scan speed = 7, average = 2, z-interval = 0.2 μm). When all regions cover the dendritic field of SAC were finished, the objective were switch to low power again to acquire a stack of the whole dentritic field of the injected cell pair, depending on the size of dendritic field, sometime the 40× objective lens was used (Zeiss, Plan-NEOFLUAR, 0.75 NA).

To avoid crosstalk, fluorescein (or Lucifer Yellow) and Alexa Fluor 555 (or Cy3, DiI) signals were

always detected separately using the multi-track function. The fluorescein (or Lucifer Yellow) labeling was excited using the 488 nm (458 nm for Lucifer Yellow) line of an argon ion laser and detected after passing a band-pass 505-530 nm emission filter. For detection of the Alexa Fluor 555 (or Cy3, DiI) signal, the 543 nm of green helium-neon laser was used in combination with a long-pass 560 nm emission filter. Furthermore, identical beam splitter HFT 488/543/633 was applied to both wavelengths to minimize the horizontal pixel shift.

2.8 Data analysis

All photomicrographs were visualized, and exported by AxioVision LE 4.3 (Zeiss) or the Zeiss LSM Image Browser 3.2. Brightness and contrast were adjusted to improve the image quality. The scales were calibrated using a micrometer (Electron Microscopy Sciences). In our experiments, the pixel size was about 0.09 μm, and the voxel height was about 0.2 μm.

In the dendritic contacts experiments, the double-labeled cell pairs were easily separated by color. Thus, contacts between the dendrites can be directly counted using Metamorph 6.1 (Universal Imaging, Media, PA). Noise of the raw image data were first removed by medium filter. The threshold for green, red, and both channels were set, and the values were measured for further calculation. For the image derived from the inhibitory synaptic connections experiments, cell dendrite and the puncta of $GABA_A$ were depicted by hand using a pen tablets (Graphire3; Wacom Technology, Vancouver, WA) before measuring. The processing of these reconstructed images was

carried out using Adobe PhotoShop CS v. 8.0.1 (Adobe Systems, Mountain View, CA).

Direction selectivity was quantified using the direction index (*DI*). For electrophysiology:

$$DI = \frac{(R_p - R_n)}{(R_p + R_n)}$$

where R_p and R_n are the spike numbers of the measured responses to moving light bars of the preferred and null directions, respectively. So, if a cell exhibits robust spike responses to the preferred direction movement, and shows weak or none responses to the motion in the null direction, the value of *DI* should be close or equal to 1. On the contrary, if two reverse directions with similar responses were chosen, the *DI* should be close or equal to 0. For dendritic contacts:

$$DI = \frac{(DCI_p - DCI_n)}{(DCI_p + DCI_n)}$$

The dendritic contact index (*DCI*) was defined as follows:

$$DCI = \frac{\frac{CONTACT}{DSGC} \times 100\%}{\frac{SAC}{AREA} \times 100\%}$$

where *AREA* is the total pixel number in an region we analyzed, *SAC* is the pixels that exceed threshold in the red channel (the SAC dendrites), *DSGC* is the pixels counted in green channel (the DSGC dendrites), and *CONTACT* is the co-existing pixels for both red and green (co-localization of the dendrites of the SAC and the DSGC). The dendritic contacts can be normalized through the computation of *DCI*. Thus, two regions may have different *DCI* value even they have the same amount of contacts, namely the *DCI* of the region with more dendrites (or larger measuring area) should be smaller, whereas the *DCI* of the less-dendrites (or smaller measuring area) region is larger. The cell pairs were divided into eight regions of different directions. The *DCI* of each region was

calculated. Suppose the response of direction selectivity is accomplished by the dendritic contacts, asymmetry of these *DCI*s should be found. The maximum *DCI* and two of its neighbor *DCI* were summed up and regarded as DCI_p, whereas the sum of the *DCI*s of the regions in the opposite direction was DCI_n.

For synaptic connections:

$$DI = \frac{(SCI_p - SCI_n)}{(SCI_p + SCI_n)}$$

The synaptic connection index (*SCI*) was defined as follows:

$$SCI = \frac{\frac{GABAR}{DSGC} \times 100\%}{\frac{SAC}{AREA} \times 100\%}$$

where *AREA* is the total pixel number in an region we analyzed, *SAC* is the pixels that exceed threshold in the red channel (the SAC dendrites), *DSGC* is the pixels counted in green channel (the DSGC dendrites), and *GABAR* is the blue pixels (the $GABA_A$ receptor puncta, and they were labeled in blue for the simplicity of calculation). The synaptic connections can be normalized through the computation of *SCI*. Thus, two regions may have different *SCI* value even they have the same amount of contacts, namely the *SCI* of the region with more dendrites (or larger measuring area) should be smaller, whereas the *SCI* of the less-dendrites (or smaller measuring area) region is larger. Likewise, the somata of SACs were positioned in the center, and the cell pairs were divided into eight regions of different directions. The *SCI* of each region was calculated. Suppose the response of direction selectivity is accomplished by the inhibitory synaptic connections, asymmetry of these *SCI*s should be found. The maximum *SCI* and two of its neighbor *SCI* were summed up and

regarded as SCI_p, whereas the sum of the *SCI*s of the regions in the opposite direction was SCI_n.