Improved retinal function in RCS rats after suppressing the over-activation of mGluR5

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Müller cells maintain retinal synaptic homeostasis by taking up glutamate from the synaptic cleft and transporting glutamine back to the neurons. To study the interaction between Müller cells and photoreceptors, we injected either DL-α-aminoadipate or L-methionine sulfoximine—both inhibitors of glutamine synthetase—subretinally in rats. Following injection, the a-wave of the electroretinogram (ERG) was attenuated, and metabotropic glutamate receptor 5 (mGluR5) was activated. Selective antagonism of mGluR5 by 2-methyl-6-(phenylethynyl)-pyridine increased the ERG a-wave amplitude and also increased rhodopsin expression. Conversely, activation of mGluR5 by the agonist, (R,S)-2-chloro-5-hydroxyphenylglycine, decreased both the a-wave amplitude and rhodopsin expression, but upregulated expression of Gᵦalpha subunit and phospholipase CβIII. Overexpression of mGluR5 reduced the inward-rectifying potassium ion channel (Kir) current and decreased the expression of Kir4.1 and aquaporin-4 (AQP4). Further experiments indicated that mGluR5 formed a macromolecular complex with these two membrane channels. Lastly, increased expression of mGluR5 was found in Royal College of Surgeons rats—a model of retinitis pigmentosa (RP). Inhibition of mGluR5 in this model restored the amplitude of ERG features, and reduced the expression of glial fibrillary acidic protein. These results suggest that mGluR5 may be worth considering as a potential therapeutic target in RP.
on the activity of rod bipolar cells\textsuperscript{17–19}. In addition, inhibition of GS by intravitreal injection of a GS inhibitor leads to decreased amplitude of the b-wave, but not the a-wave\textsuperscript{20}, which reflects the light-induced responses of photoreceptors\textsuperscript{21}. However, induction of glial dysfunction via the inhibition of GS affects the assembly and mosaic arrangement of photoreceptors\textsuperscript{22, 23}. Most importantly, the selective ablation of Müller cells results not only in apoptosis of the photoreceptors, but also in decreased amplitudes of both a- and b-waves in the ERG\textsuperscript{24, 25}. Here, we studied whether the light response of photoreceptors is affected by the inhibition of GS in Müller cells. We found that the ERG a-wave was attenuated after subretinal injection of GS inhibitor. We then investigated the cause of this a-wave attenuation. Glutamate-mediated excitotoxicity is known to be linked to photoreceptor loss in retinal degeneration\textsuperscript{26}. And over-activation of mGluRs has been reported to contribute to the pathogenesis of glaucoma and other neurological disorders\textsuperscript{11, 27}. We therefore hypothesized that mGluR5 over-activation, due to excessive synaptic glutamate, might mediate the effects of GS inhibition. Our results showed that mGluR5 was activated after GS inhibition, and a-wave amplitude was regulated by mGluR5 activity. In addition, mGluR5 was over-activated during the degeneration of RCS rats (a model of RP). Moreover, inhibition of the over-activated mGluR5 in this model restored the amplitude of the ERG. These results suggest that mGluR5 may be worth considering as a potential therapeutic target in RP.

**Figure 1.** Subretinal DL-AAA injection caused reduced ERG a-/b-wave amplitude and reduced expression of GS. (A) Representative light-evoked ERG waveforms measured at 7 days and 10 days following subretinal injection of DL-AAA (red traces) or PBS control (blue traces) with six different light intensities (from \(-4.5\) to \(1\) log(cd*m/s\(^2\))). (B) Top row: Average stimulus-response curves for a-wave amplitude in DL-AAA treated and PBS-treated eyes at 7 and 10 days post-injection (n = 6 per data point). Bottom row: The same for b-wave amplitude. (C) Expression of GS protein (green) 7 days after injection of PBS (left) or DL-AAA (right). Nuclear counterstain with DAPI (blue). Scale bar = 50 µm. (D) GS mRNA expression in Müller cells following DL-AAA treatment (n = 3 per group). (E) Top: Representative western blotting for GS protein expression following DL-AAA treatment. GAPDH as loading control. Bottom: Quantification of GS protein expression by western blotting (n = 3 per group). Data are shown as mean ± SEM. *p < 0.05, **p < 0.01. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.
Because DL-AAA has other effects on the glutamate-glutamine cycle, beyond GS inhibition 28–31, we used another specific GS inhibitor, L-methionine sulfoximine (MSO), to test the relationship between the disruption of GS and the attenuation of ERG waves (Fig. S2). Our results confirmed that both a- and b-waves decreased 7 days after subretinal injection of MSO, but the ratio of b-wave to a-wave was unchanged (Fig. S1B).

A decrease in expression of GS in Müller cells following DL-AAA injection has previously been reported32, 33. To examine this further, cryostat sections of rat retinas, 7 days after injection, were treated with fluorescent-labelled anti-GS antibodies, and the immunostaining pattern was measured using a fluorescence microscope. A decline in expression of GS protein was observed at day 7 post DL-AAA injection (e.g. Fig. 1C). These results were further supported by reverse-transcription polymerase chain reaction (RT-PCR) and western blotting experiments (Fig. 1D and E). In summary, GS expression can be reduced by DL-AAA administration, and thus the glutamate-glutamine cycle may be compromised by DL-AAA administration.

What causes the changes in the ERG amplitude after DL-AAA delivery? It has been previously demonstrated that GS activity influences the uptake of glutamate34 and the recycling of glutamate is disrupted after inhibition of the GS20, which may lead to the over-activation of group I metabotropic glutamate receptors expressed on Müller cells in a model of chronic ocular hypertension (COH) 11. To investigate the molecular pathways in Müller cells that lead to decreased ERG amplitude, we injected DL-AAA, and studied the effect on mGluR5 and mGluR1 expression using immunofluorescence staining, RT-PCR, and western blotting (Figs 2 and S3).

Using immunofluorescence staining, we found that mGluR5 expression was increased 7 days after DL-AAA injection, compared to PBS control (e.g. Fig. 2A and B, red channel). Colocalization of mGluR5 (red) and CRALBP, a fluorescent stain for Müller glia (green), was most prominent in the outer plexiform layer (OPL) and ganglion cell layer (GCL) (Fig. 2A and B, right column).

Western blotting demonstrated that mGluR5 protein expression was 87% ± 19% higher in the DL-AAA treated eyes than the PBS control eyes (p = 0.0015, by paired T-test; Fig. 2C and D). Quantitative real-time PCR (RT-PCR) showed that mGluR5 mRNA expression was 31% ± 5.3% higher in the DL-AAA group (p = 0.028 vs. PBS control, by paired T-test; Fig. 2E). In support of these results, treatment with MSO produced a similar (60%) increase in mGluR5 mRNA expression 7 days after injection (p = 0.0072 vs. PBS, by paired T-test, Fig. S2C).

By contrast, there was no significant effect of DL-AAA on either mGlur1 mRNA transcription (p = 0.7479 vs. PBS control, by paired T-test; Fig. S3), or mGluR1 protein expression (p = 0.1504 vs. PBS control, by paired...
T-test; Fig. S3). Taken together, these results indicated that inhibiting GS in Müller glia increased their expression of mGluR5, but not mGluR1.

We speculated that the significant increase in mGluR5 expression may be functionally associated with the diminished amplitude of the ERG. To confirm this, an agonist and an antagonist of mGluR5–(R,S)-2-chloro-5-hydroxyphenylglycine (CHPG) and 2-methyl-6-(phenylethynyl)-pyridine (MPEP) respectively–were injected into the subretinal space of separate groups of RDY rats.

Firstly, we injected CHPG (or PBS control) and recorded ERG (e.g. Fig. 3A) at 30 min, 2 days and 10 days post-injection (n = 6, 11, 6 rats for each time point, respectively). At 30 min (0.02 days) and 10 days post-injection, no significant differences were found between the two groups in either the a-wave or b-wave amplitude for any light intensity. At 2 days post-injection, both the a-wave and the b-wave amplitudes of the CHPG group were significantly decreased at all except the lowest light intensity (p < 0.05 or 0.01 vs. PBS control, by paired T-tests; Fig. 3B), but the ratio of b-wave to a-wave amplitude was unchanged (Fig. S1D,E).

We also performed experiments where we injected DL-AAA plus either MPEP or a PBS control in the contralateral eye, and recorded ERG (e.g. Fig. 3C). At 2 days post-injection, both the a-wave and the b-wave amplitudes of the CHPG group were significantly decreased at all except the lowest light intensity (p < 0.05 or 0.01 vs. PBS control, by paired T-tests; Fig. 3B), but the ratio of b-wave to a-wave amplitude was unchanged (Fig. S1D,E).

To explore this hypothesis, we plotted a-wave vs. b-wave amplitude for data from 93 rats treated with PBS (Fig. S1E). The data were fitted using least squares linear regression, with an R² value of 0.954.

Next, we analyzed the oscillatory potentials (OPs) of the ERG, which are generally thought to originate in the inner retina35, 36. Following either DL-AAA, CHPG or DL-AAA + MPEP treatment, no significant differences were seen in the OPs to b-wave amplitude ratio, compared to PBS control (p > 0.05 for all comparisons; Fig. S4).

We hypothesized that the changes in the ERG amplitude in response to DL-AAA, MSO, CHPG, or MPEP, were mediated by an alteration in expression of the photopigment protein, rhodopsin. To test this, we examined rhodopsin protein expression using immunofluorescence (Fig. 4A–D), and rhodopsin mRNA expression using RT-PCR, following the drug treatments already outlined above. Rhodopsin mRNA expression was significantly lower in eyes treated with DL-AAA, CHPG or MSO, compared to PBS controls (p < 0.05 vs. PBS control, by paired T-test; Figs 4E and S2D). However, rhodopsin mRNA expression was significantly increased following MPEP treatment (p = 0.0015 vs. PBS control, by paired T-test; Fig. 4E). Taken together, we concluded that the
changes seen in light responses of the outer retina were mediated by changes in rhodopsin expression, and that this was in turn regulated by mGluR5.

Group I mGluRs are found in both neurons and astrocytes, and mediate their effects partly via an intracellular second-messenger cascade that leads to increased concentration of intracellular calcium ions ([Ca^{2+}]_i) (summarized in Fig. 5A). We hypothesized that this second-messenger cascade was being activated in Müller glia by DL-AAA. To test this, we injected Rdy rats subretinally with CHPG, MPEP or DL-AAA (and PBS controls in contralateral eyes). Two days (with CHPG and MPEP) or seven days (with DL-AAA) later, we harvested the retinas, and performed western blotting for G_{αq} and PLCβIII on whole-retina lysates. Treatment with CHPG and DL-AAA led to significantly increased expression of both G_{αq} and PLCβIII (p < 0.01 for each combination vs. PBS control, by paired T-test, Fig. 5B–D), while treatment with MPEP group led to decreased expression of both

**Figure 4.** Rhodopsin expression is reduced by DL-AAA and CHPG but increased by MPEP treatment. (A) Representative immunofluorescence images showing expression of rhodopsin (green) following injection of PBS (control). Nuclear counterstain with DAPI (blue). Scale bar = 50 µm. (B) Following DL-AAA injection (7 days). (C) Following CHPG injection (2 days). (D) Following MPEP injection (2 days). (E) Rhodopsin mRNA expression following treatment with DL-AAA, MPEP or CHPG, vs PBS control (n = 3 per bar). Data are shown as mean ± SEM. *p < 0.05, **p < 0.01. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segment.
proteins (p < 0.01 for both vs. PBS control, by paired T-test, Fig. 5B–D). Taken together, these results confirm that pharmacological modulation of mGluR5 altered the expression level of downstream signaling proteins.

An AQP4/Kir 4.1/mGluR5 macromolecular complex is expressed on Müller cells. We next studied the relationship between mGluR5 and inward-rectifying potassium channels (Kir) as described previously. Firstly, we first studied the Kir channel currents using whole-cell voltage-clamp recordings of cultured rat Müller cells, both before and after delivery of DL-AAA (Fig. 6A), MPEP, or CHPG (in the extracellular bath medium). Current rose with voltage, but decreased dramatically upon addition of DL-AAA or CHPG. After washout, the amplitudes of the K+ currents in the Müller cells were almost fully restored (n = 3, Fig. 6B and C). In contrast, K+ current amplitude was increased after addition of MPEP, and this returned fully to normal after MPEP was washed out (Fig. 6D).

We next studied the expression of Kir 4.1 specifically, as well as aquaporin-4 (AQP4) which is suggested to have a close relationship with Kir 4.1 on Müller cells. Using western blotting, we found that the expression of AQP4 and Kir 4.1 was significantly decreased following subretinal injection of DL-AAA or CHPG (p < 0.01 for each vs. PBS control, by paired T-test; Fig. 7A–C). By contrast, the expression of AQP4 and Kir 4.1 was significantly increased following MPEP treatment (p < 0.01 for each vs. PBS control by paired T-test; Fig. 7A–C).

Previous studies have shown that, in the adult retina of mouse, AQP4 and Kir 4.1 proteins are tightly co-localized on the membranes of Müller cells. mGluR5 is known to interact with many molecules, including AQP4, and perform a wide array of vital tasks by forming a macromolecular complex/transporting microdomain in astrocytes. Within the retina, Müller cells are thought to have several functions similar to astrocytes. Therefore, we hypothesized that mGluR5 might also form a complex with AQP4/Kir 4.1 in retinal Müller cells. To study this, we performed immunoprecipitation assays using an mGluR5 antibody on retinal tissue. These were then immunoblotted with antibodies for AQP4 and Kir 4.1. We found that AQP4 and Kir 4.1 co-immunoprecipitated with mGluR5 (Fig. 7D), suggesting that mGluR5 does directly interact with AQP4/Kir 4.1 molecules in Müller cells.

Inhibition of mGluR5 restored the ERG b-wave and reduced the expression of GFAP in RCS rats. Our results suggested that Müller cells indirectly regulate the response of photoreceptor cells to light stimulation via modulation of mGluR5. Given the potential therapeutic implications of these findings, we next wanted to study the properties of mGluR5 in a model of RP, a condition of degenerative visual loss. To do this, we studied the Royal College of Surgeons (RCS) rat model, a classical animal model of RP, involving Mer tyrosine kinase (MerTk) gene expression defects.
We first tested the expression of mGluR5 in RCS rat retinas at post-natal day twenty (P20), P30, P40, P60 and P90, using western blotting and RT-PCR. We observed significantly increased mGluR5 protein expression in RCS rats at P60 and P90, compared to an RDY control group (p < 0.01 at both time points, by unpaired T-test) and significantly decreased expression at P20 (p < 0.01 compared to control, by unpaired T-test; Fig. 8A and B). Furthermore, mGluR5 mRNA expression in RCS rats at P30, P40, P60 and P90 was significantly higher than that in RDY rats (p < 0.05 or 0.01 at each time point, by unpaired T-test) (Fig. 8C). These results suggested that mGluR5 was overexpressed in RCS rats, particularly at the later stages of retinal degeneration.

Next, we studied the effect of mGluR5 inhibition on the ERG in RCS rats. We studied the eyes of RCS rats that had been subretinally injected with MPEP (with PBS in the contralateral eye as a control) at P35. We performed ERG studies at 2, 4, 7, 14, 21 and 28 days post-injection (n = 7 for each group). At 4 days post-injection, the...
b-wave amplitudes of the MPEP group were significantly larger than PBS controls at the five highest light intensities (p < 0.05 or 0.01, by paired T-tests; Fig. 8D and E). No significant differences were seen in a-wave amplitude. Also, at 7, 14, 21 and 28 days after injection, no significant differences in the b-wave were found between the MPEP-treated eyes and PBS-treated eyes (p > 0.05 for each time point; Fig. 8F).

Finally, we studied glial fibrillary acidic protein (GFAP), which is reported to aggravate the degeneration of neurons in the neural degenerated diseases41. For example, GFAP expression has previously been reported to be reduced in a rat model of COH after inhibition of mGluR5 11. We studied whether GFAP expression was reduced in the RCS rat retina by MPEP treatment. We found that there was reduced expression of GFAP at 4 days following MPEP injection (Fig. 8G). Furthermore, GFAP mRNA expression was also significantly reduced following MPEP treatment (p = 0.0005 vs. PBS control, by paired T-test; Fig. 8H). In contrast, the level of mRNA expression for GS, K_4.1, AQP4 and rhodopsin was significantly higher following MPEP treatment (p < 0.01 for each protein vs. PBS control, by paired T-tests; Fig. 8H).

In summary, we found that the amplitude of ERG b-waves were restored on days 2 and 4 after inhibition of the over-activated mGluR5 in RCS rats. And over the same period the expression of GFAP was reduced.

Discussion
The aim of this study was to determine whether, and how, Müller cells regulate the light response of photoreceptors following the inhibition of glutamine synthetase (GS). Our results demonstrate several findings that have not been previously reported.

The first of these findings is that the ERG a-wave exhibited changes at the various time points following the delivery of DL-AAA or MSO. It has been previously reported that DL-AAA or MSO decrease the b-wave of the ERG, but without an effect on the a-wave30,32. There may be a number of reasons why we have seen a new effect in this study. One reason may be that the toxic effect of DL-AAA is dose-dependent41. To ensure that we were effectively inhibiting Müller cells, we examined the effects of various concentrations (7, 70 and 140 μg/μl) of DL-AAA on the ERG (Fig. S5). The amplitudes of the a- and b-waves of ERGs did not change when DL-AAA was administered at the concentration of 7 μg/μl. However, when the concentration reached 140 μg/μl, the amplitudes of the ERG a- and b-waves were significantly decreased, or even disappeared. Additionally, the half-decay
time of the normalized waveform (T1/2) – which was determined from the start time to the time at which the normalized b-wave decayed to half of its peak – was delayed in the DL-AAA injected eyes. These results suggested that not only the outer but also the inner retina was affected by DL-AAA. Therefore, the optimum dose of DL-AAA required for photoreceptor disruption was determined to be 70 µg/µl. Another reason is that the effects of DL-AAA also depended on time, and the functional and morphological changes were differed at the same time point. Our ERG recordings suggested both the a- and b-waves induced by DL-AAA were significantly reduced at 7 days after administration compared with the controls. Finally, these waves began to recover approximately 10 days later. These results are consistent with results reported in carp32. However, it has been reported that there is a decrease in GS immunoreactivity at 4 days after DL-AAA injection33. Our results demonstrated that the reduction in GS activity could be viewed 1 day after DL-AAA delivery, and they were restored approximately 14 days later (Fig. S6). It was recently demonstrated that the retina fails to recover normal histological morphology following subretinal injection of DL-AAA into mice41. A possible explanation is that the retinas between rats and mice differ. Additionally, the different routes of injection and animal models have different influences on the toxic effects of DL-AAA. The most common route of drug delivery to the retina is intravitreal injection. Intravitreally injected DL-AAA causes loss of the ERG b-wave but not the a-wave in the Cyprinus carpio32. DL-AAA delivered into the intravitreal space is likely to escape from the vitreous more easily than macromolecular drugs43. To overcome the barrier properties of RPE and the retinal inner limiting membrane, subretinal injection can be utilized. Subretinal administration of DL-AAA can alter the blood-retinal barrier in rats and eliminate photoreceptors in monkeys33, 44. Our results suggested that ERG a-waves and the expression of GS reduced at 7 days after subretinal injection of 70 µg/µl DL-AAA to the rats. The effects of MSO were the same as that of DL-AAA. It has been previously reported that ERG b-waves decreased 40 min and 90 min after intravitreal injection of MSO, but with slight a-wave enlargement45. The activity of GS was also disrupted at 12 hours after injection20.

The second major finding of this study is that mGluR5 contributed to the light responses of the outer retina. In the present study, we demonstrated that mGluR5 was activated after injection of DL-AAA/MSO, which may have been as a result of increased synaptic glutamate, due to reduced glutamate uptake by Müller cells44. Selective activation of mGluR5 by CHPG reduced the ERG a-wave amplitude at two days post-injection, which suggests that the functions of photoreceptors were influenced by mGluR5 activity. Additionally, the selective inhibition of mGluR5, by MPEP, rescued the loss of a-waves caused by DL-AAA. These results suggest that mGluR5 may mediate the changes in ERG caused by DL-AAA/MSO.

Our immunofluorescence data showed that mGluR5-positive cells in the retina co-localized with Müller cell markers. In addition, our electrophysiological findings in cultured Müller cells showed that DL-AAA and CHPG decreased the amplitude of K+ currents. This is consistent with previous studies which have shown that Group I mGluRs in Müller cells from rat and salamander retina modulate K+ currents, and evoke intracellular calcium waves46, 47. These results suggest that mGluR5 is expressed by Müller cells.

Our immunohistochemical data also demonstrated that the expression of mGluR5 was localized in both the OPL and the IPL, which is consistent with a previous report that mGluR5 is present in the dendritic tips of bipolar cells of the OPL and in the amacrine cell processes of the IPL48. Therefore, subretinal injection of CHPG may not only activate mGluR5 in Müller cells, but also that expressed elsewhere in the retina. However, the unchanged OP to b-wave ratio and b-wave to a-wave ratio indicate relatively little contribution of neurons in the inner retina to the increased/decreased a-waves induced by MPEP/CHPG. Finally, we found that the expression of rhodopsin was downregulated by the activation of mGluR5, which illustrates a mechanism by which mGluR5 activation can reduce the light response of photoreceptors.

A third major finding from this study is that mGluR5 regulated both K+ currents and glutamate transporters in Müller cells, and that these proteins together formed a macromolecular complex. We demonstrated that the expression of mGluR5 was increased after subretinal injection of DL-AAA or CHPG, and the expression levels of its downstream molecules, Gq/11 and PLCβ1/3/IV, were markedly increased thereafter. These results are similar to the Ca2+-dependent PI-PLC/ PKC signaling pathway that is involved in the mGluR1-mediated suppression of K+ currents in COH rats49. Over-activation of mGluR5 was proven in our experiment to decrease the expression of K+4.1 channels, which is consistent with the statement that the expression of K+4.1 in cultured rat Müller cells is inhibited after activation of the group I metabotropic glutamate receptor40.

The decreased expression of K+4.1, caused by mGluR5, may result from the increased expression of Gq/11, because it has been previously reported that the expression of the K+4.1 channel is modulated by multiple neuro-transmitters via Gq-coupled receptors46, and both mutant Ca2+-sensing receptors and activated Gq/11 suppress the expression of K+4.1 in HEK-293 cells50. It is noteworthy that downregulation of K+4.1 and the opening of cation channels inhibit voltage-dependent glutamate uptake45.

Furthermore, we found that the activation of mGluR5 also decreased the expression of AQP4. Previous research suggests that activation of the ERK1/2 pathway decreases the expression of AQP4 in cultured astrocytes46, although further studies are needed to explore the interaction between mGluR5 and AQP4. Recent studies have reported that the uptake of glutamate is downregulated in primary cultured astrocytes from AQP4−/− mice51. Additionally, the retinas of AQP4−/− mice exhibit lower levels of GS and higher expression levels of glutamate than those of wild type mice under light damage52, 53. Therefore, we speculate that decreased expression of K+4.1 and AQP4 caused by the activation of mGluR5 resulted in a decrease in glutamate uptake, and this protected the Müller cells against the toxicity which normally results when the glutamate concentration increases dramatically in the Müller cells after the inhibition of GS20.

Fourthly, we demonstrated in our study that a macromolecular complex of mGluR5/Kir4.1/AQP4 exists in Müller cells. Previous studies have consistently shown that mGluR5 interacts with AQP4 by forming a macromolecular complex in astrocytes46, and that AQP4 and K+4.1 expression are tightly co-localized in Müller cells37, 38. However, the contribution of K+4.1 to the ERG is the slow PIII wave, which is a negative component that is secondary to the a-wave46, 54. Delayed b-waves have also been observed in patients who carry the Kir4.1 mutant55.
the right eye, and the same volume of phosphate buffered saline (PBS) was injected into the contralateral eye through the sclera at the level of the temporal peripheral retina. In each animal, 4 μm (CHPG, 200 μmol) and L-methionine sulfoximine (MSO, 20 mM) were individually administered via subretinal injection. Prior to use, CHPG was dissolved in 0.5 M NaOH and then neutralized using 0.5 M HCl. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where specifically mentioned.

In this study, we also evaluated the effects of inhibitors, (R,S)-2-chloro-5-hydroxyphenylglycine (CHPG), 200 μmol and L-methionine sulfoximine (MSO, 20 mM) on mGluR5 activation. CHPG and MSO were individually administered via subretinal injection. A 150 μl volume was injected into each eye after the subretinal injection, and the animals were then immediately sacrificed.

Electroretinography (ERG). ERG was performed as described previously. Briefly, after 12 hours of adaptation to darkness, the animals were prepared for recording under dim red-light (wavelength > 620 nm). Light stimuli were delivered at intensities of −4.5, −3.5, −2.5, −1.5, −1.0, −0.5, −0.2, and −0.1 log scotopic trolands (trolands). The inter-stimulus interval was varied between 30 and 120 s, depending on the light intensity. Data were output to a computer and processed and analyzed using MATLAB 2010b (MathWorks, MA, USA) and Microsoft Excel (Microsoft Corporation, WA, USA). The amplitude of the a-wave was calculated as the maximum negative trough below the baseline, and the b-wave was measured from the a-wave trough to the maximum subsequent positive peak. To extract oscillatory potentials (OPs), raw ERG signals were bandpass filtered at 60–300 Hz (Butterworth filter, 5th order), and the amplitude of each OP was calculated from the amplitude of the largest OP wavelet.

Immunofluorescence staining. Immunofluorescence staining of frozen sections was performed as described previously. Briefly, the retinas of RCS-rdy rats (RDY rats, postnatal day 20, 30, 40, 60 and 90; either sex) and RCS-rdy rats (RDY rats, postnatal day 20, 30, 40,60 and 90; either sex) were used in this study and were provided by the Animal Center of the Third Military Medical University, Chongqing, China. Unless specifically mentioned, all rats used in the article were control rats (RDY rats). All experiments were conducted in accordance with the ‘ARRIVE’ guidelines (NC3Rs, London, UK; https://www.nc3rs.org.uk/arrive-guidelines).

Materials and Methods
All procedures were conducted with the approval of the Third Military Medical University Animal Care and Use Committee. RCS-rdy-p+ (Royal College of Surgeons rat) and RCS-rdy-p+ rats (RDY rats, postnatal day 20, 30, 40, 60 and 90; either sex) were used in this study. All reagents and materials, were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where specifically mentioned.

Animal model and drug administration via subretinal injection. All surgical procedures were performed under anesthesia with intramuscular injection of pentobarbital sodium (10 mg/kg), and the fundus was also examined using direct ophthalmoscopy.

DL-α-aminoadipate (DL-AAA) was prepared in accordance with established procedures. Different concentrations of DL-AAA solution (7 μg/μl to 140 μg/μl) were injected into the subretinal space under dim-red light (wavelength > 620 nm). Specifically, DL-AAA was injected using a fine glass microelectrode (tip < 10 μm) through the sclera at the level of the temporal peripheral retina. In each animal, 4 μl of DL-AAA was injected into the right eye, and the same volume of phosphate buffered saline (PBS) was injected into the contralateral eye as a control.

Similarly, 2-methyl-6-(phenylethynyl)-pyridine (MPEP, 200 μmol), (R,S)-2-chloro-5-hydroxyphenylglycine (CHPG, 200 μmol) and L-methionine sulfoximine (MSO, 20 mM) were individually administered via subretinal injection. Prior to ERG, CHPG was dissolved in 0.5 M NaOH and then neutralized using 0.5 M HCl. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where specifically mentioned.

Co-immunoprecipitation. For mGluR5 co-immunoprecipitation, retinas were briefly sonicated in 2 ml radio immunoprecipitation assay (RIPA) buffer (PBS with 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), a cocktail of protease inhibitors and phosphatase inhibitors (1 mM levamisole, 2 mM Na3VO4, 1 mM NaF). Anti-mGluR5 antibody (described in Table 1) was

Only b-waves are significantly reduced in the AQP4 knockout mice, especially in the older mice. These results of previous studies demonstrate that the individual contributions of Kc.4.1 and AQP4 to the ERG a-waves can be largely ignored. The decreased light responses of the photoreceptors caused by activating mGluR5 may result from the dysfunction of glutamate uptake and degradation.

Finally, the ERG of RCS rats could be restored by inhibition of the over-activated mGluR5. We observed that mGluR5 was over-activated in the RCS rat during degeneration, especially at late stage, which may result from disrupted glutamate homeostasis. However, the protein level of mGluR5 at P20 and P30 in the RCS rats was reduced when compared with the control groups, which was not consistent with increased mGluR5 mRNA expression level. Further studies are needed to explore the reason for these differences. Most importantly, we found that the b-wave amplitude was increased after inhibiting the upregulated mGluR5 in the RCS rats, which suggests that inhibition of mGluR5 could rescue visual function of RCS rats. The increased b-wave amplitude may result from the improved light responses of photoreceptors. Although no significant differences were found in the a-wave amplitude between the two groups, we speculate that our findings may result from the amplification effects between photoreceptors and bipolar cells. Last, the expression of GFAP, characteristic of gliosis during retinal degeneration, was downregulated after MPEP delivery, which is consistent with a previous report in a COH rat model. This decreased gliosis may also contribute the increased ERG amplitude.
incubated with the crude retinal lysate and immunoprecipitated with protein-G agarose and antibodies, according to the manufacturer's instructions (shown in Table 1). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described below.

**Quantitative real-time PCR and western blotting.** To detect mRNA and protein expression levels of GS, rhodopsin, mGluR1, mGluR5, K_+, AQP4 and GFAP, we performed RT-PCR and western blotting, as has been described previously. For RT-PCR, the primer pairs used are described in Table 2. The change in expression in the target gene in the experimental group compared to the control group was calculated as a ‘fold’ change, as follows:

\[
\text{fold change} = 2^{-(\Delta \Delta CT, \text{Exp} - \Delta \Delta CT, \text{Ctrl})}
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For quantitative western blotting, an Odyssey system (V1.2.15; Li-Cor, NE, USA) was used to scan the nitrocellulose membranes for GS, rhodopsin, mGluR1, mGluR5, K_+, AQP4 and GFAP, or β-actin bands. The relative level of each of these proteins was obtained by calculating the ratio of the protein to GAPDH (or β-actin), according to the densitometry for semi-quantification (Full-length blots/gels are presented in Supplementary 2).

**Primary Müller cell culture.** The eyes from P7–8 RCS-rdy^-+^ rats were enucleated and incubated for 6–8 h in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, MA, USA). The eyecups were transferred to dissociation solution (DMEM containing 1% penicillin/streptomycin, 1% glucose and 20% fetal bovine serum (FBS)) and incubated at 37 °C in a CO₂ incubator for 1 h. Eyecups were then washed with DMEM containing 10% FBS and 1% antibiotic-antimycotic mixture, and the retinas were dissected with care to avoid contamination of the retinal pigment epithelium (RPE) and ciliary epithelium. Next, the retinas were mechanically dissociated into small aggregates and cultured in DMEM containing 10% FBS. After 7 days, the floating retinal aggregates and debris were removed, leaving the Müller cells attached to the bottom of the dish. The cells were trypsinized and cultured in DMEM containing 10% FBS for another 5 days, to obtain a further-purified population, and this process was performed twice (in total).

| Antibody | Company | Titre | Species | Cat# | Purpose |
|----------|---------|-------|---------|------|---------|
| Goq      | Santa Cruz | 1:50/1:100 | rabbit | sc-393 | IF/WB   |
| PLCβIII  | Santa Cruz | 1:50/1:100 | rabbit | sc-403 | IF/WB   |
| mGluR1   | Millipore | 1:500/1:1000 | rabbit | mab9448 | IF/WB   |
| mGluR5   | Abcam    | 1:500/1:1000 | rabbit | ab53090 | IF/WB   |
| mGluR5   | Abcam    | 1:100/1:200 | rabbit | ab27190 | IP      |
| AQP4     | Abcam    | 1:100 | mouse | ab9512 | WB      |
| K_+      | Santa Cruz | 1:100 | goat | sc-23637 | WB      |
| GFAP     | Abcam    | 1:500 | rabbit | Ab2760 | IF      |
| GS       | Millipore | 1:600 | mouse | mab302 | IF/WB   |
| CRALBP   | Abcam    | 1:200 | mouse | ab15051 | IF      |
| GAPDH    | Coelvo, China | 1:2000 | rabbit | cw0101 | Internal control |
| β-actin  | Coelvo, China | 1:1000 | mouse | cw0096 | Internal control |
| Secondary antibodies Cy3 (mouse) | Beyotime, China | 1:500 | goat | A0521 | IF      |
| Secondary antibodies Cy3 (rabbit) | Beyotime, China | 1:500 | goat | A0516 | IF      |
| Secondary antibodies Cy3 (goat) | Beyotime, China | 1:500 | donkey | A0502 | IF      |
| Secondary antibodies Cy3 (mouse) | GeneTex | 1:500 | donkey | GTX85338 | IF      |
| Secondary antibodies FITC (goat) | Santa Cruz | 1:200 | donkey | SC-2024 | IF      |
| Secondary antibodies FITC (mouse) | Zhongshan Goldenridge, China | 1:200 | goat | ZF-0312 | IF      |

**Table 1.** Antibodies used in immunofluorescence staining, western blotting and immunoprecipitation.

| Genes (rat) | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| mGluR5      | ATGGAGAAAGTGGATGGAGGCTT | ACCACCCGGGCTTTAGTTAAGTA |
| GS          | TCACAGGGACAAAATGCGAGG | GTTGATGGTGGGAGTTCGAG |
| Rhodopsin   | GCCATTTCTGCTTGAGTTGAGT | CTGGCTTTCTGAGTTGAG |
| K_+        | CAAAGATTGCCGCCAAGAAGAAGA | TGGGTTGAAAGCATGTTGAGTGC |
| AQP4        | GGACTGGAGCTTCGGAGAGTATTA | GAAATCTGAGGGCCAGTCTAG |
| GFAP        | TCTCAGGGAAAGGCAAACAGAG | CAGCCTCAGGGTGGTTCAT |
| GAPDH       | AGACACCGCGCATCCTCTGTTG | TGGATGGGAACATGTCG |

**Table 2.** Design of RT-PCR primers.
Whole-cell patch-clamp recordings. Whole-cell patch-clamp recordings were performed as previously described. Briefly, cultured Müller cells were continuously perfused with a solution containing 135 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 11 mM glucose, 1 mM sodium pyruvate, 0.5 mM tetrodotoxin (TTX), and 10 mM sucrose, adjusted to pH 7.4 with NaOH.

Patch pipettes were made by pulling borosilicate glass tubes (WPI products, FL, USA). The impedance of the pipette was typically 4–6 MΩ after filling with an internal solution containing 20 mM NaCl, 130 mM potassium gluconate, 1 mM CaCl₂, 2 mM MgCl₂, 1 mM ethylene glycol-bis(3-aminoethly ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM HEPES, 0.1 mM guanosine triphosphate-Na, and 2 mM adenosine triphosphate-Mg, adjusted to pH 7.2 with KOH.

Whole-cell membrane currents were recorded from Müller cells using an Axopatch 200B amplifier in voltage-clamp mode (Axon Instruments, CA, USA) and Clampex computer software (Molecular Devices, CA, USA). Signals were low-pass filtered at 1, 2, or 6 kHz (eight-pole Bessel filter) and digitized at 5, 10, or 30 kHz, respectively, using a 12-bit analog-to-digital converter. All recordings were performed at room temperature (20–25 °C).

Statistics. All quantitative data are presented as the mean and standard error of the mean (mean ± SEM). Means were compared statistically using paired two-tailed T-tests, except for the experiments comparing RCS to RDY (control) rats, where unpaired two-tailed Student’s T-tests were used. Values of p < 0.05 were considered significant.

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