

Müller glia cell cycle re-activation by simultaneous cyclin D1 overexpression and p27^{Kip1} knockdown promotes retinal regeneration in mice

Reviewed Preprint

v1 • August 28, 2024

Not revised

Zhifei Wu, Baoshan Liao, Julia Ying, Jan Keung, Zongli Zheng, Virpi Ahola, Wenjun Xiong 

Department of Biomedical Sciences, City University of Hong Kong, Hong Kong, China • Ming Wai Lau Centre for Reparative Medicine, Karolinska Institutet, Hong Kong, China • Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland

 https://en.wikipedia.org/wiki/Open_access
 Copyright information

Abstract

Harnessing the regenerative potential of endogenous stem cells to restore lost neurons is a promising strategy for treating neurodegenerative disorders. Müller glia (MG), the primary glial cell type in the retina, exhibit remarkable regenerative abilities in lower vertebrate species, such as zebrafish and amphibians, where injury induces MG to proliferate and differentiate into various retinal neuron types. The regenerative potential of mammalian MG is constrained by their inherent inability to re-enter the cell cycle, likely due to high levels of the cell cycle inhibitor p27^{Kip1} and low levels of cyclin D1 observed in adult mouse MG. In this study, we found that adeno-associated virus (AAV)-mediated cyclin D1 overexpression and p27^{Kip1} knockdown exerts a strong synergistic effect on MG proliferation. MG proliferation induced by this treatment was potent but self-limiting, as MG did not undergo uncontrolled proliferation or lead to retinal neoplasia. Single-cell RNA sequencing (scRNA-seq) revealed that cell cycle reactivation leads to immunosuppression and dedifferentiation of MG. Notably, scRNA-seq analysis identified a new cluster of rod-like MG cells expressing both rod and MG genes, which was further validated by RNA in situ hybridization. Cell cycle reactivation also led to de novo genesis of bipolar- and amacrine-like cells from MG. Overall, our findings suggest that AAV-mediated cyclin D1 overexpression and p27^{Kip1} knockdown stimulate MG proliferation and promote MG reprogramming. This approach may be a promising strategy, especially when combined with other regeneration-promoting factors, to enhance MG-mediated retinal repair.

eLife assessment

The manuscript presents a potentially **important** strategy to stimulate mammalian Müller glia to proliferate in vivo by manipulating cell cycle components. The findings are likely to appeal to retinal specialists and neuroscientists in general. However, the evidence that these cells become neurogenic is lacking/**incomplete**, suggesting that additional barriers exist to stimulate the regeneration of retinal neurons.

<https://doi.org/10.7554/eLife.100904.1.sa3>

Introduction

Müller glia (MG) is the last cell type generated by the retinal progenitor cells (RPCs) during development and exhibit a gene expression profile similar to that of late retinal progenitor cells (Jadhav et al., 2009; Roesch et al., 2012). In teleost fish and amphibians, MG respond rapidly to retinal injury, undergoing robust proliferation and regenerating lost retinal neurons from MG-derived progenitor cells (Hamon et al., 2016; Todd and Reh, 2022). In contrast, the proliferative and neurogenic ability of MG in response to injury in mammals is severely limited, failing to mediate retinal self-repair (Karl et al., 2008). Recent investigations have achieved great success in mammalian retinal regeneration by direct conversion of MG to retinal neurons through a single or combination of neurogenic transcription factors (Jorstad et al., 2017; Levi et al., 2021; Todd et al., 2022; Yumi et al., 2015). However, this may lead to a depletion of the MG population and cause further retinal degeneration, as MG are indispensable for retinal function and homeostasis. Other studies have shown that the quiescent state of MG can be overridden by upstream signaling pathways such as Wnt and Hippo (Hamon et al., 2019; Rueda et al., 2019; Yao et al., 2016). Activation of Wnt signaling by forced expression of β -catenin in adult mouse MG promoted spontaneous cell cycle re-entry in uninjured retinas (Yao et al., 2016). Moreover, it was demonstrated that bypassing the Hippo pathway in mouse MG led to spontaneous re-entry into the cell cycle and reprogramming into a progenitor cell-like state (Hamon et al., 2019; Rueda et al., 2019). These findings suggest that the re-entry of MG into the cell cycle, which is the first step of MG-mediated retinal regeneration in zebrafish, could be unlocked in mammalian retinas.

The cell cycle of MG is mainly regulated by cyclins and cyclin-dependent kinases (CDKs). Cyclins bind to CDKs to form cyclin-CDK complexes to promote cell cycle progression. During retinal development, the expression of D-type cyclins (cyclin D1, D2, and D3) is tightly regulated (Barton and Levine, 2008; Dyer and Cepko, 2001; Trimarchi et al., 2008). Among these, cyclin D1, encoded by the *Ccnd1* gene, is the predominant D-type cyclin in the developing retina and is highly expressed in the RPCs but absent in differentiated cells (Barton and Levine, 2008; Trimarchi et al., 2008). Mice lacking *Ccnd1* have small eyes and hypocellular retinas due to reduced RPC proliferation (Fantl et al., 1995; Sicinski et al., 1995), which cannot be compensated by *Ccnd2* and *Ccnd3* (Das et al., 2012, 2009). Negative regulators of the cell cycle are CDK inhibitors (CDKIs), which include the INK4 (p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}) and CIP/KIP families (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) (Reynisdóttir et al., 1995). The CDKIs inhibit cell cycle progression by binding to and inactivating the cyclin-CDK complexes (Besson et al., 2008), and they regulate the proliferation in distinct retinal progenitor cells (Dyer and Cepko, 2000a, 2001; Levine et al., 2000). p27^{Kip1} inhibits the cyclin D-CDK complex to enter of the S phase. Following acute retinal damage in mice, a very small number of MG re-enter the cell cycle, co-incident with the downregulation of p27^{Kip1} or upregulation of cyclin D1 (Dyer and Cepko, 2000b; Hamon et al., 2019; Rueda et al., 2019; Yao et al., 2016). However, this process is transient, as cyclin D1 expression rapidly returns to the basal level (Dyer and Cepko, 2000; Hamon et al., 2019a; Rueda et al., 2019).

In this study, we propose that targeting the two key regulators of the cell cycle, cyclin D1 and p27^{Kip1}, can effectively induce MG cell cycle re-entry. Our findings demonstrate that simultaneously reducing p27^{Kip1} and increasing cyclin D1 in the MG using a single AAV vector has a strong synergistic effect on promoting MG proliferation in uninjured adult mouse retinas. MG proliferation induced by this treatment is robust and self-limiting, as MG undergo a single round of cell division rather than unlimited proliferations. Through single-cell RNA sequencing (scRNA-seq), we observed that cell cycle reactivation leads to immunosuppression and dedifferentiation of the MG. Interestingly, scRNA-seq analysis also showed that a new cluster of rod-like MG cells that express both rod and MG genes was induced by the treatment.

RNA in situ analysis further confirmed that MG cells in the outer nuclear layer (ONL) express high levels of rod gene *Rho* and *Gnat1* while maintaining the expression of MG gene *Glul*. Additionally, a few EdU+ MG daughter cells in the inner nuclear layer (INL) express the bipolar cell marker *Otx2* or the amacrine cell marker *HuC/D*, suggesting de novo neurogenesis from MG. These results indicate that cell cycle reactivation also promotes MG reprogramming. Importantly, MG cell cycle reactivation does not disrupt retinal structure or impair retinal function, as the treatment did not deplete the MG from the retina. In summary, our results showed that MG cell cycle reactivation by downregulating p27^{Kip1} and upregulating cyclin D1 stimulates MG proliferation and promotes cell fate reprogramming, and it is possible to combine this approach with other factors that promote regeneration in order to enhance retinal repair mediated by MG.

Results

Simultaneous p27^{Kip1} downregulation and cyclin D1 overexpression drive robust MG proliferation in the uninjured mouse retina

To test the hypothesis that adult mouse MG are kept in a quiescent state by high levels of p27^{Kip1} and low levels of cyclin D1, we examined whether spontaneous MG proliferation can be activated by directly changing the levels of these two downstream cell cycle regulators. In this study, we used adeno-associated virus (AAV) serotype 7m8 vector and a promoter sequence cloned from the human *glial fibrillary acidic protein* (GFAP) gene to drive MG-specific gene expression (Fig. 1A, Supplementary Fig. 1) (Lee et al., 2008). AAV7m8 vectors were injected intravitreally into the eyes of C57BL/6 mice on postnatal day 6 (P6), when MG finish proliferation and start differentiation, and intraperitoneal injections of 5-ethynyl-2'-deoxyuridine (EdU) were given for five consecutive days to label the MG that had entered the S phase of the cell cycle (Fig. 1B). In the control retinas infected with AAV7m8-GFAP-GFP- nontarget (NT) short hairpin RNA (shRNA) virus, all MG, which were identified by Sox9 expression aligning in the inner nuclear layer (INL), were negative of EdU labeling (Fig. 1C). When the retina was infected by AAV7m8-GFAP-mCherry-p27^{Kip1} shRNA, which expresses a highly efficient p27^{Kip1} shRNA, a small number of MG cells re-entered the cell cycle (Fig. 1D, G, Supplementary Fig. 2); however, these proliferated MG accounted for less than 1% of the total MG population. Similarly, overexpressing cyclin D1 alone through AAV7m8-GFAP- cyclin D1 infection stimulated a subset of MG cells to proliferate, resulting in a three-fold increase in the number of EdU+ MG cells compared to p27^{Kip1} knockdown (Fig. 1E, G, Supplementary Fig. 3). Finally, the AAV7m8-GFAP-cyclin D1-p27^{Kip1} shRNA vector, which simultaneously overexpressed cyclin D1 and suppressed p27^{Kip1}, had the most significant impact on MG proliferation, with a five-fold increase in EdU+Sox9+ cells compared to cyclin D1 overexpression alone (Fig. 1F, G, whole retinal cross-section shown in Supplementary Fig. 4). These findings suggest that both low levels of p27^{Kip1} and high levels of cyclin D1 are required for postmitotic MG to re-enter the cell cycle. Henceforth, we refer to this AAV vector that enables robust MG proliferation through concurrent p27^{Kip1} knockdown and cyclin D1 overexpression as the cell cycle activator (CCA).

A transgenic mouse line *Glast-Cre^{ERT2}; Sun1:GFP*, in which MG nuclei were labeled by nuclear membrane-bound GFP (Supplementary Fig. 5), was used to quantify the percentage of MG that re-entered the cell cycle. In the retinal area where virus infection rate was the highest, approximately 45% of Sun1:GFP+ MG were EdU positive (Fig. 1H), and the total number of MG increased by about 50% (Fig. 1I), indicating that nearly half of the MG cells re-entered the cell cycle. Previous research has shown that the ability of retina regeneration by various stimuli declines with the age of the mice (Löffler et al., 2015; Ueki et al., 2015). We compared the efficiency of CCA in driving MG proliferation in young (P28) and aged (>8 months) mouse retinas to that in P6 pups.

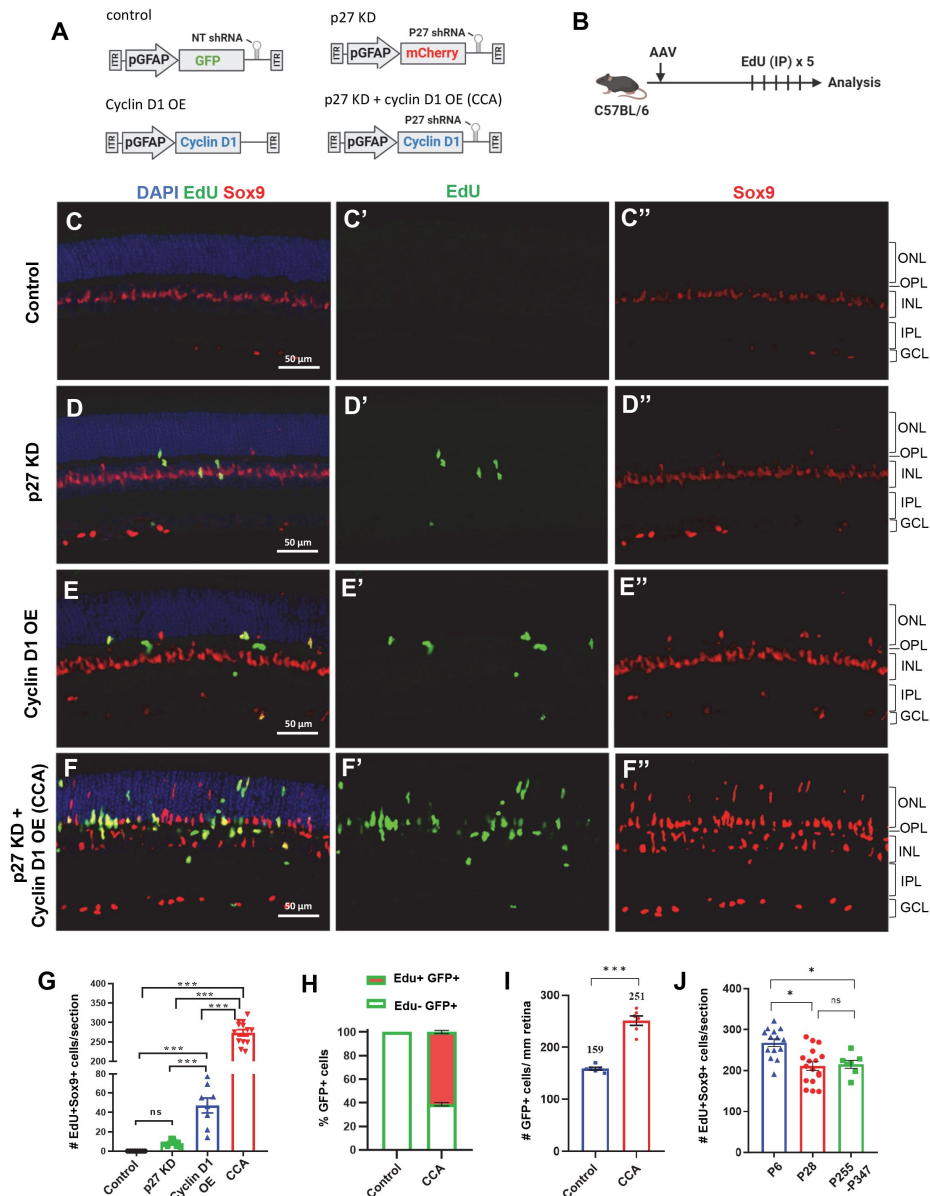


Figure 1.

Simultaneous p27^{Kip1} downregulation and cyclin D1 overexpression drive robust MG proliferation in the uninjured mouse retina

(A) Schematic of AAV vectors used in this study. (B) Experimental design. (C-F) Analysis of EdU incorporation. Uninjured mouse eyes were injected with (C) AAV-GFAP-mCherry-non target (NT) shRNA (control), (D) AAV-GFAP-mCherry-p27 shRNA, (E) AAV-GFAP-cyclin D1, and (F) AAV-GFAP-cyclin D1-p27 shRNA (CCA) at P6 and harvested at P18 after 5-day EdU intraperitoneal injection. Retinal sections were co-labeled for MG marker Sox9. (G) Quantification of EdU+ Sox9+ cells. AAV-GFAP-mCherry-NT shRNA control virus (n=8), AAV-GFAP-mCherry-p27shRNA (n=11), AAV-GFAP-cyclin D1 (n=8), and CCA (n=14). (H) Quantification of the percentages of EdU+GFP+ and EdU-GFP+ cells in the area with efficient virus infection in the *Glast-CreERT2*; *Sun1:GFP* mouse retina injected with the control or CCA vector. (I) Quantification of the total GFP+ MG in the *CreERT2*; *Sun1:GFP* retina infected by CCA. Retinal area (250 μ m in width) with the most efficient virus infection in the middle retina region was selected for quantification. (J) Quantification of EdU+ Sox9+ cells in the young (CCA injection at P6, n=13), adult (CCA injection at P28, n=17), and aged retinas (CCA injection at P255-P347, n=7). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Data are presented as mean $\pm$ SEM. * $P < 0.05$; *** $P < 0.001$; ns=not significant by one-way ANOVA analysis with Tukey's post-hoc test (G, J) and two-tail unpaired student t-test (I).

Remarkably, MG proliferation induced by CCA remained robust in young adult and aged mice, with more than 200 EdU+Sox9+ cells per section (**Fig. 1J**). These findings suggest that CCA efficiently drives MG proliferation in both young and aged animals.

MG proliferation driven by CCA is self-limiting

Concerned that p27^{Kip1} suppression and cyclin D1 overexpression may lead to uncontrolled cell proliferation and retinal tumorigenesis, we analysed the proliferative capacity of MG driven by CCA. Firstly, we examined the duration of MG proliferation after CCA treatment by a time-course EdU incorporation assay. On various days after the CCA injection, EdU was administrated to label the cells that were undergoing proliferation (**Fig. 2A**). The result revealed that MG proliferation started as quickly as the third day after CCA injection, reached its peak around the fifth day, followed by a gradual decrease (**Fig. 2A**). By two weeks post CCA injection, only a few MG were observed to have re-entered the cell cycle. Two months post-CCA injection, MG proliferation had mostly ceased (**Fig. 2A**). These findings suggest that MG proliferation was largely completed within 2 weeks post-CCA treatment, probably due to the dilution of AAV episomal genome copies in the dividing cells. In addition, an EdU/BrdU double-labeling assay was performed to examine whether MG undergoes one or multiple cell divisions after CCA treatment. A single injection of EdU was given at 7 days post CCA treatment, followed by BrdU injection 24 hours later (**Fig. 2B**). Retinas were collected 2 days after BrdU injection to evaluate if any MG continuously entered the S phase of the cell cycle. While there were a number of EdU+ cells and BrdU+ cells, few cells were co-labeled with EdU and BrdU (**Fig. 2B,C**), indicating that MG undergoes only one cell division subsequent to CCA treatment.

Next, we examined the distribution of the proliferated MG in the retina over time. As the labelling efficiencies of MG by Tamoxifen-induced reporter expression and by EdU varied among experiments and individual animals, we used the percentages of EdU+GFP+ MG in each retinal layer to assess the distribution of the proliferated MG. During retina development, proliferating retinal progenitor cells migrate in an apical-basal manner, whereas post-mitotic cells migrate basally to reach their final laminar position (Lee and Norden, 2013). The proliferating MG undergo similar interkinetic migration towards the apical surface of the retina, as majority of the EdU+GFP+ MG nuclei were observed in the ONL and OPL (~53% and ~22%, respectively) at one week after CCA treatment, which is around the peak time of MG proliferation (**Fig. 2D,E**). To further explore whether the ONL MG daughter cells eventually return to the INL over an extended period, we examined the distribution of EdU+ MG cells at three weeks and four months post-CCA treatment. We found that while the percentage of INL EdU+ MG increased slightly over time, there was still about half of EdU+ MG remained in the ONL and in the OPL (**Fig. 2D,E**). Across the three time points examined, the absence of EdU+GFP+ MG in the IPL and GCL suggests that migration of MG daughter cells to the GCL does not occur, at least without injury (**Fig. 2D,E**). These findings led us to investigate whether the long-term localization of MG daughter cells in the ONL and OPL is associated with any change in their cell fate.

Cell cycle re-activation drives downregulation of IFN pathway and MG dedifferentiation

To unbiasedly assess the cell fate of MG and their daughter cells, we performed single-cell RNA-Seq (scRNA-Seq) analysis on the mouse retinas that received CCA treatment. AAV7m8- GFAP-cyclin D1-p27^{Kip1} shRNA and AAV7m8-GFAP-GFP-NT shRNA (9:1 mixed) were co- injected to stimulate MG proliferation in 4-week-old *Glast-Cre^{ERT2};tdTomato* mice (**Fig. 3A**), and 10% AAV7m8-GFAP-GFP-LacZsh was added to serve as an indicator of infection success. The control group was injected with AAV7m8-GFAP-GFP-NT shRNA to account for any non- specific effect caused by virus injection and/or shRNA expression. Three weeks after virus injection, the retinas with successful viral transduction were collected, and tdTomato+ cells were isolated by fluorescence-activated cell sorting (FACS), followed by scRNA-seq (**Fig. 3A**). As previous studies demonstrated that N-methyl-D-aspartate (NMDA)-induced retinal damage and histone deacetylase inhibitor Trichostatin

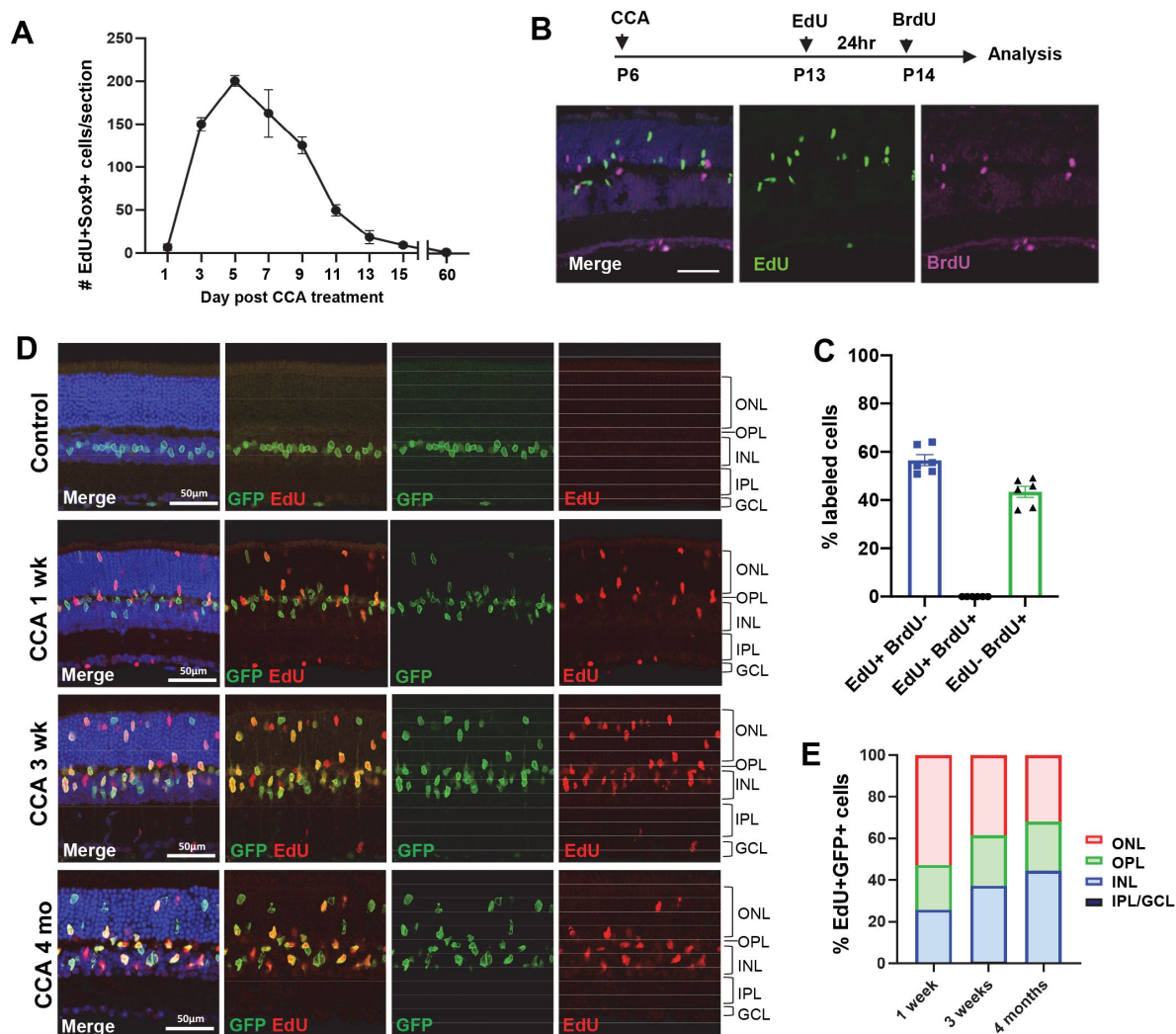


Figure 2.

MG proliferation driven by CCA is self-limiting

(A) Time-course analysis of MG proliferation after CCA injection. EdU was administered for two consecutive days, starting from different days after the CCA injection, and samples were harvested for analysis one day after the second EdU injection. Data are presented as mean $\pm$ SEM, $n \geq 4$. (B) Analysis of EdU and BrdU labeled cells. (C) Quantification of the number of EdU+BrdU-, EdU-BrdU+ and EdU+ BrdU+ cells. Data are presented as mean $\pm$ SEM. $n \geq 4$. (D) Representative retinal sections of *Glast-CreERT2*; *Sun1:GFP* mice at one week, two weeks, and four months post CCA injection. (E) Quantification of MG daughter cell (EdU+GFP+) distribution in each retinal layer. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Data are presented as mean $\pm$ SEM, $n=3$.

A (TSA) improve MG proliferation and reprogramming (Hoang et al., 2020 [DOI](#); Jorstad et al., 2017 [DOI](#); Rueda et al., 2019 [DOI](#)), a group of CCA- treated mice also received NMDA on day 7 and TSA on day 9 post-CCA injection (CCA+NMDA+TSA, referred to as CCANT) to enhance the reprogramming effect, if any, of CCA.

After quality control, 3,758 cells were profiled in the control group, 3,890 cells in the CCA group, and 3,278 cells in the CCANT group (Supplementary Figures 6 and 7). Clustering analysis separated the cells into six different clusters (**Fig. 3B** [DOI](#)), which are quiescent MG, reactivated MG, MG in G2/M phase, MG in S phase, rod-like MG, and rods, as annotated by the known retinal cell type markers (**Fig. 3C** [DOI](#)). In the control group, the vast majority of MG (>90%) remained as quiescent MG which express high levels of MG genes such as *Glul* and *Kcnj10* (**Fig. 3C-H** [DOI](#)). Cell cycle state analysis showed that a small percentage of cells are in the G2/M or S phase in the CCA group, suggesting that there was still a small number of proliferating MG at three weeks after CCA treatment (**Fig. 3H** [DOI](#), Supplementary Figure 7D,E). In the CCA group, the majority of MG (~70%) formed a separate cluster, which we refer to as reactivated MG. In this cluster of cells, we observed downregulation of MG genes (such as *Glul* and *Kcnj10*) and upregulation of reactive gliosis gene *Gfap* and cell cycle inhibitor *Btg2* compared with the quiescent MG cluster (**Fig. 3H** [DOI](#)). Interestingly, the top differentially expressed genes (DEGs) between reactivated MG and quiescent MG are the interferon (IFN) pathway genes, including *Stat1*, *Stat2*, *Irgp1*, *Irgm1* and *Igtp*, which were downregulated in the reactivated MG (**Fig. 2F-H** [DOI](#), Supplementary Fig. 8), while *Stat3* was not downregulated concurrently with *Stat1* and *Stat2* (Supplementary Fig. 8). Here, downregulation of the IFN pathway in the reactivated MG may be a direct response to cyclin D1 overexpression and/or p27^{Kip1} knockdown. A small percentage of MG (~8%) of reactivated MG was also present in the control virus-injected group, which may be due to retinal injury caused by virus injection and inflammatory response caused by the AAV virus (**Fig. 3D, E** [DOI](#)). The cell clusters of the CCANT group largely overlapped with the CCA group, with CCANT having fewer proliferating cells and more reactivated MG.

CCA upregulates rod gene expression in a subset of MG

Interestingly, the rods and rod-like MG were two unexpected clusters that appeared in the scRNA-seq analysis. Upon close examination of the retina of *Glast-Cre^{ERT2}; R26-tdTomato* mice without Tamoxifen induction, we found that very few rods (14 Tdt+ rods in 128 whole retinal sections of eight mouse retina samples screened) were mislabelled by leaky Tdtomato expression in a Cre-independent manner (Supplementary Figure 9). The small rod clusters are likely the true rods mislabelled by Tdt, which is presented in all three groups with a similar proportion. The rod-like MG cluster resides between the reactivated MG cluster and the rod cluster, and it is significantly enriched in the CCA and CCANT groups. The rod-like MG expressed most rod genes, including *Rho*, *Gnat1*, *Rcvrn*, *Crx*, and *Nrl* (**Fig. 3G, H** [DOI](#), Supplementary Fig. 10), although the expression levels of the rod-specific genes were lower than those of natural rods. However, not all the rod genes are evenly upregulated, as the upregulation of transcription factor *Otx2* that is crucial for rod cell fate specification and the genes related to neuron projection and photoreceptor cell cilium such as *Syt1* and *Cacnb2* were less significant (Supplementary Figure 10). In addition, the rod-like cells expressed a high level of MG genes, while natural rods did not (**Fig. 3G, H** [DOI](#)). When comparing the rod-like MG cluster with the reactivated MG cluster, gene ontology (GO) analysis showed significant gene enrichment in the pathways related to visual system development, neuron differentiation, visual perception, and neuron projection (Supplementary Fig. 10). And GO was significantly enriched in the genes associated with neuron projection and neuron differentiation in the rod cluster as compared to the rod-like cluster (Supplementary Fig. 10), suggesting that rod-like MG were not fully differentiated rods. When comparing the CCA- and CCANT-treated groups, NMDA and TSA increased the percentages of reactivated MG and rod-like MG, but they did not induce the fully differentiated rods or any other new cell clusters. In the following study, we focused solely on the CCA treatment.

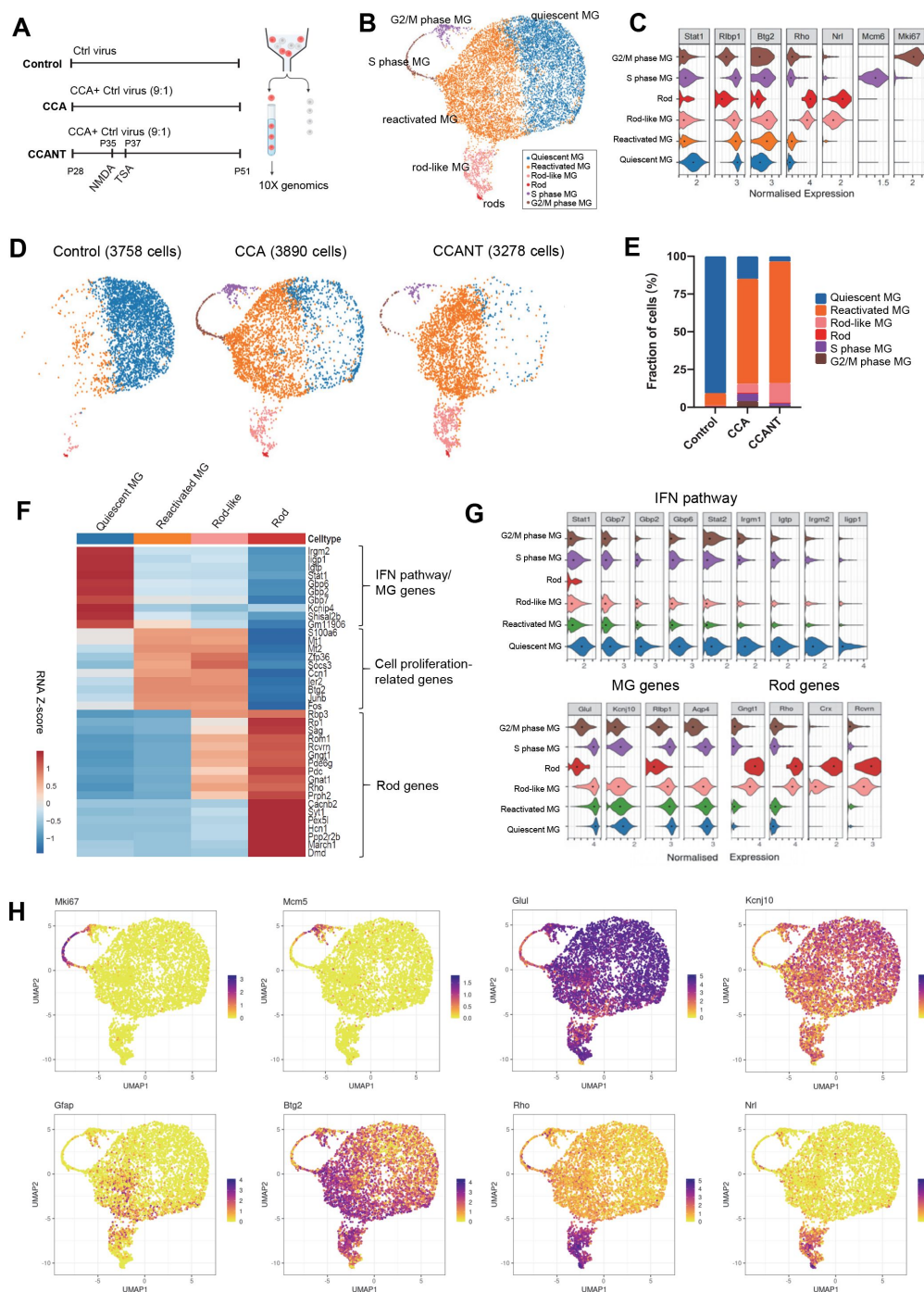


Figure 3.

scRNA-seq analysis of MG at three weeks post CCA treatment

(A) Schematic illustration of scRNA-seq experiment. (B) UMAP plot of scRNA-seq data for MG treated with CCA, CCANT, and control virus, with clusters identified by the known marker gene expression. (C) Expression of retinal cell markers in different cell clusters. (D) Split UMAP plots of the control, CCA, and CCANT groups. (E) The proportion of cell clusters in the control, CCA, and CCANT groups. (F) Heatmap of top DEG genes between cell clusters. Cells are shown in columns, and genes are in rows. Color scale denotes Z score of the normalized gene expression levels. (G) The violin diagram shows the expression of IFN pathway, MG and Rod genes in different cell clusters. (H) Feature plots of cell cycle regulator (*Mik67*, *Mcm5*), glial (*Glul*, *Kcnj10*), gliosis (*Gfap*), cell proliferation (*Btg2*), and rod (*Rho* and *Nr1*) normalized gene expression in different cell clusters. CCA, cell cycle activator; CCANT, cell cycle activator + NMDA + TSA.

Rod-like MG in the ONL expresses both rod and MG genes

To exclude the possibility that the rod-like MG are fused MG with rod cell debris during retina isolation for scRNA-seq analysis, we examined the expression of rod gene expression in the rod-like MG on the retinal sections of the CCA-treated *Glast-Cre^{ERT2};Sun1:GFP* mice. As the immunoreactivity for rod proteins such as RHO and GNAT1 is present in rod outer segment (ROS), which are densely packed and distal from rod nuclei, we performed RNA in situ hybridization on retinal sections using the RNAscope assay, which allowed us to analyse target RNA abundance in rod nuclei with the preservation of the spatial information. We examined the mRNA levels of the *Rho* and *Gnat1* genes at three weeks post CCA treatment, which is the same harvest time point as in the scRNA-seq data. The signals of both probes are specific as they are only detected in the ONL layer (Fig. 4A,B). In the control retinas, the mRNA of the two rod genes was abundantly present in the rods in the ONL (Fig. 4A,C), while there was minimal *Gnat1* and *Rho* mRNA in the MG in the INL (Fig. 4A,E). In the CCA-treated retina, the MG in the ONL showed clear signals of both *Gnat1* and *Rho* mRNA (Fig. 4B,D), while the INL MG maintained very low levels of *Gnat1* and *Rho* mRNA (Fig. 4B,F). We counted the number of *Gnat1* fluorescent dots, which served as a reliable indicator of *Gnat1* mRNA levels. The result showed a significant upregulation of *Gnat1* mRNA levels in the MG-derived cells in the ONL and that its level was comparable to the natural rods (Fig. 4G). The MG in the OPL had intermediate levels of *Gnat1* mRNA, while the MG in the INL is similar to the untreated MG controls (Fig. 4G). The *Rho* mRNA levels were quantified by measuring the pixel intensity due to the fusion of numerous *Rho* fluorescent dots, which showed a similar trend of *Rho* mRNA expression as that of *Gnat1* (Fig. 4H). The RNAscope result demonstrated that the MG-derived cells in the ONL express high level of rod genes, those in the OPL express intermediate level of rod genes, while all MG in the INL retained the no/low rod gene expression, suggesting that the expression level of rod genes in the MG daughter cells is associated their position within the retinal layers.

We further examined the expression level of the MG marker gene *Glul*, which encodes Glutamate synthetase, in the MG cells located in different retinal layers (Fig. 5A-D). After CCA treatment, the INL MG continue to express *Glul*, although the level is decreased (Fig. 5E), which is consistent with the downregulation of MG gene in the majority of the MG by scRNA-seq. The MG-derived cells in the ONL expressed lower level of *Glul*, suggesting that they retain the MG identity as well. The RNAscope results align with the scRNA-seq analysis, indicating that the MG-derived cells in the ONL express both rod and MG genes at three weeks post CCA treatment.

To find out whether the ONL MG could differentiate into mature rods if given an extended period of time, we examined the morphology of the EdU+ MG in the ONL. Despite the extensive search, we did not identify any EdU+ Tdt+ cells in the ONL with a ROS, which is an important functional structure for rod phototransduction (Fig. 6A,B). Mature mouse rods have a unique nuclear architecture, where heterochromatin localizes in a single large chromocenter surrounded by euchromatin (Solovei et al., 2009). After CC treatment, the majority of the ONL MG nuclei do not resemble the rod nuclei (Supplementary Figure 11A-D). In addition, we labeled the MG sparsely with a low dose of Tamoxifen before CCA treatment and found that most of the ONL MG daughter cells retained the apical processes (Supplementary Figure 11E). We examined the mRNA expression of rod and MG genes again at four months after CCA treatment. *Rho*, *Gnat1*, and *Glul* mRNA expression do not display significant change at four months post-CCA treatment (Supplementary Fig. 12). Therefore, we conclude that the ONL MG do not develop to mature rods despite the continuous expression of rod gene and that they retained MG properties.

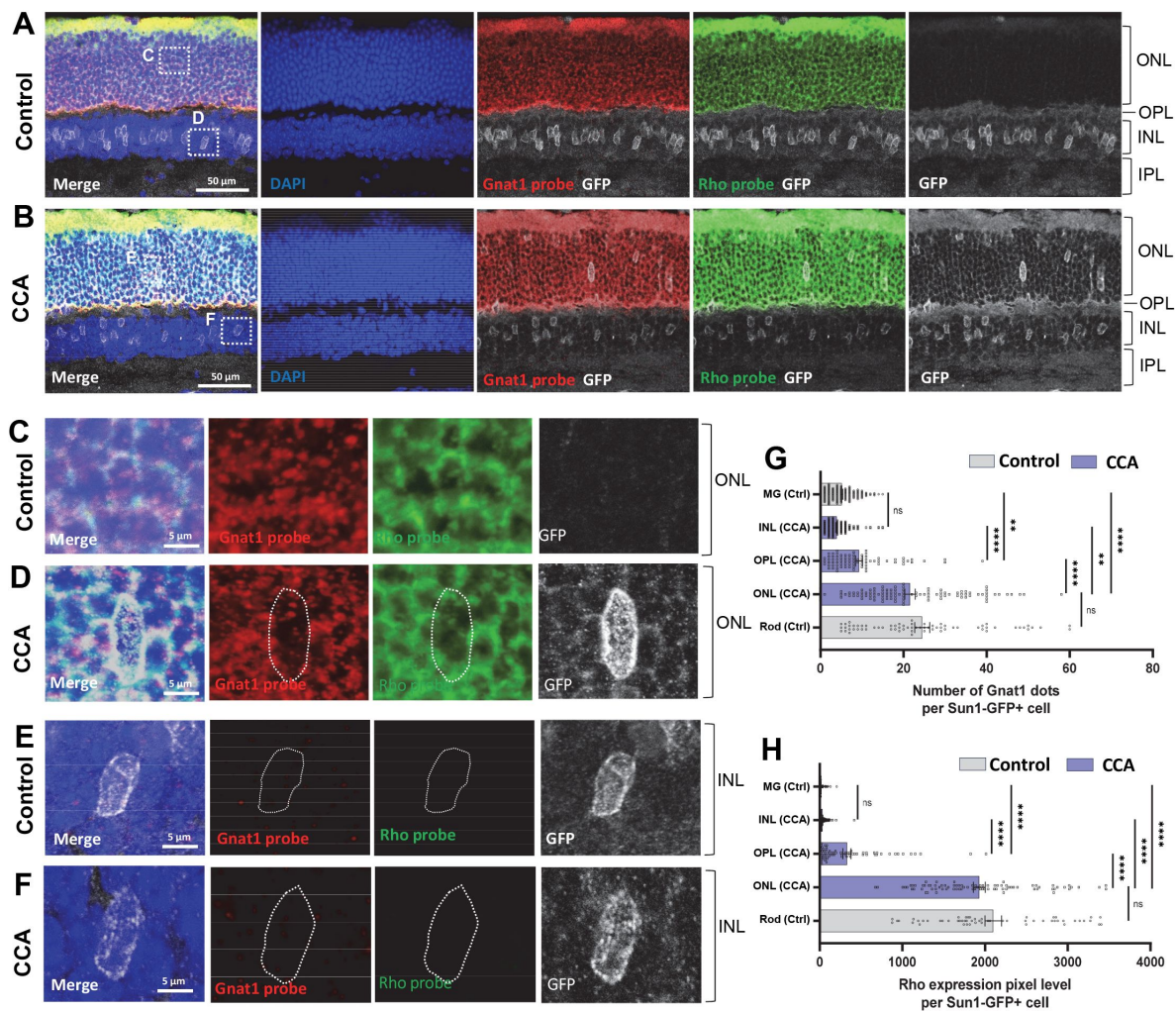


Figure 4.

Gnat1 and *Rho* mRNA are expressed in the ONL and OPL MG

(A-B) *Gnat1* and *Rho* mRNA in situ hybridization in the control (A) and the *Glast-CreERT2;Sun1:GFP* mouse retinas harvested at three weeks post CCA injection (B). (C-F) Magnified views of the highlighted regions in (A) and (B). (G) The number of *Gnat1* mRNA dots per GFP+ cell and (H) the average pixel level of *Rho* mRNA per GFP+ cell. $n=3$ mice, data are presented as mean $\pm$ SEM. ns=not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$, by one-way ANOVA with Tukey's post hoc test (G, H).

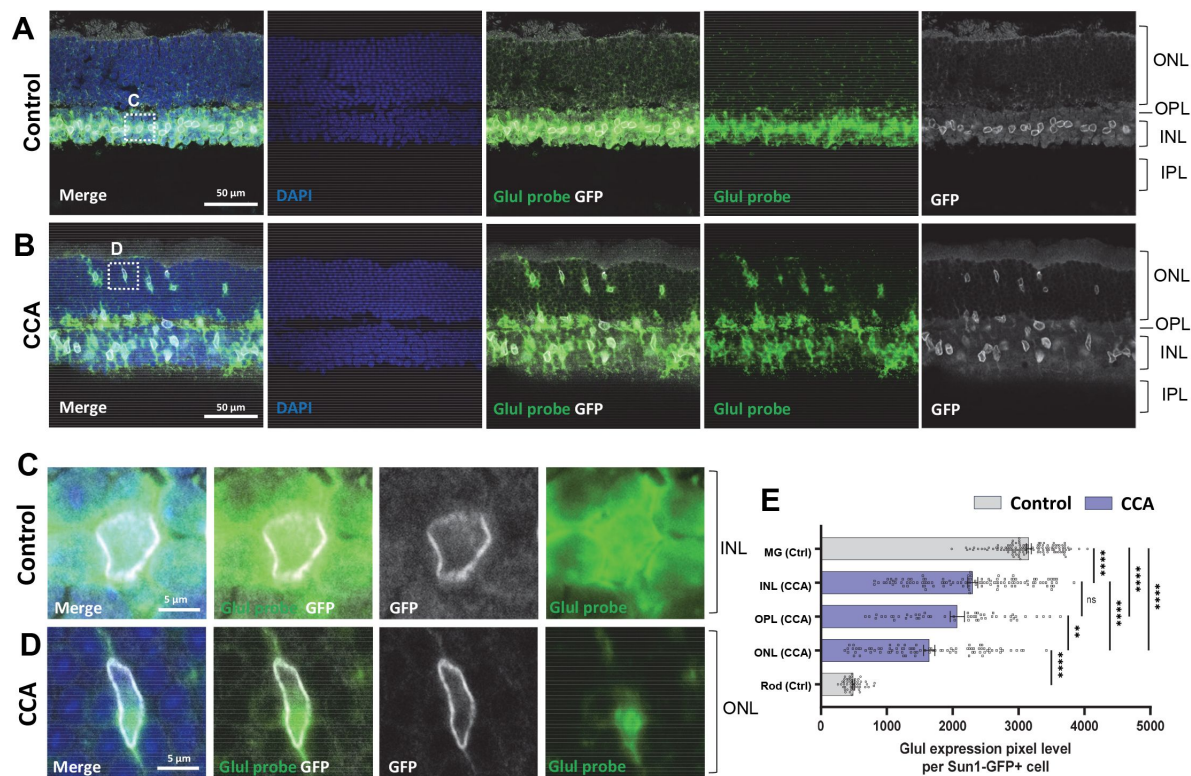


Figure 5.

Glul mRNA level is decreased in the ONL and OPL MG

(A-B) *Glul* mRNA in situ hybridization. (C-D) Magnified views of the highlighted regions in (A) and (B). (E) The average pixel level of *Glul* mRNA per GFP+ cell. n=3 mice, data are presented as mean $\pm$ SEM. ns=not significant, * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001, by one-way ANOVA with Tukey's post hoc test (E).

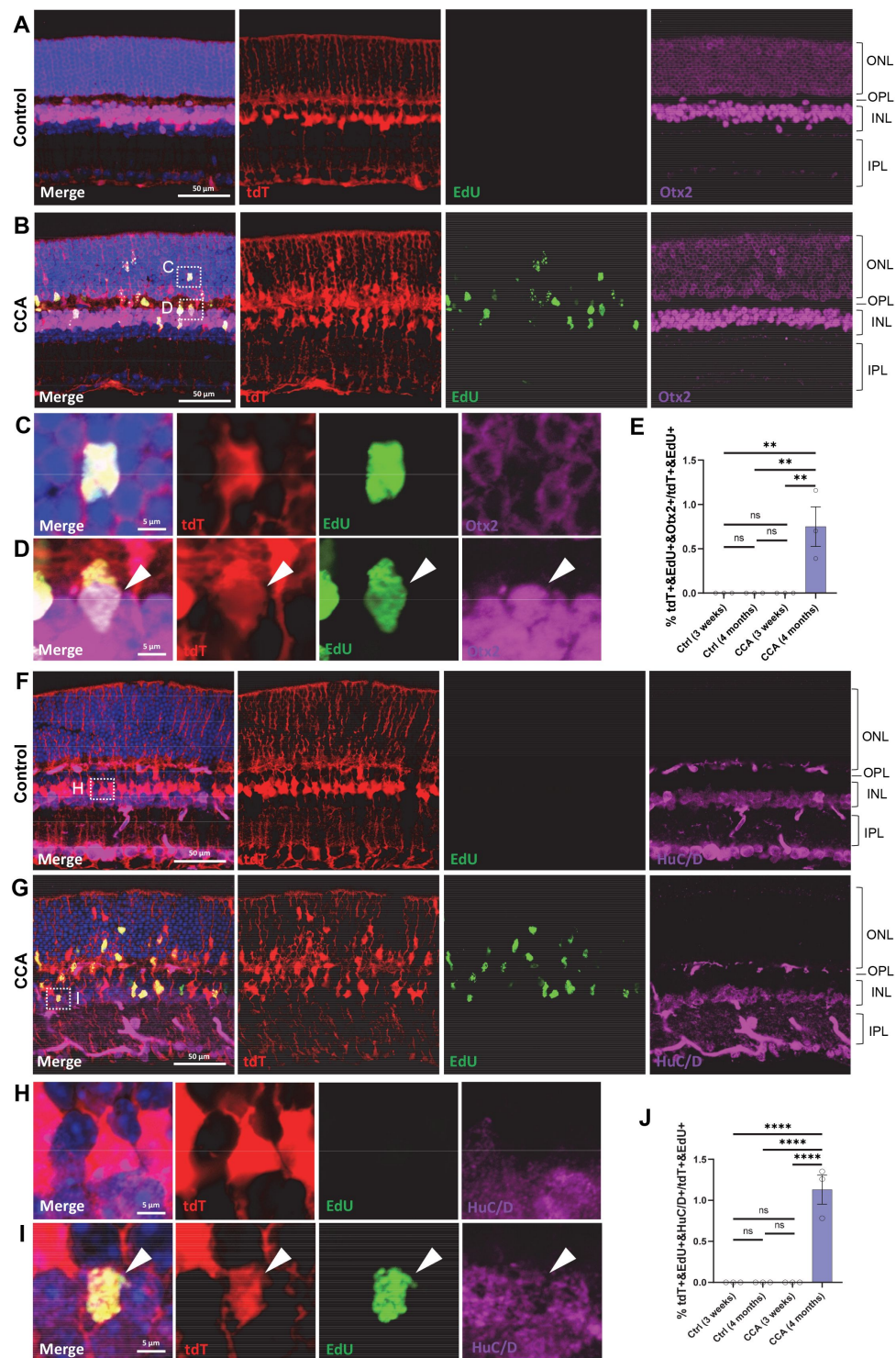


Figure 6.

CCA induces de novo genesis of Otx2+ BP-like and HuC/D+ AC-like cells from MG

(A-B) Representative retinal sections of *Glast-CreERT2; tdTomato* mice at four months post injection of CCA. Sections were immunostained with EdU and Otx2. (C-D) Magnified views of the highlighted regions in (B). (E) Quantification of tdT+EdU+Otx2+/tdT+EdU+%. (F-G) Representative retinal sections immunostained with EdU and HuC/D. (H-I) Magnified views of the highlighted regions in (F) and (G). (J) Quantification of tdT+EdU+HuC/D+/tdT+EdU+%. n=3 mice, data are presented as mean ± SEM. *p<0.05; **p<0.01, by unpaired two-tailed Student's t-test (E, J).

Few bipolar and amacrine-like cells are regenerated from MG

Bipolar (BP) and amacrine cells (AC) are two retinal neuron types that have been shown to be regenerated from MG (Hoang et al., 2020 [DOI](#); Jorstad et al., 2020 [DOI](#), 2017 [DOI](#)). Although the scRNA-seq data did not reveal other retinal neuron-like clusters derived from MG after CCA treatment, we performed immunostaining of the bipolar marker Otx2 or the ganglion/amacrine maker HuC/D to examine whether there are rare MG-derived cells expressing either marker. In order to specifically identify *de novo* neurogenesis from MG and to exclude any mislabelled neurons in the reporter mouse line, we focused our analysis on the EdU+Tdt+ MG in the *Glast-Cre^{ERT2}; R26-tdTomato* mice. At four months post-CCA treatment, we observed less than 1% of the total EdU+Tdt+ cells expressing Otx2, whereas these cells were not present at three weeks post-CCA treatment (**Fig. 7A-E** [DOI](#)). We also found rare HuC/D+EdU+Tdt+ cells in the lower INL where the HuC/D+ amacrine cells naturally reside (**Fig. 7F-J** [DOI](#)). Whether these cells express other BP or AC markers and whether they are functional interneurons that connect with the retinal circuitry needs further investigation. Nonetheless, our findings suggest that cell cycle re-activation alone can increase the plasticity of MG daughter cell fate, which allows some of them to become BP or AC-like neurons.

CCA does not cause retinal neoplasia or functional deficit

To assess the long-term effect of CCA treatment, a cohort of C57BL/6 mice were observed for a year following CCA injection (**Fig. 7** [DOI](#)). Visual function assessments showed no significant differences in visual acuity and electroretinography (ERG) between CCA-treated eyes and uninjected control eyes one year after CCA treatment (**Fig. 7A-C** [DOI](#)). Upon examining all the harvested retinas from the CCA-treated group, no retinal neoplasia was observed. The retinal structure remained intact, with no malignancy or disruptions in the retinal layers or stratification (**Fig. 7D,G** [DOI](#)). These results indicate that CCA treatment did not have a detrimental impact on retinal structure or function in mice, nor did it induce retinal tumours.

We used the MG marker Sox9 to examine the MG population in the retina sections harvested at a year post CCA treatment. In the control retina, Sox9+ MG cells were aligned in the INL (**Fig. 7D-E** [DOI](#)). In the CCA-treated retinas, there was a significant expansion in the number of Sox9+ MG cells, distributed across the ONL, OPL, and INL (**Fig. 7F-G** [DOI](#)). The large population of Sox9+ MG remained to support the retinal homeostasis, which explains the relatively unaffected retinal structure and function observed on the CCA-treated eyes. We further made comparison of the numbers of Sox9+ cells after one year of CCA treatment and after 2 weeks of CCA treatment. The total numbers of Sox+ cells were similar between these two time points (**Fig. 7H** [DOI](#)), indicating no significant MG loss after MG proliferation. To directly assess whether CCA treatment may lead to the cell death of the MG, especially the MG displaced in the ONL and OPL, we examined the CCA-treated retinas with TUNEL assay and did not observe many TUNEL+ cells (Supplement Figure 13). Since we could only examine cell death at single time points, we cannot completely exclude the possibility that there were small numbers of MG-derived cells dying without being detected. The fact that the numbers of MG in the ONL and OPL did not decrease in the retinas harvested at one-year post-CCA treatment suggests that these MG-derived cells persist without dying or losing the MG identity (**Fig. 7I** [DOI](#)).

Discussion

MG proliferation is a key step towards MG-mediated regeneration in the retina. Previously, it was shown that activation of the canonical Wnt pathway by AAV-mediated overexpression of β -catenin stimulates MG proliferation through the Lin28/let7 pathway (Yao et al., 2016 [DOI](#)), and cyclin D1 is a

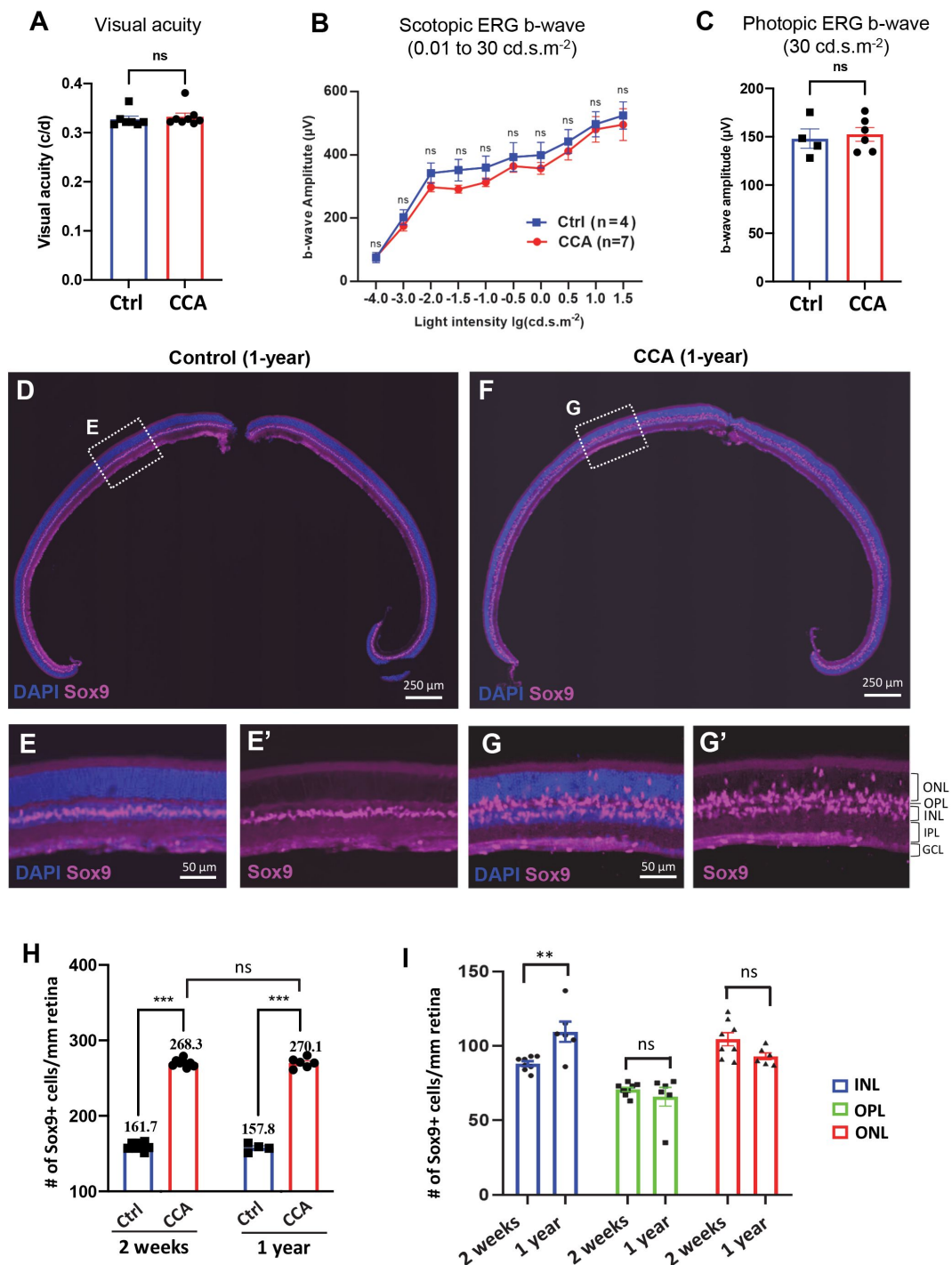


Figure 7.

CCA does not lead to retinal neoplastic transformation

(A-C) Optomotor and electroretinography (ERG) tests were performed on wild type mice at 1 year after CCA injection (n≥4). Visual acuity by the optomotor test (A), and b-wave amplitudes of the scotopic ERG under different light intensity in (B) and photopic ERG under 30 $\text{cd} \times \text{s.m}^{-2}$ in (C), respectively. Data are presented as mean ± SEM. ns=not significant by two-tail unpaired Student's t-test (A-C). (D-E) Sox9 immunofluorescence in untreated control mice (D) and WT mice one year after CCA injection (E). (F-G) Zoom-in images of the indicated area in (D) and (E). (H) Quantification of the number of Sox9+ cells in retinas at two weeks or one year after CCA injection and in age-corresponding WT control retinas. (I) Quantification of the number of Sox9+ cells in each retinal layer. Data are presented as mean ± SEM. ns=not significant, **p ≤ 0.01, ***p ≤ 0.001 by one-way ANOVA with Tukey's post hoc test (H) and two-tail unpaired Student's t-test (I).

direct target gene of both Wnt signaling and Lin28/let7 (Li et al., 2012 [↗](#); Shtutman et al., 1999 [↗](#); Tetsu and McCormick, 1999 [↗](#)). Suppression of the Hippo pathway by transgenic expression of YAP5SA induces MG proliferation, accompanied by an increase in cyclin D1 expression (Hamon et al., 2019 [↗](#); Rueda et al., 2019 [↗](#)). However, the Hippo and Wnt pathways have downstream genes besides cyclin D1 and serve different cellular functions. It is not clear whether cyclin D1 activation is necessary or sufficient to drive MG proliferation in these contexts. In this study, we demonstrated that cyclin D1 overexpression alone can promote MG proliferation and that the combination of cyclin D1 overexpression and p27^{Kip1} KD is the most potent to drive MG proliferation in mouse retina without injury stimulus.

The strategy to stimulate the MG cell cycle for the purpose of retinal regeneration must be carefully weighed against the concern of potential tumorigenesis. p27^{Kip1} and cyclin D1 are gatekeepers of cell cycle regulation, and any abnormalities in their expression may impact cell division, potentially leading to the development of tumours. Previous studies have provided evidence that mice lacking p27^{Kip1} are approximately 30% larger and generally do not exhibit malignancies, except for pituitary tumours that develop spontaneously (Fero et al., 1996 [↗](#)). Cyclin D1 is frequently overexpressed in a significant fraction of human cancers, such as breast and respiratory tract tumours (Callender et al., 1994 [↗](#); Zukerberg et al., 1995 [↗](#)). The concern regarding retinal tumour formation due to CCA treatment may be mitigated, as the *in vivo* gene transfer was performed using AAV vectors. The advantage of using AAV vector is that AAV episomal genome becomes diluted after cell division, reducing the risk of tumor formation due to continuous cyclin D1 overexpression and p27^{Kip1} KD. Consistent with this, the rate of MG proliferation was observed to decrease gradually over time, and the majority of MG cells did not undergo multiple cell divisions (Fig. 2 [↗](#)). Additionally, the retinal structure was normal without neoplasia (Fig. 6 [↗](#), 7 [↗](#)). These results suggest that CCA is a potent and viable treatment to stimulate MG proliferation and may be a promising approach to be used in combination with other neurogenic factors to promote MG-mediated retinal regeneration.

Interestingly, we found that the IFN pathway genes are enriched in the top downregulated genes in the reactivated MG cluster. The IFN pathway, mediated by STAT1 and STAT2, is an important component of the innate immune system against viral infection and is also known as antitumor cytokine signaling which facilitates immunosurveillance in tumor cells (Durbin et al., 1996 [↗](#); Kaplan et al., 1998 [↗](#)). This pathway has also been implicated in suppressing regeneration in mice (Jorstad et al., 2020 [↗](#); Todd et al., 2020 [↗](#)). STAT activation was shown to reduce the ASCL1-induced retinal regeneration (Jorstad et al., 2020 [↗](#)), and decreased IFN signaling could be induced by microglia depletion, which improves MG neurogenic capacity (Todd et al., 2020 [↗](#)). In this study, we showed that the IFN pathway genes were downregulated in the MG after CCA treatment. This would be a direct response to the upregulation of cyclin D1 and/or downregulation of p27^{Kip1} in the MG cell-autonomously, or it could indirectly result from the microglial responses elicited by the treatment. Investigating whether and how the downregulation of IFN signaling plays a role in CCA-induced retinal regeneration would be interesting questions to explore further.

The upregulation of rod genes in the MG residing in the ONL is an unexpected outcome of MG cell cycle activation by CCA, as this was not reported in the other studies focusing on MG proliferation (Hamon et al., 2019 [↗](#); Rueda et al., 2019 [↗](#); Yao et al., 2016 [↗](#)). In those studies, a very small subset of proliferative MG differentiated into BP and/or AC-like, but no photoreceptor genesis was reported. In our study, a significant subset of MG expressed the mRNA of *Rho* and *Gnat1* by scRNA-seq and RNAscope. However, the rod-like MG is unlikely a result of MG-to-rod cell fate reprogramming, as we did not see many cells overexpressing neurogenic genes, such as *Ascl1* and *Dll3*, or rod precursor gene *prdm1* (Supplementary Figure 14A). scRNA-seq analysis at earlier time points after CCA treatment would be helpful to further exclude this possibility. How does CCA activate rod gene expression if it is not through cell fate reprogramming? One hypothesis is that cyclin D1 or p27^{Kip1} regulates rod gene expression in addition to their well-characterized functions in cell cycle regulation. It was shown that cyclin D1 possesses transcriptional functions by

recruiting CREB-binding protein histone acetyltransferase in developing mouse retinas (Bienvenu *et al.*, 2010 [DOI](#)). Cyclin D has been shown to activate retinoblastoma protein (Rb) by mono-phosphorylation (Narasimha *et al.*, 2014), and Rb in turn promotes rod maturation, which is independent of its function for RPC cell cycle exit (Zhang *et al.*, 2004). In the *Rb1*^{-/-} retinal explants, many genes expressed in rods, such as *Nrl* and *Nr2e3*, were significantly downregulated (Zhang *et al.*, 2004). Additionally, *p27^{xic1}*, the *Xenopus* homolog of mammalian *p27^{kip1}*, has been shown to promote glial cell fate independent of its CDK binding activity (Ohnuma *et al.*, 1999 [DOI](#)). Therefore, besides cell cycle activation, cyclin D1 overexpression and/or *p27^{kip1}* KD may promote rod gene expression directly or indirectly, which may explain the rod-like MG population observed following CCA treatment.

Although rod-like MG express rod genes, they are, by no means, mature rods. First of all, they lack ROS and nuclear architecture of the mature rods. In addition, their gene expression profile was different from that of natural rod. We compared gene expression profiles of rod-like cells with natural rods and found that rod-like cells still lacked the expression of some genes related to neuron projection and photoreceptor cell cilium such as *Syt1* and *Cacnb2* (Supplementary Figure 10), indicating that rod-like cells were not fully differentiated. In addition, the rod-like MG still expressed MG genes such as *Glul*. By comparing differentially expressed genes associated with cell differentiation, we found that Notch-related genes, including *Notch1* and its downstream gene *Hes1*, remain highly expressed in the reactivated and rod-like MG (Supplementary Figure 14B). Notch signaling pathway is involved in regulating both cell proliferation and cell fate specification during retinal development (Jadhav, Mason and Cepko, 2006; Yaron *et al.*, 2006). Genetic removal of *Notch1*, or Notch downstream effectors such as *Hes1* and *RBP-J* leads to precocious cell cycle exit and increased photoreceptors production during early retinogenesis (Tomita *et al.*, 1996; Jadhav, Mason and Cepko, 2006; Riesenberger *et al.*, 2009; Zheng *et al.*, 2009). In addition, *Notch1* functions in postmitotic cell fates to repress the rod fate (Mizeracka, Demaso and Cepko, 2013). Therefore, we conjecture that the high activity of Notch signaling may be the reason why rod-like cells fail to become full differentiated rods. A recent preprint has shown that suppressing Notch signaling in the MG and deletion of nuclear factor I factors a, b, and x (*Nfia/b/x*) synergize to promote neurogenesis of MG into retinal bipolar and amacrine interneurons in adult mice after NMDA-induced retinal injury (Le *et al.*, 2023 [DOI](#)).

Limitations of the study

One unresolved question is how to further enhance rod regeneration by combining CCA with other factors. It remains to be tested whether inhibiting Notch signaling or overexpressing other neurogenic factors, in combination with CCA treatment, can result in the full differentiation of new rod photoreceptors. Additionally, while the CCA approach exhibited potent mitogenic activity in uninjured mouse retinas, its ability to promote MG proliferation after retinal injury is still unknown. Further investigation is needed to test the effects of CCA in mouse models of retinal degeneration. Finally, it is yet to be investigated how the CCA treatment affects the promoter accessibility of rod genes in the rod-like MG cells. Single-cell ATAC-seq or ChIP-seq analyses could provide insights into the epigenetic changes that underlie the rod-like phenotype acquired by the proliferated MG cells.

Materials and Methods

Animals

Wild-type mice (strain C57BL/6J), Rosa26-tdTomato reporter mice (strain B6.Cg-Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze/J}), Rosa26-Sun1:GFP reporter mice (Strain B6.129-Gt(ROSA)26Sor^{tm5.1(CAG-Sun1/sfGFP)Nat/MmbeJ}), and *Glast-CreER* reporter mice (strain Tg(*Slc1a3-cre/ERT*)1Nat/J) mice were purchased from the Jackson Laboratory and were kept on a 12 hour light/12 hour dark cycle. All animal procedures performed were approved by the Hong Kong Department of Health under Animals Ordinance Chapter 340, and animal care was carried out in accordance with institutional guidelines.

AAV plasmids and vectors

pAAV-GFAP-GFP vector plasmid was cloned by replacing the CMV promoter with a 681bp ABC1D region of the human *GFAP* gene promoter (Lee et al., 2008 [\[1\]](#)) into the pAAV-CMV- GFP vector (Supplementary Figure 1A). cDNAs encoding mouse cyclin D1 were cloned into AAV plasmids by Gibson ligation. AAV-shRNA vectors were cloned by replacing the GFP sequence with mCherry-shRNA in the pAAV-GFAP-GFP vector. pAAV-GFAP-cyclinD1-P27 shRNA and pAAV-GFAP-GFP-LacZ shRNA were cloned by replacing mCherry sequence with cyclinD1 and GFP from the pAAV-GFAP-mCherry-P27 shRNA1 and pAAV-GFAP- mCherry-LacZ shRNA, respectively (Supplementary Figure 2A). pAAV rep/Cap 2/2, and Adenovirus helper plasmids were obtained from the University of Pennsylvania Vector Core, Philadelphia. pAAV7m8 plasmid (#64839) was purchased from addgene.

AAV was produced in HEK293T cells (HCL4517; Thermo Scientific) by AAV vector, rep/cap packaging plasmid, and adenoviral helper plasmid co-transfection followed by iodixanol gradient ultracentrifugation. Purified AAVs were concentrated with Amicon 100K columns (EMD Millipore) to a final titer higher than 5×10^{13} genome copies/mL. Protein gels were run to determine virus titers.

Tamoxifen injection

Intraperitoneal injections of tamoxifen (Sigma) in corn oil were administered to induce the expression of Cre recombinase. Starting from P20, tamoxifen was given at a dosage of 50 mg/kg daily for 5 consecutive days to label as many MG as possible. For the purpose of sparse labeling, a single dose of tamoxifen at 15 mg/kg was administered on P20.

AAV injection

Intravitreal injection was performed using a pulled angled glass pipette controlled by a FemtoJet (Eppendorf). The tip of the needle was passed through the sclera at the equator, near the dorsal limbus of the eyeball, and entered into the vitreous cavity. The injection volume of AAVs (5×10^{13} genome copies/mL) was 0.5µl per eye for P6 injection and 1µl per eye for adult mouse injection.

In situ RNA hybridization

In situ RNA hybridization was performed using the RNAscope Multiplex Fluorescent Detection Reagents V2 kit (Advanced Cell Diagnostics) following the commercial protocols. In brief, retinas were dissected, 4% PFA fixed, dehydrated in sucrose solution and embedded in optimal cutting temperature (OCT) medium. Retinas were cryosectioned into 20µm and mounted on SuperFrost Plus glass slides (Eppredia). After OCT removal by PBS and further dehydration by ethanol, retinal sections were stained with GFP antibody (AB_2307313; Aves Labs) at 4 °C overnight. After three times wash with PBST (PBS with 0.1% Tween-20), retinal sections were hybridized with RNA probes (Advanced Cell Diagnostics Cat.No. 474801-C3 for Mm *Rho*, 524881-C2 for Mm *Gnat1*, 426231-C2 for Mm *Glul*) for 2 hours at 40 °C for two hours. Following in situ RNA hybridization steps, slides were stained using secondary antibodies (Jackson ImmunoResearch) and DAPI for two hours at room temperature. The fluorescent signals were visualized and captured using Nikon

A1HD25 High speed and Large Field of View Confocal Microscope. The mRNA levels inside the GFP labelled nuclei membrane were quantified as number of RNA dots or measured as signal intensity level by ImageJ.

Immunohistochemistry

Retinas were dissected and fixed in 4% formaldehyde in PBS for 30 min at room temperature and sectioned at 20- μ m thickness by cryostat. Retinal sections were blocked in 5% BSA in PBST (PBS with 0.1% Triton X-100), stained with primary antibodies at 4 °C overnight, and washed three times with PBST. Primary antibodies used in this study included rabbit anti- p27^{kip1} (1:200, PA5-16717; Thermofisher); rabbit anti-cyclin D1 antibody (1:300, 26939-1-AP; Proteintech), goat anti-Otx2 antibody (1:200, AF 1979; R&D systems), mouse anti-HuC/D antibody (1:200, A21271; Thermofisher), rabbit anti-GFAP antibody (Z0334, 1:500, DAKO), rabbit anti-Sox2 antibody (1:2000, ab97959; abcam) and rabbit anti-Sox9 antibody (1:1000, AB5535; Millipore). Sections were stained using secondary antibodies (Jackson ImmunoResearch) for two hours at room temperature. Cell nuclei were counterstained with DAPI (Sigma). TUNEL was performed using an TUNEL BrightRed Apoptosis Detection Kit (Vazyme) according to the manufacturer's protocol.

EdU incorporation and BrdU detection assay

5'-ethynyl-2'-deoxyuridine (EdU, 50mg/kg, Abcam ab146186) or 5-bromo-2' deoxyuridine (BrdU, 100mg/kg, Abcam 142567) was intraperitoneally injected to label the cells in the S phase. EdU staining was performed using the Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit (C10337). For BrdU detection, the retinal sections were incubated with 2 M HCl for 1 hour at room temperature. The sections were rinsed with PBST and incubated with a blocking buffer containing 5% BSA in PBST for 2 hours at room temperature. Primary antibody mouse anti- BrdU (1:300, ab8152; Abcam) was stained overnight at 4 °C, and secondary antibodies (Jackson ImmunoResearch) were stained for 2 hours at room temperature.

Single-cell RNA-seq

Library preparation and sequencing of single cells

The FACS-sorted cells from each sample were filtered through 40 μ m strainer (pluriStrainer) and processed with Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1 (1000269), Chromium Next GEM Chip G Single Cell Kit (1000127) and Chromium Controller (10x Genomics) according to the manufacturer's protocol. The constructed libraries were sent to Novogene (Beijing) for NovaSeq paired-end 150 bp sequencing and produced 330 Gb of raw data.

Preprocessing, filtering and clustering of scRNA data

Sequencing results were preprocessed with 10x Genomics Cell Ranger 6.1.1. (Zheng et al., 2017 [\[1\]](#)) for demultiplexing, barcode assignment, and unique molecular identifier (UMI) quantification using a standard pipeline for 10X for each of the three samples. We used indexed mouse genome mm10 with added tdTomato and GFP transgenes as a reference. Reads mapping to introns covered more than 34% of all reads. Therefore, they were included in feature counts by using 'include_introns' option in cellranger count step of the pipeline. Cell Ranger aggregation was applied for merging runs from three samples.

Quality control, filtering, dimensional reduction, and clustering of the data were carried out using the Seurat package in R (Stuart et al., 2019 [\[2\]](#)). The cells with less than 2,000 expressed features, total UMI count higher than 100,000 and percentage of mitochondrial transcripts more than 15% were disregarded. Features of the Y chromosome, as well as *Xist* and *Tsix* genes of the X chromosome, were excluded to avoid confusing effects of gender.

Further filtering of cells was carried out by only including cells expressing tdTomato (Supplementary Figure 6). Potential cell duplicates were estimated using DoubletFinder method (McGinnis et al., 2019 [DOI](#)) implemented in R (with parameter settings PC=10, pK=0.28, pN=0.3). The threshold for the expected doublet formation rate was set to 10% to reflect the assumption that cell duplicates can appear relatively commonly in the MG cells. Detected duplicates were excluded from the data (Supplementary Figure 6G,H).

For the remaining cells, Uniform Manifold Approximation and Projection (UMAP) dimension reduction based on eight principal components (PCs) was applied, and cells were clustered using the graphical clustering method in Seurat. Cell types were identified using known marker genes (Clark et al., 2019 [DOI](#); Hoang et al., 2020 [DOI](#)). After removing doublets, cell types in clusters 4-9 other than MG, Rod-like, and Rod included only a few cells, and 10-30% of the cells were from the control sample, suggesting that these cells are likely to be contaminated (Supplementary Figure 6I,J). After excluding cells in clusters 4-9, the new UMAP and clustering (Supplementary Figure 7A) revealed two subclusters of Rod-like cells (Clusters C3- C4, Supplementary Figure 7A). The cells in the subcluster C4 exhibited similar proportional contributions from all three groups and expressed lower level of rod-specific genes such as *Rho* (Supplementary Figure 7B,C). These cells were again defined as contaminated and excluded from the data. The final UMAP was constructed using seven PCs, and the same PCs were used for clustering of cells (Figure 3B [DOI](#)).

Analysis of scRNA-seq data

The cell cycle state of each cell was defined by the Cell Cycle Scoring function in the Seurat R package. The cells with G2/M and S scores lower than 0.1 were defined to be proliferating (Supplementary Figure 7D,E). Differential gene expression analysis between different cell types or three treatment groups was performed using Deseq2 (Supplementary table 1) (Love et al., 2014 [DOI](#)). Enrichment analysis of top 200 up- and downregulated genes was performed for Gene Ontologies (GO), KEGG and Reactome pathways using Gprofiler2 R interface to g:Profiler (Raudvere et al., 2019 [DOI](#)).

The optomotor and electroretinography tests

Mouse visual acuity was measured using an Optometry System (Cerebral Mechanics Inc.) following the published protocol (Douglas et al., 2005 [DOI](#)). Testing was done with a grating of 12 degrees/second drifting speed and 100% contrast. The injected right eyes (CCA treated) and uninjected left eyes (Ctrl) were tested independently for counterclockwise and clockwise grating rotations, respectively. A staircase procedure was used, in which the observer tested low to high visual acuity. Each animal was tested for about 10-15 minutes per session.

ERG was measured using an Espion E3 System (Diagnosys LLC Inc.) as previously described (Hoang et al., 2023 [DOI](#)). Animals were dark-adapted overnight before the test. After inducing anesthesia with a ketamine: xylazine injection intraperitoneally, the pupils of both eyes were dilated using Mydrin®-P Ophthalmic Solution. Once the pupils were fully dilated, the eyes were maintained moist using a topical gel. The measurement electrodes were then applied to the cornea of both eyes, while the ground electrodes were attached to the mouth and tail. All these steps were performed in the darkroom under dim red light. For scotopic ERG recordings, a multiple 530nm light with different intensities (increments from 0.01 cd.s/m² to 30 cd.s/m²) were elicited to stimulate scotopic responses in a specific time interval. For photopic ERG recordings, 5 mins exposure under 10 cd.s/m² light intensity was adopted to inhibit the rod function. The photopic response was measured by multiple flashes of 30 cd.s/m² intensity in the illuminated background (10 cd.s/m²). The average amplitude and implicit time of a- and b- wave were recorded and exported for further analysis. The b-wave amplitude of the step-wise scotopic responses and that of photopic response at 30 cd.s/m² were shown in figure.

Statistics

Data were represented as mean $\pm$ SEM in all figures. Sample sizes and statistical analysis were indicated for each experiment in figure legend. ANOVA analysis with Tukey test was performed to compare multiple groups, and Student's t-test to compare two groups. A P value of $P < 0.05$ was considered statistically significant. GraphPad Prism was used to perform statistical analysis and make figures.

Acknowledgements

This research was funded by Research Grants Council Hong Kong Project (11103819, 11102922, and 11100723), Hong Kong Health and Medical Research Fund Project (05160276 and 06172466), TUNG Biomedical Sciences Foundation, and Ming Wai Lau Center for Reparative Medicine Research Associate Program.

Conflict of interest

The authors declare that no conflict of interest exists.

References

- Barton KM, Levine EM (2008) **Expression patterns and cell cycle profiles of PCNA, MCM6, cyclin D1, cyclin A2, cyclin B1, and phosphorylated histone H3 in the developing mouse retina** *Dev Dyn* **237**:672–682
- Besson A, Dowdy SF, Roberts JM (2008) **CDK inhibitors: cell cycle regulators and beyond** *Dev Cell* **14**:159–169
- Bienvenu F *et al.* (2010) **Transcriptional role of cyclin D1 in development revealed by a genetic-proteomic screen** *Nature* **463**:374–378
- Callender T, el-Naggar AK, Lee MS, Frankenthaler R, Luna MA, Batsakis JG. (1994) **PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma** *Cancer* **74**:152–158
- Clark BS *et al.* (2019) **Single-cell RNA-seq analysis of retinal development identifies NFI factors as regulating mitotic exit and late-born cell specification** *Neuron* **102**:1111–1126
- Das G, Choi Y, Sicinski P, Levine EM (2009) **Cyclin D1 fine-tunes the neurogenic output of embryonic retinal progenitor cells** *Neural Dev* **4**
- Das G, Clark AM, Levine EM (2012) **Cyclin D1 inactivation extends proliferation and alters histogenesis in the postnatal mouse retina** *Dev Dyn* **241**:941–952
- Douglas RM, Alam NM, Silver BD, McGill TJ, Tschetter WW, Prusky GT (2005) **Independent visual threshold measurements in the two eyes of freely moving rats and mice using a virtual- reality optokinetic system** *Vis Neurosci* **22**:677–684
- Durbin JE, Hackenmiller R, Simon MC, Levy DE (1996) **Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease** *Cell* **84**:443–450
- Dyer MA, Cepko CL (2001) **p27Kip1 and p57Kip2 regulate proliferation in distinct retinal progenitor cell populations** *J Neurosci* **21**:4259–4271
- Dyer MA, Cepko CL (2000) **p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina** *Development* **127**:3593–3605
- Dyer MA, Cepko CL (2000) **Control of Müller glial cell proliferation and activation following retinal injury** *Nat Neurosci* **3**:873–880
- Fantl V, Stamp G, Andrews A, Rosewell I, Dickson C (1995) **Mice lacking cyclin D1 are small and show defects in eye and mammary gland development** *Genes Dev* **9**:2364–2372
- Fero ML *et al.* (1996) **A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27 -deficient mice** *Cell* **85**:733–744
- Hamon A, García-García D, Ail D, Bitard J, Chesneau A, Dalkara D, Locker M, Roger JE, Perron M (2019) **Linking YAP to Müller Glia quiescence exit in the degenerative retina** *Cell Rep* **27**:1712–1725

- Hamon A, Roger JE, Yang X-J, Perron M (2016) **Müller glial cell-dependent regeneration of the neural retina: An overview across vertebrate model systems** *Dev Dyn* **245**:727–738
- Hoang DA, Liao B, Zheng Z, Xiong W (2023) **Mutation-independent gene knock-in therapy targeting 5'UTR for autosomal dominant retinitis pigmentosa** *Signal Transduct Target Ther* **8**
- Hoang T *et al.* (2020) **Gene regulatory networks controlling vertebrate retinal regeneration** *Science* **370**
- Jadhav AP, Roesch K, Cepko CL (2009) **Development and neurogenic potential of Müller glial cells in the vertebrate retina** *Prog Retin Eye Res* **28**:249–262
- Jorstad NL, Wilken MS, Grimes WN, Wohl SG, VandenBosch LS, Yoshimatsu T, Wong RO, Rieke F, Reh TA (2017) **Stimulation of functional neuronal regeneration from Müller glia in adult mice** *Nature* **548**:103–107
- Jorstad NL *et al.* (2020) **STAT signaling modifies Ascl1 chromatin binding and limits neural regeneration from Muller Glia in adult mouse retina** *Cell Rep* **30**:2195–2208
- Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD (1998) **Demonstration of an interferon γ -dependent tumor surveillance system in immunocompetent mice** *Proc Natl Acad Sci U S A* **95**:7556–7561
- Karl MO, Hayes S, Nelson BR, Tan K, Buckingham B, Reh TA (2008) **Stimulation of neural regeneration in the mouse retina** *Proc Natl Acad Sci U S A* **105**:19508–19513
- Le N, Vu T-D, Palazzo I, Pulya R, Kim Y, Blackshaw S, Hoang T. (2023) **Robust reprogramming of glia into neurons by inhibition of Notch signaling and NFI factors in adult mammalian retina** *bioRxiv* <https://doi.org/10.1101/2023.10.29.560483>
- Lee HO, Norden C (2013) **Mechanisms controlling arrangements and movements of nuclei in pseudostratified epithelia** *Trends Cell Biol* **23**:141–150
- Lee Y, Messing A, Su M, Brenner M (2008) **GFAP promoter elements required for region-specific and astrocyte-specific expression** *Glia* **56**:481–493
- Todd L, Hooper MJ, Haugan AK, Finkbeiner C, Jorstad N, Radulovich N, et al (2021) **Efficient stimulation of retinal regeneration from Müller glia in adult mice using combinations of proneural bHLH transcription factors** *Cell Rep*
- Levine EM, Close J, Fero M, Ostrovsky A, Reh TA (2000) **p27(Kip1) regulates cell cycle withdrawal of late multipotent progenitor cells in the mammalian retina** *Dev Biol* **219**:299–314
- Li N *et al.* (2012) **Lin-28 homologue A (LIN28A) promotes cell cycle progression via regulation of cyclin-dependent kinase 2 (CDK2), cyclin D1 (CCND1), and cell division cycle 25 homolog A (CDC25A) expression in cancer** *J Biol Chem* **287**:17386–17397
- Löffler K, Schäfer P, Völkner M, Holdt T, Karl MO (2015) **Age-dependent Müller glia neurogenic competence in the mouse retina** *Glia* **63**:1809–1824
- Love MI, Huber W, Anders S (2014) **Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2** *Genome Biol* **15**

- McGinnis CS, Murrow LM, Gartner ZJ (2019) **DoubletFinder: Doublet detection in single-cell RNA sequencing data using artificial nearest neighbors** *Cell Syst* **8**:329–337
- Ohnuma S, Philpott A, Wang K, Holt CE, Harris WA (1999) **p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina** *Cell* **99**:499–510
- Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H, Vilo J (2019) . **g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update)** *Nucleic Acids Res* **47**:W191–W198
- Reynisdóttir I, Polyak K, Iavarone A, Massagué J (1995) **Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta** *Genes Dev* **9**:1831–1845
- Roesch K, Stadler MB, Cepko CL (2012) **Gene expression changes within Müller glial cells in retinitis pigmentosa** *Mol Vis* **18**:1197–1214
- Rueda EM, Hall BM, Hill MC, Swinton PG, Tong X, Martin JF, Poché RA (2019) **The Hippo pathway blocks mammalian retinal Müller glial cell reprogramming** *Cell Rep* **27**:1637–1649
- Shtutman M, Zhurinsky J, Simcha I, Albanese C, D’Amico M, Pestell R, Ben-Ze’ev A (1999) **The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway** *Proc Natl Acad Sci U S A* **96**:5522–5527
- Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H, Haslam SZ, Bronson RT, Elledge SJ, Weinberg RA (1995) **Cyclin D1 provides a link between development and oncogenesis in the retina and breast** *Cell* **82**:621–630
- Solovei I, Kreysing M, Lanctôt C, Kösem S, Peichl L, Cremer T, Guck J, Joffe B (2009) **Nuclear architecture of rod photoreceptor cells adapts to vision in mammalian evolution** *Cell* **137**:356–368
- Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, Hao Y, Stoeckius M, Smibert P, Satija R. (2019) **Comprehensive integration of single-cell data** *Cell* **177**:1888–1902
- Tetsu O, McCormick F (1999) **Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells** *Nature* **398**:422–426
- Todd L, Finkbeiner C, Wong CK, Hooper MJ, Reh TA (2020) **Microglia suppress Ascl1- induced retinal regeneration in mice** *Cell Rep* **33**
- Todd L *et al.* (2022) **Reprogramming Müller glia to regenerate ganglion- like cells in adult mouse retina with developmental transcription factors** *Sci Adv* **8**
- Todd L, Reh TA (2022) **Comparative biology of vertebrate retinal regeneration: Restoration of vision through cellular reprogramming** *Cold Spring Harb Perspect Biol* **14**
- Trimarchi JM, Stadler MB, Cepko CL (2008) **Individual retinal progenitor cells display extensive heterogeneity of gene expression** *PLoS One* **3**
- Ueki Y, Wilken MS, Cox KE, Chipman L, Jorstad N, Sternhagen K, Simic M, Ullom K, Nakafuku M, Reh TA (2015) **Transgenic expression of the proneural transcription factor Ascl1 in Müller glia stimulates retinal regeneration in young mice** *Proc Natl Acad Sci U S A* **112**:13717–13722

Yao K, Qiu S, Tian L, Snider WD, Flannery JG, Schaffer DV, Chen B (2016) **Wnt regulates proliferation and neurogenic potential of Müller glial cells via a Lin28/let-7 miRNA-dependent pathway in adult mammalian retinas** *Cell Rep* **17**:165–178

Yumi MS, Wilken KE, Cox L, Chipman N, Jorstad K, Sternhagen M, Simic K, Ullom M, Nakafuku TA (2015) **Transgenic Expression of the Proneural Transcription Factor Ascl1 in Müller Glia Stimulates Retinal Regeneration in Young Mice** *Proceedings of the National Academy of Sciences of the United States of America* **112**:13717–13722

Zheng GXY *et al.* (2017) **Massively parallel digital transcriptional profiling of single cells** *Nat Commun* **8**

Zukerberg LR, Yang WI, Gadd M, Thor AD, Koerner FC, Schmidt EV, Arnold A (1995) **Cyclin D1 (PRAD1) protein expression in breast cancer: approximately one-third of infiltrating mammary carcinomas show overexpression of the cyclin D1 oncogene** *Mod Pathol* **8**:560–567

Editors

Reviewing Editor

Xiaorong Liu

University of Virginia, Charlottesville, United States of America

Senior Editor

Lois Smith

Boston Children's Hospital, Boston, United States of America

Reviewer #1 (Public Review):

Summary:

In this manuscript, Wu et al. introduce a novel approach to reactivate the Muller glia cell cycle in the mouse retina by simultaneously reducing p27Kip1 and increasing cyclin D1 using a single AAV vector. The approach effectively promotes Muller glia proliferation and reprogramming without disrupting retinal structure or function. Interestingly, reactivation of the Muller glia cell cycle downregulates IFN pathway, which may contribute to the induced retinal regeneration. The results presented in this manuscript may offer a promising approach for developing Müller glia cell-mediated regenerative therapies for retinal diseases.

Strengths:

The data are convincing and supported by appropriate, validated methodology. These results are both technically and scientifically exciting and are likely to appeal to retinal specialists and neuroscientists in general.

Weaknesses:

There are some data gaps that need to be addressed.

- (1) Please label the time points of AAV injection, EdU labeling, and harvest in Figure 1B.
- (2) What fraction of Müller cells were transduced by AAV under the experimental conditions?
- (3) It seems unusually rapid for MG proliferation to begin as early as the third day after CCA injection. Can the authors provide evidence for cyclin D1 overexpression and p27 Kip1

knockdown three days after CCA injection?

(4) The authors reported that MG proliferation largely ceased two weeks after CCA treatment. While this is an interesting finding, the explanation that it might be due to the dilution of AAV episomal genome copies in the dividing cells seems far-fetched.

<https://doi.org/10.7554/eLife.100904.1.sa2>

Reviewer #2 (Public Review):

This manuscript by Wu, Liao et al. reports that simultaneous knockdown of P27Kip1 with overexpression of Cyclin D can stimulate Muller glia to re-enter the cell cycle in the mouse retina. There is intense interest in reprogramming mammalian muller glia into a source for neurogenic progenitors, in the hopes that these cells could be a source for neuronal replacement in neurodegenerative diseases. Previous work in the field has shown ways in which mouse Muller glia can be neurogenically reprogrammed and these studies have shown cell cycle re-entry prior to neurogenesis. In other works, typically, the extent of glial proliferation is limited, and the authors of this study highlight the importance of stimulating large numbers of Muller glia to re-enter the cell cycle with the hopes they will differentiate into neurons. While the evidence for stimulating proliferation in this study is convincing, the evidence for neurogenesis in this study is not convincing or robust, suggesting that stimulating cell cycle-reentry may not be associated with increasing regeneration without another proneural stimulus.

Below are concerns and suggestions.

Intro:

(1) The authors cite past studies showing "direct conversion" of MG into neurons. However, these studies (PMID: 34686336; 36417510) show EdU+ MG-derived neurons suggesting cell cycle re-entry does occur in these strategies of proneural TF overexpression.

(2) Multiple citations are incorrectly listed, using the authors first name only (i.e. Yumi, et al; Levi, et al;). Studies are also incompletely referenced in the references.

Figure 1:

(3) When are these experiments ending? On Figure 1B it says "analysis" on the end of the paradigm without an actual day associated with this. This is the case for many later figures too. The authors should update the paradigms to accurately reflect experimental end points.

(4) Are there better representative pictures between P27kd and CyclinD OE, the EdU+ counts say there is a 3 fold increase between Figure 1D&E, however the pictures do not reflect this. In fact, most of the Edu+ cells in Figure 1E don't seem to be Sox9+ MG but rather horizontally oriented nuclei in the OPL that are likely microglia.

(5) Is the infection efficacy of these viruses different between different combinations (i.e. CyclinD OE vs. P27kd vs. control vs. CCA combo)? As the counts are shown in Figure 1G only Sox9+/Edu+ cells are shown not divided by virus efficacy. If these are absolute counts blind to where the virus is and how many cells the virus hits, if the virus efficacy varies in efficiency this could drive absolute differences that aren't actually biological.

(6) According to the Jax laboratories, mice aren't considered aged until they are over 18months old. While it is interesting that CCA treatment does not seem to lose efficacy over maturation I would rephrase the findings as the experiment does not test this virus in aged retinas.

(7) Supplemental Figure 2c-d. These viruses do not hit 100% of MG, however 100% of the P27Kip staining is gone in the P27sh1 treatment, even the P27+ cell in the GCL that is likely an astrocyte has no staining in the shRNA 1 picture. Why is this?

Figure 2

(8) Would you expect cells to go through two rounds of cell cycle in such a short time? The treatment of giving Edu then BrdU 24 hours later would have to catch a cell going through two rounds of division in a very short amount of time. Again the end point should be added graphically to this figure.

Figure 3

(9) I am confused by the mixing of ratios of viruses to indicate infection success. I know mixtures of viruses containing CCA or control GFP or a control LacZ was injected. Was the idea to probe for GFP or LacZ in the single cell data to see which cells were infected but not treated? This is not shown anywhere?

(10) The majority of glia sorted from TdTomato are probably not infected with virus. Can you subset cells that were infected only for analysis? Otherwise it makes it very hard to make population judgements like Figure 3E-H if a large portion are basically WT glia.

(11) Figure 3C you can see Rho is expressed everywhere which is common in studies like this because the ambient RNA is so high. This makes it very hard to talk about "Rod-like" MG as this is probably an artifact from the technique. Most all scRNA-seq studies from MG-reprogramming have shown clusters of "rods" with MG hybrid gene expression and these had in the past just been considered an artifact.

(12) It is mentioned the "glial" signature is downregulated in response to CCA treatment. Where is this shown convincingly? Figure H has a feature plot of Glul, which is not clear it is changed between treatments. Otherwise MG genes are shown as a function of cluster not treatment.

Figure 4

(13) The authors should be commended for being very careful in their interpretations. They employ the proper controls (Er-Cre lineage tracing/EdU-pulse chasing/scRNA-seq omics) and were very careful to attempt to see MG-derived rods. This makes the conclusion from the FISH perplexing. The few puncta dots of Rho and GNAT in MG are not convincing to this reviewer, Rho and GNAT dots are dense everywhere throughout the ONL and if you drew any random circle in the ONL it would be full of dots. The rigor of these counts also comes into question because some dots are picked up in MG in the INL even in the control case. This is confusing because baseline healthy MG do not express RNA-transcripts of these Rod genes so what is this picking up? Taken together, the conclusion that there are Rod-like MG are based off scRNA-seq data (which is likely ambient contamination) and these FISH images. I don't think this data warrants the conclusion that MG upregulate Rod genes in response to CCA.

Figure 5

(14) Similar point to above but this Glul probe seems odd, why is it throughout the ONL but completely dark through the IPL, this should also be in astrocytes can you see it in the GCL? These retinas look cropped at the INL where below is completely black. The whole retinal section should be shown. Antibodies exist to GS that work in mouse along with many other MG genes, IHC or western blots could be done to better serve this point.

Figure 6

(15) Figure 6D is not a co-labeled OTX2+/ TdTomato+ cell, Otx2 will fill out the whole nucleus as can be seen with examples from other MG-reprogramming papers in the field (Hoang, et al. 2020; Todd, et al. 2020; Palazzo, et al. 2022). You can clearly see in the example in Figure 6D the nucleus extending way beyond Otx2 expression as it is probably overlapping in space.

Other examples should be shown, however, considering less than 1% of cells were putatively Otx2+, the safer interpretation is that these cells are not differentiating into neurons. At least 99.5% are not.

(16) Same as above Figure 6I is not convincingly co-labeled HuC/D is an RNA-binding protein and unfortunately is not always the clearest stain but this looks like background haze in the INL overlapping. Other amacrine markers could be tested, but again due to the very low numbers, I think no neurogenesis is occurring.

(17) In the text the authors are accidentally referring to Figure 6 as Figure 7.

Figure 7

(18) I like this figure and the concept that you can have additional MG proliferating without destroying the retina or compromising vision. This is reminiscent of the chick MG reprogramming studies in which MG proliferate in large numbers and often do not differentiate into neurons yet still persist de-laminated for long time points.

General:

(19) The title should be changed, as I don't believe there is any convincing evidence of regeneration of neurons. Understanding the barriers to MG cell-cycle re-entry are important and I believe the authors did a good job in that respect, however it is an oversell to report regeneration of neurons from this data.

(20) This paper uses multiple mouse lines and it is often confusing when the text and figures switch between models. I think it would be helpful to readers if the mouse strain was added to graphical paradigms in each figure when a different mouse line is employed.

<https://doi.org/10.7554/eLife.100904.1.sa1>

Author response:

Public Reviews:

Reviewer #1 (Public Review):

Summary:

In this manuscript, Wu et al. introduce a novel approach to reactivate the Muller glia cell cycle in the mouse retina by simultaneously reducing p27Kip1 and increasing cyclin D1 using a single AAV vector. The approach effectively promotes Muller glia proliferation and reprogramming without disrupting retinal structure or function. Interestingly, reactivation of the Muller glia cell cycle downregulates IFN pathway, which may contribute to the induced retinal regeneration. The results presented in this manuscript may offer a promising approach for developing Müller glia cell-mediated regenerative therapies for retinal diseases.

Strengths:

The data are convincing and supported by appropriate, validated methodology. These results are both technically and scientifically exciting and are likely to appeal to retinal specialists and neuroscientists in general.

Weaknesses:

There are some data gaps that need to be addressed.

(1) Please label the time points of AAV injection, EdU labeling, and harvest in Figure 1B.

We thank the reviewer for highlighting the lack of clarity in our experimental design. We will label all experiment timelines in the figures where appropriate in the revised version.

(2) What fraction of Müller cells were transduced by AAV under the experimental conditions?

We apologize for not clearly conveying the transduction efficiency. The retinal region adjacent to the injection site, typically near the central retina, exhibits a transduction efficiency of nearly 100%. In contrast, the peripheral retina shows a lower transduction efficiency compared to the central region. We will include the quantification of AAV transduction efficiency in the revised manuscript.

The quantification of Edu+ MG or other markers was conducted in the area with the highest efficiency.

(3) It seems unusually rapid for MG proliferation to begin as early as the third day after CCA injection. Can the authors provide evidence for cyclin D1 overexpression and p27 Kip1 knockdown three days after CCA injection?

In our pilot study, we tested the onset time of GFP expression from AAV-GFAP-GFP following intravitreal injection. We observed GFP expression in MG as early as two days post-infection. These findings will be included in the revised manuscript. Additionally, we plan to perform qPCR or Western blot analysis to confirm cyclin D1 overexpression and p27kip1 knockdown at the onset of Müller glia proliferation, which will also be included in the revised manuscript.

(4) The authors reported that MG proliferation largely ceased two weeks after CCA treatment. While this is an interesting finding, the explanation that it might be due to the dilution of AAV episomal genome copies in the dividing cells seems far-fetched.

We believe that the lack of durability in high Cyclin D1 and low p27kip1 levels in MG contributes to the cessation of their proliferation. A potential reason for the loss of high Cyclin D1 overexpression and p27kip1 knockdown during MG proliferation could be the dilution of the AAV episomal genome. However, testing this hypothesis is challenging. Instead, we plan to provide direct evidence in the revised manuscript by examining the levels of Cyclin D1 and p27kip1 in the retina treated with CCA before and after the peak of MG proliferation.

Reviewer #2 (Public Review):

This manuscript by Wu, Liao et al. reports that simultaneous knockdown of P27Kip1 with overexpression of Cyclin D can stimulate Muller glia to re-enter the cell cycle in the mouse retina. There is intense interest in reprogramming mammalian muller glia into a source for neurogenic progenitors, in the hopes that these cells could be a source for neuronal replacement in neurodegenerative diseases. Previous work in the field has shown ways in which mouse Muller glia can be neurogenically reprogrammed and these studies have shown cell cycle re-entry prior to neurogenesis. In other works, typically, the extent of glial proliferation is limited, and the authors of this study highlight the importance of stimulating large numbers of Muller glia to re-enter the cell cycle with the hopes they will differentiate into neurons. While the evidence for stimulating proliferation in this study is convincing, the evidence for neurogenesis in this study is not convincing or robust, suggesting that stimulating cell cycle-reentry may not be associated with increasing regeneration without another proneural stimulus.

Below are concerns and suggestions.

Intro:

(1) The authors cite past studies showing "direct conversion" of MG into neurons. However, these studies (PMID: 34686336; 36417510) show EdU+ MG-derived neurons suggesting cell cycle re-entry does occur in these strategies of proneural TF overexpression.

We thank the reviewer for pointing this out. We will revise the statement to "MG neurogenesis," which encompasses both direct conversion and Müller glia proliferation followed by neuronal differentiation.

(2) Multiple citations are incorrectly listed, using the authors first name only (i.e. Yumi, et al; Levi, et al;). Studies are also incompletely referenced in the references.

We apologize for the mistake with the reference. We will fix these mistakes in the revised version.

Figure 1:

(3) When are these experiments ending? On Figure 1B it says "analysis" on the end of the paradigm without an actual day associated with this. This is the case for many later figures too. The authors should update the paradigms to accurately reflect experimental end points.

We thank the reviewer for highlighting the lack of clarity in our experimental design. We will label all experiment timelines in the figures where appropriate in the revised version.

(4) Are there better representative pictures between P27kd and CyclinD OE, the EdU+ counts say there is a 3 fold increase between Figure 1D&E, however the pictures do not reflect this. In fact, most of the Edu+ cells in Figure 1E don't seem to be Sox9+ MG but rather horizontally oriented nuclei in the OPL that are likely microglia.

Thanks to the reviewer for pointing this out. We will replace the image of Cyclin D1 which a better representative image.

(5) Is the infection efficacy of these viruses different between different combinations (i.e. CyclinD OE vs. P27kd vs. control vs. CCA combo)? As the counts are shown in Figure 1G only Sox9+/Edu+ cells are shown not divided by virus efficacy. If these are absolute counts blind to where the virus is and how many cells the virus hits, if the virus efficacy varies in efficiency this could drive absolute differences that aren't actually biological.

Because the AAV-GFAP-Cyclin D1 and AAV-GFAP-Cyclin D1-p27kip1 shRNA viruses do not carry a fluorescent reporter gene, we cannot easily measure viral efficacy in the same experiment. We believe that variations in viral efficacy cannot account for the significant differences in MG proliferation for two reasons: 1) We injected the same titer for all three viruses, and 2) Viral infection efficacy is very high, approaching 100% in the central retina. Nonetheless, to rule out the possibility that the differences in MG proliferation among the Cyclin D overexpression, p27kip1 knockdown, and CCA groups are due to variations in viral efficacy, we will include the p27kip1 knockdown and Cyclin D1 overexpression efficiencies for all four groups using qPCR and/or Western blot analysis in the revised manuscript.

(6) According to the Jax laboratories, mice aren't considered aged until they are over 18 months old. While it is interesting that CCA treatment does not seem to lose efficacy over maturation I would rephrase the findings as the experiment does not test this virus in aged retinas.

Thank you to the reviewer for bringing this to our attention. We will void using “aged mice” in our revised manuscript.

(7) Supplemental Figure 2c-d. These viruses do not hit 100% of MG, however 100% of the P27Kip staining is gone in the P27sh1 treatment, even the P27+ cell in the GCL that is likely an astrocyte has no staining in the shRNA 1 picture. Why is this?

For Supplementary Figure 2c-d, we focused on the central area where knockdown efficiency was high, approaching 100%. We will replace this image with one that includes both high and low Müller glia transduction efficiency regions, clearly demonstrating the complete loss of p27kip1 staining in the area of high transduction efficiency.

Figure 2

(8) Would you expect cells to go through two rounds of cell cycle in such a short time? The treatment of giving Edu then BrdU 24 hours later would have to catch a cell going through two rounds of division in a very short amount of time. Again the end point should be added graphically to this figure.

We thank the reviewer for raising this important point. While the typical cell cycle time for human cells is approximately 24 hours, we hypothesized that 24 hours would be the most likely timepoint to capture cells continuously progressing through the cell cycle. However, we acknowledge that we cannot exclude the possibility of some cells entering a second cell cycle at much later timepoints.

In the revised manuscript, we will carefully qualify our conclusion to state that the majority of MG do not immediately undergo another cell division, rather than making a definitive statement. This more cautious phrasing will better reflect the limitations of the 24-hour timepoint and allow for the potential of a small subset of cells proceeding through additional rounds of division at later stages.

Figure 3

(9) I am confused by the mixing of ratios of viruses to indicate infection success. I know mixtures of viruses containing CCA or control GFP or a control LacZ was injected. Was the idea to probe for GFP or LacZ in the single cell data to see which cells were infected but not treated? This is not shown anywhere?

The virus infection was not uniform across the entire retina. To mark the infection hotspots, we added 10% GFP virus to the mixture. Regions of the retina with low infection efficiency were removed by dissection and excluded from the scRNA-seq analysis. We apologize for not clearly explaining this methodological detail in the original text, and will update the Methods section accordingly.

(10) The majority of glia sorted from TdTomato are probably not infected with virus. Can you subset cells that were infected only for analysis? Otherwise it makes it very hard to make population judgements like Figure 3E-H if a large portion are basically WT glia.

This question is related to the last one. Since the regions with high virus infection efficiency were selectively dissected and isolated for analysis, the percentage of CCA-infected MG should constitute the majority in the scRNA-seq data.

(11) Figure 3C you can see Rho is expressed everywhere which is common in studies like this because the ambient RNA is so high. This makes it very hard to talk about "Rod-like" MG as this is probably an artifact from the technique. Most all scRNA-seq studies from MG-reprogramming have shown clusters of "rods" with MG hybrid gene expression and these had in the past just been considered an artifact.

We agree that the low levels of Rho in other MG clusters (such as quiescent, reactivated, and proliferating MG) are likely due to RNA contamination. However, the level of Rho in the rod-like MG is significantly higher than in the other clusters, indicating that this is unlikely to be solely due to contamination.

As shown in Supplementary Figure 7A-C, a cluster of MG-rod hybrid cells (cluster C4) was present in all three experimental groups at similar ratios, and this hybrid cluster was excluded from further analysis. In contrast, the rod-like Müller glia (cluster C3) were predominantly found in the CCA and CCANT groups, suggesting a genuine response to CCA treatment.

Furthermore, we will conduct Rho and Gnat1 RNA in situ hybridization on the dissociated retinal cells to further support the conclusion that rod-specific genes are upregulated in a subset of MG in the revised manuscript.

(12) It is mentioned the "glial" signature is downregulated in response to CCA treatment. Where is this shown convincingly? Figure H has a feature plot of Glul, which is not clear it is changed between treatments. Otherwise MG genes are shown as a function of cluster not treatment.

We will add box plots of several MG-specific genes to better illustrate the downregulation of the glial signature in the relevant cell cluster in the revised manuscript.

Figure 4

(13) The authors should be commended for being very careful in their interpretations. They employ the proper controls (Er-Cre lineage tracing/EdU-pulse chasing/scRNA-seq omics) and were very careful to attempt to see MG-derived rods. This makes the conclusion from the FISH perplexing. The few puncta dots of Rho and GNAT in MG are not convincing to this reviewer, Rho and GNAT dots are dense everywhere throughout the ONL and if you drew any random circle in the ONL it would be full of dots. The rigor of these counts also comes into question because some dots are picked up in MG in the INL even in the control case. This is confusing because baseline healthy MG do not express RNA-transcripts of these Rod genes so what is this picking up? Taken together, the conclusion that there are Rod-like MG are based off scRNA-seq data (which is likely ambient contamination) and these FISH images. I don't think this data warrants the conclusion that MG upregulate Rod genes in response to CCA.

We performed RNA in situ hybridization on retinal sections because we aimed to correlate cell localization with rod gene expression. We understand the reviewer's concern that the punctate signals of Rho and GNAT1 in the ONL MG may actually originate from neighboring rods. In the revised manuscript, we will conduct RNAscope on dissociated retinal cells to avoid this issue.

Figure 5

(14) Similar point to above but this Glul probe seems odd, why is it throughout the ONL but completely dark through the IPL, this should also be in astrocytes can you see it in the GCL? These retinas look cropped at the INL where below is completely black. The whole retinal section should be shown. Antibodies exist to GS that work in mouse along with many other MG genes, IHC or western blots could be done to better serve this point.

Indeed, the GCL was cropped out in Figure 5 A-B. We have other images with all retinal layers, which we will use in the revised manuscript. Additionally, we will perform the GS antibody staining to demonstrate partial MG dedifferentiation following CCA treatment.

Figure 6

(15) Figure 6D is not a co-labeled OTX2+/ TdTomato+ cell, Otx2 will fill out the whole nucleus as can be seen with examples from other MG-reprogramming papers in the field (Hoang, et al. 2020; Todd, et al. 2020; Palazzo, et al. 2022). You can clearly see in the example in Figure 6D the nucleus extending way beyond Otx2 expression as it is probably overlapping in space. Other examples should be shown, however, considering less than 1% of cells were putatively Otx2+, the safer interpretation is that these cells are not differentiating into neurons. At least 99.5% are not.

We have additional examples of Otx2+ Tdt+ Edu+ cells, which suggest that MG neurogenesis to Otx2+ cells does occur, despite the low efficiency. We will include these images in the revised manuscript.

(16) Same as above Figure 6I is not convincingly co-labeled HuC/D is an RNA-binding protein and unfortunately is not always the clearest stain but this looks like background haze in the INL overlapping. Other amacrine markers could be tested, but again due to the very low numbers, I think no neurogenesis is occurring.

We have additional examples of HuC/D+ Tdt+ Edu+ cells, which we will show in the revised manuscript.

(17) In the text the authors are accidentally referring to Figure 6 as Figure 7.

We thank the reviewer for pointing out the mistake. We will correct the mistake in the revised manuscript.

Figure 7

(18) I like this figure and the concept that you can have additional MG proliferating without destroying the retina or compromising vision. This is reminiscent of the chick MG reprogramming studies in which MG proliferate in large numbers and often do not differentiate into neurons yet still persist de-laminated for long time points.

General:

(19) The title should be changed, as I don't believe there is any convincing evidence of regeneration of neurons. Understanding the barriers to MG cell-cycle re-entry are important and I believe the authors did a good job in that respect, however it is an oversell to report regeneration of neurons from this data.

We thank the reviewer for the suggestion. We will consider changing the title in the revised manuscript.

(20) This paper uses multiple mouse lines and it is often confusing when the text and figures switch between models. I think it would be helpful to readers if the mouse strain was added to graphical paradigms in each figure when a different mouse line is employed.

We will label the mouse lines used in each experiment in the figures where appropriate.

<https://doi.org/10.7554/eLife.100904.1.sa0>