Supplemental information

Experimental Procedures

Animals

All experimental procedures were granted ethical approval by the animal care committee of the RIKEN Center for Biosystems Dynamics Research (BDR) and were carried out following local guidelines and adhering to the ARVO statement regarding the use of animals in ophthalmic and vision research. The C57BL/6J-Pde6brd1-2/rd1-2J/J mouse line (JAX stock #004766) were used as the model of end-stage retinal degeneration and crossed with B6;FVB-Tg(Pcp2-EGFP)2Yuza/J mice (JAX stock #004690) to generate the rd1;L7-GFP mouse line used in the current study (Tomomura et al., 2001) Unless otherwise specified, mice aged 8 to 13 weeks and of both sexes were used for transplantation. 2 animals were used for correlative array tomography, and 12 animals were used for Multielectrode Array (MEA) and confocal imaging.

ES cell lines

ROSA26$^{+/Nrl\text{-}CtBP2:tdTomato}$ is a *Rosa26* knock-in construct for expression of the CtBP2-tdTomato fusion protein under the Nrl promoter and was used to establish the Thy1-GCaMP6f;Ribeye-reporter ES cell line (Mandai et al., 2017). This cell line is referred to as “wildtype” cell line. Bhlhb4-/- and Islet1-/- lines were generated as previously described (Matsuyama et al., 2021). Briefly, a part of Bhlhb4 or Islet1 genomic region including the start codon was deleted using the CRISPR-Cas9 system (pSpCas9(BB)-2A-Puro (PX459), Addgene) in the wildtype ES cell line, and cells with homologous deletion were selected under puromycin.

Transplantation of 3D retinas

Maintenance, differentiation, and preparation of optic vesicles for transplantation of ES cell-derived retinas were the same as previously described (Assawachananont et al., 2014). Briefly, the neural retina (DD10-15), a characteristic continuous homogeneous epithelium region on the optic vesicle structure of organoids, was cut out at around 0.5 mm × 1 mm area on the day of transplantation. The small piece of neural retina was inserted subretinally with 1 mM valproic acid and x7 Viscoat (Alkcon) into the ventrotemporal side of the rd1;L7-GFP mouse eye, approximately 0.5-1 mm away from the optic disc, using a sharpened glass micropipette with a tip opening of approximately 500 μm diameter. Indomethacin (10 mg/L) was supplied in the drinking water of all transplanted mice starting on the day of transplantation. Optical coherence tomography and fundus images of transplanted eyes were acquired before sampling, and samples with unsuccessful transplantation such as apparent graft leakage in the vitreous space were excluded from the following analyses.

Correlative array tomography

Animals were anesthetized with sevoflurane and perfused with phosphate buffered saline (PBS) followed by fixation solution (4% paraformaldehyde (PFA) with 0.1% glutaraldehyde). After removal of the cornea and iris, the eyecups were immersion-fixed overnight at 4℃ with the same fixation solution.

For confocal imaging, the fixed eyecups were rinsed with PBS, embedded in 3% agarose-PBS, and sliced at 50 μm thickness using a Linear Slicer Pro7 (Dosaka EM). The sections mounted between cover slips with grid (Matsunami) were counter-stained with DAPI in PBS for confocal imaging before being replaced with fixation solution. For the control retina, we stained the retina with mouse anti-CtBP2 antibody (1:500, BD, #612044) at 4℃ for 5 days, washed with PBS , then stained with donkey A546 anti-mouse antibody (1:1000, Invitrogen, # A10036) at 4℃ for 2 days. Confocal imaging was performed using the Leica TCS SP8 microscope with 63X objective lens and zoom factor of 1.0-2.0. The resolution was 4096 × 4096, and the speed was set at 600 with a pinhole of 0.6 AU and the Z-step size was set as system-optimized.

For resin embedding, the fixed samples on the cover slips with grid were post-

fixed with 1% osmium and stained with 0.5% uranyl acetate overnight at room temperature, followed by dehydration with 50-100% ethanol and infiltration with TAAB 812 resin. The infiltration involved multiple steps, including 30% resin in propylene oxide, followed by 100% resin, and a final step of 100% resin with degassing. After infiltration, the samples were placed in embedding capsules and cured at 60℃ for 48 hours. Finally, serial semi-thin sections (500 nm thickness) were cut using a Histo knife (DIATOME) on a ultramicrotome (Leica Ultracut UCT) and placed on the slide glasses. For histological observation, the sections were stained with toluidine blue (TB). After that the sections were stained with 0.4% uranyl acetate and lead nitrate, followed by osmium coating using the osmium coater (HPC-SW: Vacuum Device). The sections were examined with a field emission scanning electron microscope (FE-SEM: SU 8220) with MirrorCLEM system (Hitachi High-Tech) by YAG-BSE, 5kV. For correlative array tomography technique, see also Toyooka and Shinozaki-Narikawa, 2019.

Electrophysiology: MEA recording and data analysis

The multi-electrode array (MEA) recordings were performed using the USB-MEA60-Up-System (Multi Channel Systems) as previously described (Tu et al., 2019; Watari et al., 2023; Yamasaki et al., 2022). Briefly, transplanted rd1 mice were used 8-9 weeks after transplantation. Mice were dark-adapted for 1-3 days and sacrificed by inhalation of isoflurane or sevoflurane immediately before cervical dislocation. Eyecups were prepared under dim red LED (peak wavelength 700 nm) and kept in oxygenated Ames' medium (Sigma-Aldrich) in the dark until use. The retina was carefully isolated with residual vitreous cleaned, and then mounted on the MEA probe (60MEA200/30iR-Ti-Gr) with the RGC side down. The engrafted area was recognized by its white, dotted appearance to be placed on top of the electrodes. Full-field light stimuli with 1 sec duration at different intensities covering low to high mesopic range were generated using a white LED (NSPW500C; Nichia Corp) and repeated before, during, and after L-AP4 (10 μM; Wako). Bright photopic stimuli (15.48 log photons/cm^2/s) were applied at the end of recordings to confirm the host RGC viability. Data were collected at a 20-kHz sampling rate without online filtering. The recorded spikes were sorted offline

using Spike 2 (version 7.2; CED). Recordings with the same stimulus intensity taken before, during and after L-AP4 treatment were merged to follow the spike trains from the same set of cells of each sample. The detected cells were then arbitrarily determined to be light responsive or unresponsive (including those having spontaneous spiking activity without clear correlation to the light stimulation onset/offset) according to their light response pattern during the full recording procedure spanning 3-4 hours in average.

Immunohistochemistry

Retinas after recording were carefully removed from the MEA probe and fixed in 4% PFA for 10-15 mins at room temperature. The retinas were then incubated with sheep mGluR6 antibody diluted in PBS containing 3% Triton X-100 at 4°C for 4 to 7 days, followed by 2 days for corresponding secondary antibody together with DAPI, then mounted with VECTASHIELD (H-1000, Vector). All staining procedures were performed with gentle mixing on a rocker. The mGluR6 antibody was a generous gift from Jeanie Chen, originally developed by Kirill Martemyanov lab (Cao et al., 2009). Fluorescence images were acquired with a Leica TCS SP8 confocal microscope and reconstructed in 3D image using Imaris Microscopy Image Analysis Software (http://www.bitplane.com/) and Fiji (ImageJ).

Semi-automated quantification of host-graft synapse

Images obtained from confocal imaging was imported to Imaris software for host-graft synapse quantification. For pre-synaptic marker detection, “spot” detection function was used for Ctbp2 channel to identify the center coordinates within 2 μm diameters with automated threshold. The threshold for intensity was manually adjusted for individual images for optimal visualization of the fluorescent signal. Next, we used “surface” function to define the surface of L7-GFP positive host rod bipolar cells. We used the following parameters for this step: surface area detail was 0.6, and the background selection was 2-12 depending on the signal intensity. The auto threshold was used with mild adjustment manually to match the intensity of each image. We then used the surface of L7-GFP to trim the post-synaptic marker mGluR6 channel. This step allows us to identify the mGluR6

signals expressed by host rod bipolar cells. The trimmed mGluR6 channel was used for identifying the post-synaptic marker coordinates. “Spot” detection function was used for trimmed mGluR6 channel to identify the center coordinates using 1.5 μm diameters with automated threshold followed by manual adjustment. Finally, we used “coloc” function to identify the pre- and post- synaptic marker pairs that were within 1 μm of each other and exported the coordinates as host-graft synapse. The host-graft synapses were checked manually to exclude the obvious errors.

Statistical analysis for synapse-function correlation

The spearman’s rank correlation coefficient between the host-graft synapse numbers within 75 μm radius of MEA electrode and the number of responsive RGCs from the electrode was calculated using R software.

References

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