Starburst amacrine cells amplify optogenetic visual restoration through gap junctions

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Abstract

Ectopic induction of optogenetic actuators, such as channelrhodopsin, is a promising approach to restore vision in the degenerating retina. However, the cell type-specific response of ectopic photoreception has not been well understood. It is not easy to obtain efficient gene expression in a specifically targeted cell population by a transgenic approach. In the present study, we established retinal ganglion cell (RGC)- and amacrine cell gene induction in a murine model with high efficiency using an improved tetracycline transactivator-operator bipartite system (KENGE-tet system). To investigate the cell type-specific visual restoration effect, we expressed the channel rhodopsin gene into RGCs and amacrine cells using this system. Then, enhancement of the visual restoration effect was observed by gene transfer not only to RGCs but also to starburst amacrine cells. It was suggested that photoresponse from amacrine cells enhanced the maintained response of ganglion cells and furthered the visual restoration effect.

Introduction

Inherited retinal degeneration is one of the major causes of vision loss. More than 2 million people worldwide are blind due to this disease¹, and there is still almost no effective treatment. Previous studies have reported visual restoration effects in animal models using various molecules as optogenetic actuators^{2–9}. In addition, clinical trials have also started using channelrhodopsin 2 (RST-001, ClinicalTrials.gov Identifier: NCT01648452), and Chrimson R (GS-030, ClinicalTrials.gov Identifier: NCT03326336), with gene transduction into RGCs by intravitreal injection of recombinant adeno-associated virus (rAAV). Although the visual reconstruction effects by optogenetic gene transfer, such as channelrhodopsin-2 into RGCs¹⁰ and ON-bipolar cells^{3,5,6,8,11}, have been shown, interactions between other types of cells in the retinal neural circuits in optogenetic visual restoration have not been well understood. Channelrhodopsin 2 conductance was reported to be 50–250 fS¹², indicating the need for sufficient gene expression to control the membrane potential. In this study, we employed a tetracycline-controllable gene expression system (tet system)¹³ in which the amount of gene expression has been much improved (KENGE-tet system)¹⁴, and we established sufficient gene induction in RGCs and amacrine cells simultaneously. To investigate cell type-specific visual restoration effects, channelrhodopsin2(E123T/T159C) was ectopically expressed into these cells by this system.

Then, we revealed that amacrine cells played a role in retinal neuronal circuits via gap junctions to enhance the optogenetic visual restoration effect.

Results

Gene induction of RGCs and SACs in M4-YC and RGCs in 5B-YC

We employed two different mouse lines that express the gene encoding the tetracycline transactivation (tTA) protein under the control of a cell-type-specific promoter, muscarinic acetylcholine receptor 4 (*Chrm4*)¹⁵ or 5-hydroxytryptamine (5-HT, serotonin) 5B receptor (*Htr5b*)¹⁴ control region: *Chrm4*-tTA or *Htr5b*-tTA. These mice were further crossed with another transgenic mouse line containing the yellow Cameleon-Nano 50 (YC) fluorescent gene connected into the downstream of the tet operator (tetO) promoter¹⁶. The YC gene expression was induced only by the presence of tTA protein in the double transgenic mice (*Chrm4*-tTA::tetO-YC (M4-YC) or *Htr5b*-tTA::tetO-YC (5B-YC)) (Figure S1A, B). The expression of YC was observed in the double transgenic mouse retina with a fluorescence microscope. In the M4-YC mouse retina, we identified the expression of YC in RGCs (Figure 1A-D, Figure S2A-E) and amacrine cells (Figure 1A-D, Figure S1E-F) with *in vivo* fluorescence microscopy and in sections. In the 5B-YC mouse retina, we identified the expression of YC in RGCs (Figure 1E-H) and the corneal stromal layer (Figure S2G-M) with *in vivo* fluorescence microscopy and in sections. In both lines, no expression was found

in displaced amacrine cells, only RGCs were labeled in the ganglion cell layer, and the percentage of YC-positive cells in RGC marker RBPMS¹⁷-positive cells was 35.4% (n=5) in M4-YC and 36.5% (n=5) in 5B-YC from RGCs, which were equivalent (Figure 1I-L, O-R, Figure S2M-Q). All of the amacrine cell expression in the M4 line was choline acetyltransferase (ChAT) staining-positive and consisted of starburst amacrine cells (SACs) (Figure 1M-T, Figure S3 A-L).

As a visual restoration model, the *Chrm4*-tTA and *Htr5b*-tTA line were crossed with tetO-channelrhodopsin2(E123T/T159C) (tetO-ChR2) mice¹⁸ (Figure S1C). *Chrm4*-tTA::tetO-ChR2 (M4-ChR2) drove ChR2 expression in both RGCs and amacrine cells and *Htr5b*-tTA::tetO-ChR2 (5B-ChR2) only in RGCs in the retina. In the retinas of these double transgenic mice, photoreceptor degeneration was induced by intraperitoneal injection of N-methyl-N-nitrosourea (MNU)¹⁹. MNU is an alkylating agent that causes DNA methylation in the O6 position of guanine, resulting in apoptosis, and it has been widely used to induce a pharmacological animal model of retinitis pigmentosa (RP). Two weeks after 75 mg/kg MNU administration into the mice at the age of 8 weeks, the outer nuclear layer containing photoreceptors was largely absent (Figure S4A, B), and the light-evoked response from photoreceptors was not detected by electroretinography (ERG) (Figure S4C).

M4-ChR2 mouse shows higher visual restoration effect.

To evaluate the function of the channelrhodopsin ectopically induced in the transgenic mouse retinas, we performed multi-electrode array (MEA) recording, which can record extracellular potentials of RGCs (Figure S4D). As a result of photoreceptor degeneration induced with MNU treatment, the control retina without channel-rhodopsin expression (tetO-ChR2) showed no response from RGCs detected by MEA (Figure 2A). In contrast, the M4-ChR2 (Figure 2B) and 5B-ChR2 (Figure 2C) retinas showed obvious light-induced responses. After filtering with the Gaussian function (Figure 2D, E), the maintained response after the peak was significantly greater in M4-ChR2 mice than in 5B-ChR2 mice (Figure 2F), indicating that the light-evoked ON response in RGCs could be modified through the response from amacrine cells.

To investigate whether light reception on the retina is transmitted to the visual cortex, we then examined visual evoked potentials (VEPs) from the visual cortex (Figure 2G). The output from the retinal ganglion cells is sent through the axons of the retinal ganglion cells (optic nerve) to the lateral geniculate nucleus (LGN) of the thalamus, which is a region of the mesencephalon, from the LGN to the primary visual cortex (V1) in the occipital lobe of the cerebral cortex. VEPs were not detected in the control tetO-ChR2 mice with MNU

treatment (Figure 2H, I). In contrast, VEPs were observed in both M4-ChR2 and 5B-ChR2 mice treated with MNU (Figure 2H, I). In response to the light stimulus at 4000 cds/m², the average of the VEP amplitude in MNU-treated M4-ChR2 mice was significantly higher (143.6 μ V; n=12, P=0.03) than in MNU-treated 5B-ChR2 (79.6 mV; n = 12) and the same level as in the mice without MNU treatment (155 μ V; n=4). However, the shapes of the waveforms were irregular in both models compared to controls, and their physiological roles are unknown. Thus, there is a limitation in this direct comparison.

To validate the model system, we examined the MEA, ERG and VEP of M4-ChR2 and 5B-ChR2 in the absence of retinal degeneration (no MNU treatment and no mutation) as well. As a result of MEA, the peak response was significantly reduced in both lines and was further significantly reduced with MNU treatment (Figure S4E). A similar trend was observed for the maintained response, whereas it was maintained in the M4 line (Figure S4F). This finding suggests that channelrhodopsin gene induction into healthy RGCs interferes with the physiological retinal light response. In addition, in the control mice, not only the ON response but also the OFF response was confirmed. In contrast, the OFF response was significantly lost in the M4 line (Figure S4H). It is known that SACs in the INL are connected to off bipolar cells²⁰, which might be related to this change. As a result

of ERG and VEP, both lines tended to have shorter latencies, especially in photopic examinations (Figure S5E, I, L, O), likely because of the channelrhodopsin response to strong light⁹ and its photoreception in RGCs resulting in short latency²¹. Although the amplitude of both ERG and VEP tended to be small in the 5B line (Figure S5C, G, M, N), these measurements were not significant, and there was no significant change in the shape of the waveforms.

Next, light-dark transition testing (LDT) was performed to investigate whether ectopic expression of channelrhodopsin in degenerative retinas leads to behavioral changes according to visual restoration. Rodents tend to stay in dark places according to their visual function since they are nocturnal and feel uneasy in bright environments (Figure 2J), while the visually disturbing MNU-treatment (Figure 2K) resulted in almost half of the staying time in bright and dark places (ratio in bright half at 10 min was closer to 0.5). In contrast, both the M4-ChR2 and 5B-ChR2 mouse lines with each MNU-treatment showed decreased staying time in bright places, indicating that visual restoration was confirmed in these models. Furthermore, M4-ChR2 mice showed significantly higher visual restoration effects than 5B-ChR2 mice (Figure 2K).

We also examined the optokinetic response (OKR) to investigate whether light receptivity

149 in the retina restored by ectopic channelrhodopsin expression led to central reflex movement
150 output. In OKR, a rotating striped pattern was displayed to the head-fixed mice to induce
151 eye movements, and the velocity was measured to evaluate the integrity of the subcortical
152 reflex circuitry of the mice (Figure S6A). OKR was not detected in the mice with MNU
153 administration except for the M4 line (4.80 deg/sec, n=6) (Figure S6B).

154 This outcome seems to be due to lack of ChR2 expression in SACs, which play a key role
155 in OKR²², in the retinas of 5B-ChR2 mice. We also examined *rdl* mice as a genetic animal
156 model of RP. These mice have a nonsense mutation in the *Pde6b* gene leading to rapid
157 degeneration of rod, photoreceptors followed by loss of cones^{23,24}. We used blind *rdl*
158 mutation mice at the age of 10-12 weeks for the following experiments. In the cases of *rdl*
159 mutant mice, no detectable OKR was observed in any combinations with each transgenic
160 line, including M4-ChR2. To investigate the dissociation of OKR between the MNU-
161 treatment and *rdl* models, the retinal thickness was compared under the conditions used for
162 the experiment. As a result, the total retinal thickness of *rdl* mice (78.3 μ m; n=9) was
163 significantly thinner than that of MNU-treated mice (97.2 μ m; n=10) evaluated on optical
164 coherence tomography (OCT) (Figure S6C, D). This outcome was thought to be because
165 the inner retinal layer was thinner of the *rdl* mouse (Figure S6E). Indeed, the inner nuclear
166 layer of the *rdl*, *rdl*;5B-ChR2 and *rdl*;M4-ChR2 mice was significantly thinner than that

of the MNU-treated mice (Figure S6F), suggesting that SACs in the inner layer were degenerated. This result suggests that residual INL thickness and its durability might be potential limitations of optogenetic gene therapy in inherited retinal disease patients clinically.

Acetylcholine and gap junctions were involved in the maintained response.

The M4-ChR2 mouse line, which showed transgene expression in amacrine cells in addition to RGCs, showed a higher maintained response and more effective visual restoration (Figure 2) than the 5B-ChR2 mice. Therefore, we investigated the neurocircuit pathway responsible for the enhanced response due to amacrine cells using neurotransmitter blockers on the MEA recording. Administration L-2-amino-4-phosphonobutyric acid (L-AP4), which is an agonist for group III metabotropic glutamate receptors, including mGluR6 working as a blocker of retinal ON-bipolar cells, did not show significant changes in either MNU-treated M4-ChR2 (Figure 3A-C) or 5B-ChR2 (Figure 3D-F) retinas, indicating that the photoresponse was not derived from photoreceptors. In contrast, administration of mefenamic acid (MFA) or carbenoxolone, an inhibitor of gap junctions, induced a significant decrease in the maintained response but not the peak of the response in the MNU-treated M4-ChR2 retina (Figure 3G-I, Figure S7A-C). This change was recovered after

washout and not observed in the 5B-ChR2 retina (Figure S7D-F). SACs release GABA and acetylcholine²⁵. Thus, we also examined an inhibitor of GABA receptor (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA), and a cholinergic antagonist, atropine. As a result, no significant change was observed with administration of TPMPA (Figure S7G-I), but atropine administration showed a similar decrease in maintained response and in peak response in the MNU-treated M4-ChR2 mice (Figure 3J-L). In addition, to determine whether this change occurred via bipolar cells, an AMPA receptor antagonist, 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX), and an NMDA receptor antagonist, D(-)-2-amino-5-phosphonopentanoic acid (D-AP5), were administered. As a result, no statistically significant difference was observed, although there was a tendency toward a decrease in the maintained response (Figure S7J-L). SACs form synapses with bipolar cells, RGCs, and other amacrine cells²⁵⁻²⁷. It is also known that amacrine cells are directly connected with RGCs through gap junctions^{28,29}, regulating neural circuits in the retina³⁰. Therefore, these results indicated that SACs enhanced the maintained response of RGCs directly or indirectly through acetylcholine and gap junctions.

MEA showed a visual restoration effect induced by ectopic ChR2 expression in the degenerative retina (Figure 2). These photoresponses obtained from MEA were all ON responses, as previously reported¹⁰³¹³²³³. Several slow photoresponses obtained from RGCs

were considered to have an ipRGC origin ³⁴, and they were excluded from the data. The MNU-treated M4-ChR2 retina showed a significant larger response in the maintained time phase compared to 5B-ChR2. As a result of a neurotransmitter blocking test (Figure 4), inhibition of acetylcholine and gap junctions decreased the maintained response of only M4-ChR2 retinas. The difference between M4-ChR2 and 5B-ChR2 was the presence or absence of ChR2 gene expression in SACs. Therefore, it was suggested that photoresponse from SACs enhanced the maintained response of RGCs, enhancing the visual restoration effect via acetylcholine and gap junctions.

Induction efficiency into SACs tended to affect visual restoration.

Finally, to investigate its clinical applicability, the effect of SACs on visual restoration was examined using rAAV2, which is already approved for gene therapy ³⁵. We injected rAAV2-CAG-ChR2-tdimer2 intravitreally into *rd/rd* mice and performed MEA, VEP, and LDT (Figure 4A, Figure S8A, B). In addition, we counted the number of transfected RGCs and SACs and analyzed their relevance to visual restoration effects. As a result of immunohistochemical labeling using RBPMS, the infection efficiency to RGCs was 24%, which was equivalent to previous reports (21-23%) ^{10,36,37}, and significantly less than those of our transgenic mice (Figure 4B, C). The efficiency to SACs was 12%, which was also

less than that of transgenic mice (Figure 4D, E). As a result of MEA recording, the peak response in the rAAV model was equivalent to that of both transgenic lines (Figure S8C), and the maintained response and its rate were equal to that of the M4 line (Figure S8D, E), perhaps because the input from various cell types other than SACs is also included in the rAAV model. This outcome suggests that the increase in the maintained response is not specific to gene transfer in SACs. Each average of the VEP amplitude in the *rdl*;M4-ChR2, *rdl*;5B-ChR2, and rAAV-treated *rdl* mice in response to the light stimulus at 4000 cds/m² was 72.7 μ V (n=10), 70.4 μ V (n=12) and 32.2 μ V (n=12), respectively. These responses were significantly higher than those in *rdl*;tetO-ChR2 mice (19.1 mV; n=8) but smaller than those in the 8-week-old wild-type C57BL/6J mice (155 μ V; n=4) (Figure 4F, G). There was no significant difference between *rdl*;5B-ChR2 and *rdl*;M4-ChR2 mice, and the VEPs of rAAV treated *rdl* mice were smaller than those of them (Figure 4F, G). Even in the LDT results, the restoration effect of rAAV-treated *rdl* mice tended to be lower than that of transgenic mice (Figure 4H), perhaps due to its infection efficiency, or the higher maintained response might not lead directly and simply to better visual restoration. In addition, the correlation between the infection efficiency of SACs in each rAAV-treated *rdl* mouse and the visual restoration effect was investigated. The results showed a significant, positive correlation between the number of transfected SACs in the inner nuclear layer (INL) and

the maintained to peak ratio in MEA (Figure 4I), a positive correlation between the VEP amplitude (Figure 4J) and a significant, negative correlation between the time spent in the bright half in LDT (Figure 4K). This outcome suggests that the maintained response in MEA is derived from SACs and that, clinically, including not only RGCs but also SACs is more effective for visual restoration. Although there is a limitation because infection efficiency is a confounding factor, there was no correlation between the number of transfected RGCs in GCL and the maintained to peak ratio in MEA (Figure S8F), and the correlation of the number of transfected SACs in the inner nuclear layer (INL) with the VEP amplitude (Figure 4J) and the time spent in the bright half in LDT (Figure 4K) was stronger than that with infection efficiency into RGCs (Figure S8G, H).

Discussion

In this study, we established transgenic mouse lines inducing gene induction in specific retinal cells using the tet-system (Figure S1). We identified that gene induction occurred in RGCs and SACs under the control of a muscarinic acetylcholine receptor *Chrm4* promoter, whereas a serotonin (5-HT) *Htr5b* receptor control region led to induction only in RGCs in the retina (Figure 2). Among acetylcholine receptors expressed by all types of neurons in the retina³⁸⁻⁴³, the *Chrm4* muscarinic receptor is expressed in RGCs and amacrine cells^{38,44}.

258 All of the amacrine cell expression in the M4 line was consisted of type-a SACs (Figure
259 1M-T, Figure S3 A-L). There are two subtypes of SACs, type-a and type-b (displaced
260 SACs)^{45,46}. This would be the first report of a transgenic line specific to only type-a but not
261 type-b SACs. The 5-HT *Htr5b* receptor is expressed in rodents but not in humans ⁴⁷.
262 Although several types of 5HT receptors are expressed in the mouse retina ⁴⁸, the retinal
263 distribution of *Htr5b* receptor has not been previously described to our knowledge. We
264 found that these gene inductions in both mouse retinas indicated that these strain are useful
265 for examining the functions of RGCs and SACs.
266
267 Accumulating data have shown that the visual restoration strategy induced by optogenetic
268 genes is a promising therapy for degenerative retinal diseases. Most of the amacrine cells
269 are inhibitory neurons in the vertebrate retina, which have not been regarded much in the
270 elementary visual restoration target. The results of this study showed that SACs increase
271 the maintained response through gap junctions and contribute to enhancing the visual
272 restoration effect. In particular, since the restoration effect of LDT and OKR was more
273 significant than that of VEP, it might contribute to sustained behavior and direction
274 recognition rather than transient response, consistent with the role of the maintained
275 response in RGCs ⁴⁹. Including the results using rAAV, in the current situation, viral

276 delivery in primates is limited³¹, and gene transfer involving amacrine cells, for example,
 277 using ubiquitous promoters, might be more effective in visual restoration than limiting
 278 RGCs in the clinical setting.

279

280 **Methods**

281 **Key Resources Table**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
N-Nitroso-N-methylurea	Toronto Research Chemicals	M325815
Domitor® (medetomidine hydrochloride)	Zenoac	
Antisedan® (atipamezole)	Zenoac	
Midazolam Sandoz™	Sandoz	
Vetorphal® (butorphanol)	Meiji Seika Pharma	
Ames' Medium	Sigma	A1420
L-AP4	Abcam	ab120002
Meclofenamic acid sodium salt	Sigma	M4531
TPMPA	TOCRIS	1040
CNQX	Abcam	ab120044
D-AP5	Abcam	ab120003
Carbenoxolone	MERCK	C4790
Atropine	MERCK	A0132
DAPI	Life Technologies	D21490
Anti-RBPMS antibody	Abcam	ab194213
Anti-Choline Acetyltransferase Antibody	Abcam	ab114p
Anti-VGluT2	Frontier Institute	VGluT2-Rb-Af720
Anti-Syntaxin antibody	Abcam	ab3265
Anti-Tyrosine Hydroxylase antibody	Abcam	ab112
Anti-Prox1 antibody	AngioBio	11-002P

pAAV CAG Chr2 E123T T159C 2AAddgene	#85399
tDimer	
Experimental Models: Organisms/Strains	
Mouse: C3H/HeJJcl	CLEA Japan
Mouse: tetO-YCnano50	RIKEN BRC
Mouse: tetO-ChR2 (E123T/T159C)	RIKEN BRC
Mouse: <i>Chrm4</i> -tTA	RIKEN BRC
Mouse: <i>Htr5b</i> -tTA	RIKEN BRC
Oligonucleotides	
Genotyping primer: tet-Foward: CATGAAGCAGCACGACTTCTT	
Genotyping primer: tet-Reverse: TTCTTACTTGTACAGCTCGTCCA	
Genotyping primer: M4-Foward: AAGCACCAAGTTCTCTCCCGTCTT	
Genotyping primer: M4-Reverse: CGGAGTTGATCACCTTGGACTTGT	
Genotyping primer: 5B-Foward: GCTCCAGGAAACCACAATGCCTTT	
Genotyping primer: 5B-Reverse: CGGAGTTGATCACCTTGGACTTGT	
Genotyping primer: <i>rd1</i> -Forward: AAGCTAGCTGCAGTAACGCCATTT	
Genotyping primer: wild-Forward: ACCTGCATGTGAACCCAGTATTCTATC	
Genotyping primer: <i>rd1</i> wild common-Reverse: CTACAGCCCCTCTCCAAGGTTTATAG	

282

283 **Animals**

284 Transgenic mice used for the experiments and their genotyping protocols were generated as

285 previously reported¹⁴. Mice homozygous for the retinal degeneration alleles *Pde6b^{rdl}*

(C3H/HeJcl, *rdl*) and WT C57BL/6J were obtained from CLEA Japan, Inc. Animals were maintained under 12-h light:12-h-dark conditions. For animals bred in house, littermates of the same sex were randomized to experimental groups. Mice used for the experiments were heterozygous for the tTA and tetO genes and homozygous for the *rdl* gene. All of the animal experiments were conducted in accordance with protocols approved by Institutional Animal Care and Use Committee of Keio University School of Medicine.

tetO-YC (RBRC09550), tetO-ChR2 (RBRC05843), *Chrm4*-tTA (RBRC09551), and *Htr5b*-tTA (RBRC05445) are available from RIKEN Bioresource Center in Japan.

Study approval: All of the animal experiments were conducted in accordance with protocols approved by Institutional Animal Care and Use Committee of Keio University School of Medicine (#2808).

Immunohistochemistry

The protocol for immunohistochemistry was previously described. The retinas were incubated in PBS with 1% Triton X-100 and 0.5% Tween 20 for 1 h at room temperature and in 4% BSA for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies: RBPMS (1:500, Abcam, Cambridge, UK), ChAT (1:100, Abcam), VGLUT2 (1:100, Frontier Institute, Hokkaido, Japan), syntaxin (1:100, Abcam), tyrosine

hydroxylase (1:100, Abcam) and Prox1 (1:100, AngioBio, San Diego, CA, USA) in blocking buffer. Secondary anti-rabbit, mouse and goat IgG, conjugated with Alexa TM488, TM594 and 633, respectively (1:1000; Molecular Probes), were applied for 1 h at room temperature. The retinas then were flat mounted, and the sections were mounted on slide glass.

Vector production and purification

pAAV-CAG-ChR2(E123T/T159C)-tdimer2-WPRE (Addgene, Watertown, MA) was used. Type 2 serotypes of rAAV vectors were prepared using the AAV Helper Free Packaging System (Cell Biolabs, San Diego, CA, USA). The serotypes were produced in HEK 293 cells using a helper virus-free system and were purified using two CsCl₂ density gradients and titrated by quantitative polymerase chain reaction. Final preparations were dialyzed against phosphate-buffered saline (PBS) and stored at -80°C.

Virus injection

The mice were anesthetized with a combination of midazolam, medetomidine and butorphanol tartrate at doses of 4 mg/kg, 0.75 mg/kg and 5 mg/kg of body weight and placed on a heating pad that maintained their body temperatures at 35–36°C throughout the

experiments. An aperture was made next to the limbus through the sclera with a 30-gauge disposable needle, and a 33-gauge unbeveled blunt-tip needle on a Hamilton syringe was introduced through the scleral opening into the vitreous space for intravitreal injections and introduced through the scleral opening along the scleral interior wall into the subretinal space for subretinal injections. Each eye received 1 μ l of vehicle (PBS) or vector at a titer of 2.0×10^{11} vg/ml.

Multielectrode array recordings

All of the procedures were performed under dim red light. The mice were anesthetized and euthanized by quick cervical dislocation. Following enucleation, the retina was dissected at room temperature in Ames' medium bubbled with 95% O₂/5% CO₂ (A 1420; Sigma-Aldrich). The separated retina was placed on a cellulose membrane, and RGC was directed to the electrode and was gently contacted against MEA (MEA2100-Systems; Multi Channel Systems, Reutlingen, Germany) under suction pressure. During the experiment, the retinas were continuously perfused with Ames' medium bubbling at 34 ° C. at a rate of 1-2 ml/min. Recorded signals were collected, amplified, and digitized using MC Rack software (Multi Channel Systems). Retinas were perfused for 30 min in darkness before recording responses. A 470-nm blue LED light at 1.0×10^{16} photons·cm⁻²·s⁻¹ stimulus was used. L-(+)-2-amino-

4-phosphonobutyric acid (L-AP4, ab120002; Abcam), (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA, Cat. No. 1040; Tocris Bio-Science), meclofenamic acid sodium salt (MFA, M4531; Sigma-Aldrich), atropine (A0132; MERCK) and carbenoxolone (C4790; MERCK) were newly diluted to 20 μ M, 20 μ M, 100 μ M, 20 μ M and 100 μ M respectively. Stimulation was presented for 1 seconds at 60-second intervals. Signals were filtered between 200 Hz (low cutoff) and 20 kHz (high cutoff). A threshold of 40 μ V was used to detect action potentials, and action potentials from individual neurons were determined via a standard expectation–maximization algorithm using Off-line Sorter software (Plexon, Dallas, TX, USA). The results were plotted using NeuroExplorer software (Nex Technologies Colorado Springs, CO, USA). Maintained-to-peak amplitude ratio was calculated by dividing the maintained response amplitude in maintaining time frame (0.4 to 1.0 seconds after light stimulation) by the peak amplitude (this ratio quantifies the sustenance of the response).

ERG analyses

Scotopic ERGs were recorded according to a previous report⁵⁰. Animals were dark-adapted for 12 h and prepared under dim red illumination. The mice were anesthetized with a combination of midazolam, medetomidine and butorphanol tartrate at doses of 4 mg/kg,

0.75 mg/kg and 5 mg/kg of body weight, respectively and were placed on a heating pad that maintained their body temperature at 35–36°C throughout the experiments. The pupils were dilated with a mixed solution of 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P; Santen, Osaka, Japan). The ground electrode was a subcutaneous needle in the tail, and the reference electrode was placed subcutaneously between the eyes. The active contact lens electrodes (Mayo, Inazawa, Japan) were placed on the corneas. Recordings were performed with a PuREC acquisition system (Mayo). Responses were filtered through a bandpass filter ranging from 0.3 to 500 Hz to yield a- and b-waves. White LED light stimulations of 10.0 log cd-s/m² were delivered via a Hemisphere LS-100 Stimulator (Mayo).

VEP analyses

The measuring electrodes were placed more than one week before the measurement. The mice were anesthetized with a combination of midazolam, medetomidine and butorphanol tartrate at doses of 4 mg/kg, 0.75 mg/kg and 5 mg/kg of body weight, respectively. The animals were placed in a stereotaxic holder. A stainless-steel screw (M1.0×6.0 mm) inserted through the skull into the both visual cortex (1.5 mm laterally to the midline, 1.5 mm anterior to the lambda), penetrating the cortex to approximately 1 mm, served as a measuring electrode.

376 At the time of the measurement, the mice were anesthetized again with the same doses.
377 Visual stimuli were generated by a white LED placed 3 cm from the eye. It was stimulated
378 with 100-ms pulses of white LED 4000 cds/m² light stimulus intensity. Signals were
379 acquired and analyzed with a PuREC acquisition system (Mayo). Signals were low-pass
380 filtered at 300 Hz and averaged over the 60 trials.

381

382 **OKR recording system**

383 Protocols for eye movement recording and visual stimulation were previously described⁵¹⁵².
384 Eye movements were recorded from both eyes of each animal separately. During the
385 recording, the contralateral eye was covered by aluminum foil. The head of the mouse was
386 fixed to an experimental steel board by the head-mounted stick for the LIM lens frames.
387 The reflected images through a hot mirror (43957-J, Edmund) were recorded using an
388 infrared CCD camera (BS-GV200, Libraly Inc., Tokyo, Japan). The images of the eye
389 movements were processed and analyzed using software (Move-tr/2D, Libraly Inc., Tokyo,
390 Japan). The sampling rate of the image was 200 Hz. The center of the pupil was detected by
391 software. We calculated the speed of the eye movements on two-dimensional images and
392 converted them to angular speeds using the AL of each eye. The spatial frequency was set
393 as 0.125 cycles/degree, and the temporal frequency of the visual stimulus was 1.5 Hz. The

motion onset delay (MOD) was set as 333 msec. Continuing the MOD, sinusoidal grating started to move clockwise in 5 sec. The intervals of visual stimulus were 60 sec. Eye movements were recorded three times for each experiment to exclude shaking images due to excessive body movements. Average velocities of the eye movements were calculated in the slow speed phase of their nystagmus.

LDT recording

Mice were tested in a 30 × 45 × 30-cm box, containing equally sized light and dark chambers connected by a 5 × 5-cm opening via which mice could move freely. The bright half of the box was illuminated from above by a white fluorescent light with intensity of 200 lux measured at the floor level. The animals were placed in the bright half and movement recorded (HD Pro Webcam C920, Logitech, Lausanne, Switzerland). A trial lasted 10 min, and then the testing apparatus was dismantled and cleaned with 70% ethanol. Videos were analyzed using ANY-maze tracking software and were validated by comparison with manual analysis. Time spent in the bright half was recorded.

Preparation of whole-mount samples and cryosections of retinas

Enucleated eyes were fixed for 20 min in 4% paraformaldehyde (PFA) in PBS and then dissected as previously described⁵³. The obtained tissues were post-fixed overnight in 4% PFA in PBS and stored in methanol at −20°C. Cryosections of retinas (12 mm) were prepared as previously described⁵⁴, after the eyeballs were immersed overnight in 4% PFA. Retinal sections were observed using a confocal microscope (LSM710; Carl Zeiss, Jena, Germany).

OCT Imaging

The thickness of the retina was analyzed by an SD-OCT system (Envisu R4310; Leica, Wetzlar, Germany) tuned for mice. The imaging protocol entailed a 3 mm×3 mm perimeter square scan sequence producing a single en-face image of the retina through a 50-degrees field of view from the mouse lens, following mydriasis. The en-face image consisted of 100 B-scan tomograms with each B-scan consisting of 1000 A-scans. The retinal thickness of 150 μm from the optic disc of each quadrant was measured.

Quantification and Statistical Analysis

427 All of the results are expressed as the mean $\pm$ SEM. The averaged variables were compared
 428 using the Student's 2-tailed t-test and the Kruskal Wallis one-way ANOVA test. P-values of
 429 less than 0.05 were considered statistically significant.

430

431 **Data and Software Availability**

432 Raw MEA spike data were sorted offline to identify single units using Offline Sorter
 433 software (version 4.4.0)(Plexon). Spike-sorted data were analyzed with NeuroExplorer 5
 434 software (version 5.115) (Nex Technologies).

435

Author Contribution

Y.K. and T.K. designed the research, wrote the manuscript, and performed the retinal histology, MEA, ERG and VEP recordings, and LDT experiments. K.K. performed AAV production. Y.K. and H.K. performed OKR recording. Y.K. performed data processing and analysis. H.O and T.K. made critical revisions of the manuscript. T.K. supervised the research.

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Declaration of Interests

The authors declare no competing interests.

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592

593

594 **Figures**

595 **Figure 1. Gene induction of RGCs and SACs in M4-YC and RGCs in 5B-YC**

596

597 In the M4-YC mouse retina, we identified the expression of YC (green) in RGCs and
598 amacrine cells within sections (A-D). In the 5B-YC mouse retina, we identified the
599 expression of YC (green) in RGCs in sections (E-H). Coexpression of the RGC marker
600 RBPMS in flat mounted retinas of M4-YC(I) and 5B-YC(J). Percentage of YC-positive
601 cells in RBPMS-positive (K) or DAPI-positive cells (L) and RBPMS-positive cells in YC-
602 positive cells (L) in both lines from confocal flat mounted GCL (n = 3 retinas each). Regions
603 were chosen in each quadrant, and we obtained RBPMS, DAPI-positive, YC-positive, and
604 co-labeled cells. Coexpression of the starburst amacrine cell marker ChAT in flat mounted
605 retinas of M4-YC in INL (M-O) and GCL (Q-S). Percentage of ChAT-positive cells in YC-
606 positive cells in M4-YC mice from INL (P) and GCL (R) (n = 3 retinas each). Error bars
607 represent the SEMs. INL, inner nuclear layer; GCL, ganglion cell layer, ONL, outer nuclear
608 layer.

609 Scale bar: 50 μm in (E). 100 μm in (B-D), (H), (I), (K), (M), (Q) and (R). 400 μm in (N).
610 500 μm in (F), (G), (O) and (P).
611

612 **Figure 2. M4-ChR2 mouse shows higher visual restoration effect.**

613

(A-C) Raster plots and peri-stimulus time histogram (PSTH) of light stimulation from MNU-treated tetO-ChR, M4-ChR2 and 5B-ChR2 mice. Light intensity was 13.6 log photons/cm²/s, and the duration was 1.0 second. (D, E) The averaged rate histogram after filtering with the Gaussian function from M4-ChR2 and 5B-ChR2 mice. The gray areas around the averaged traces represent the SEM. (F) Maintained-to-peak ratio of the spiking responses recorded. Maintained time frame is 0.4 to 1.0 seconds from light stimulation as shown in H (n = 7 retinas, 164 cells in MNU-treated M4-ChR2 mice, and n = 4 retinas, 127 cells in MNU-treated 5B-ChR2 mice). Error bars represent SEMs. ***p < 0.001. Student's 2-tailed t-test. (G) Schematic image of VEP measurement. (H) Representative VEP traces from MNU-injected and control mice. (I) The average amplitude of the VEPs in the control tetO-ChR mice (n = 4), MNU-treated tetO-ChR mice (n = 8), M4-ChR2 mice (n = 14), and 5B-ChR2 mice (n = 12) at 10 weeks of age. It was stimulated with 100-ms pulses of white LED 4,000 cds/m² light stimulus intensity. Signals were low-pass filtered at 300 Hz and averaged over the 60 trials. (J) Schematic image of the LDT. Mice were tested in a 30 × 45 × 30-cm box, containing equally sized bright (200 lx at ground level) and dark chambers connected by a 5 × 5-cm opening via, across which the mice could move freely. Visible mice feel uneasy in bright places, so staying time in the bright half gets shorter. (K) % time in bright half at 10 min of control (tetO-ChR2 mice) (n = 4), MNU-injected tetO-ChR2 mice

632 (n = 8), MNU injected M4-ChR2 mice (n = 15), and MNU-injected 5B-ChR2 mice (n = 8)
 633 measured from LDT. All error bars represent the SEMs. *p < 0.05, **p < 0.01, ***p < 0.001.
 634 One-way ANOVA testing.

Figure 3. Acetylcholine and gap junctions were involved in the maintained response.

(A, D, G, J) Mean $\pm$ SEM of exemplar cell response firing rate recorded during normal Ames' medium superfusion (left), in synaptic block (middle), and after washout (right). MNU-treated M4-ChR2 mice with L-AP4 block ($n = 3$ retinas, 47 cells) (A), MNU-treated 5B-ChR2 mice with L-AP4 block ($n = 3$ retinas, 45 cells) (D), MNU-treated M4-ChR2 mice with MFA block ($n = 3$ retinas, 85 cells) (G), MNU-treated M4-ChR2 mice with atropine

642 block (n = 3 retinas, 33 cells) (J). The gray areas around the averaged traces represent the
 643 SEM. (B, C, E, F, H, I, K, L) Averaged normalized peak firing rate and maintained rate.
 644 Maintained time frame is 0.4 to 1.0 seconds from light stimulation. Light intensity was 13.6
 645 log photons/cm²/s.
 646 All error bars represent the SEM. ***p < 0.001. One-way ANOVA and Tukey's test.
 647

Figure 4. Induction efficiency into SACs tended to affect visual restoration.

649

650 (A) The rAAV2-CAG-ChR2-tdimer2-WPRE expression cassette: ITR, inverted
 651 terminal repeat; WPRE, woodchuck hepatitis virus posttranscriptional regulatory
 652 element. (B-E) Coexpression of the RGC marker RBPMS (B) and starburst amacrine
 653 cell marker ChAT (D) and tdimer2 in flat mounted retinas of rAAV2-CAG-tdimer2-

654 WPRE treated retinas in *rdl* mice. (C, E) Percentage of YC-positive cells in M4-YC
655 and 5B-YC mice and tdimer2-positive cells in rAAV-treated mice in RBPMS-positive
656 (C) or ChAT-positive cells (E) from confocal flat mounted retina (n = 3 retinas each).
657 Regions were chosen in each quadrant, and we obtained RBPMS, ChAT-positive,
658 YC/tdimer2-positive, and co-labeled cells. (F, G) Average VEP traces (F) and
659 quantification of its amplitudes (G) from control (tetO-ChR2) (n = 3), *rdl::tetO-ChR2*
660 (n = 6), *rdl*;M4-ChR2 (n = 12), *rdl*;5B-ChR2 (n = 12) and rAAV treated *rdl::tetO-*
661 ChR2 mice (n = 12) at 10 weeks of age. It was stimulated with 100-ms pulses of white
662 LED 4,000 cds/m² light stimulus intensity. Signals were low-pass filtered at 300 Hz and
663 averaged over the 60 trials. (H) % time in bright half at 10 min in control (tetO-ChR2)
664 (n = 3), *rdl::tetO-ChR2* (n = 6), *rdl*;M4-ChR2 (n = 12), *rdl*;5B-ChR2 (n = 12) and
665 rAAV-treated *rdl::tetO-ChR2* mice (n = 12) measured from LDT. (I-K) Correlation
666 between transfection efficiency into SACs (tdimer2-positive cells/ChAT-positive cells
667 in INL) and maintained to peak rate (I) (n = 24), VEP amplitude (J) (n = 24) and % time
668 in bright half in LDT (K) (n = 12).

669 All error bars represent the SEMs. INL, inner nuclear layer; GCL, ganglion cell layer.
670 Scale bars, 50 μ m in (B), (D), n.s.: not significant, *p < 0.05, **p < 0.01, ***p < 0.001.
671 unpaired t-test (E), Games-Howell test (C, G, H), Pearson's correlation coefficient (I-K).