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Retinoic Acid-signaling regulates the proliferative and neurogenic capacity of Müller glia-derived progenitor cells in the avian retina

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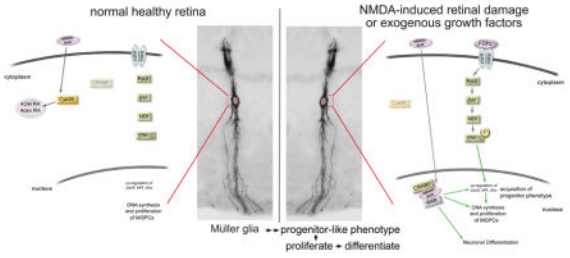
Abstract

In the retina, Müller glia have the potential to become progenitor cells with the ability to proliferate and regenerate neurons. However, the ability of Müller glia-derived progenitor cells (MGPCs) to proliferate and produce neurons is limited in higher vertebrates. Using the chick model system, we investigate how retinoic acid (RA) -signaling influences the proliferation and the formation of MGPCs. We observed an up-regulation of cellular retinoic acid binding proteins (CRABP) in the Müller glia of damaged retinas where the formation of MGPCs is known to occur. Activation of RA-signaling was stimulated, whereas inhibition suppressed the proliferation of MGPCs in damaged retinas and in FGF2-treated undamaged retinas. Furthermore, inhibition of RA-degradation stimulated the proliferation of MGPCs. Levels of Pax6, Klf4, and cFos were up-regulated in MGPCs by RA agonists and down-regulated in MGPCs by RA antagonists. Activation of RA-signaling following MGPC proliferation increased the percentage of progeny that differentiated as neurons. Similarly, the combination of RA and IGF1 significantly increased neurogenesis from retinal progenitors in the circumferential marginal zone (CMZ). In summary, RA-signaling stimulates the formation of proliferating MGPCs and enhances the neurogenic potential of MGPCs and stem cells in the CMZ.

Graphical Abstract



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Author Contributions:

LT designed and executed experiments, gathered data, constructed figures and contributed to writing the manuscript. LS and CQ executed experiments, gathered data, constructed figures and contributed to writing the manuscript. AJF designed experiments, constructed figures and contributed to writing the manuscript. No competing interests declared by any of the authors.

Introduction

The capacity for retinal regeneration varies substantially across vertebrate species. In response to injury, the teleost fish is able to regenerate a functional retina, whereas birds and mammals are unable to mount a significant regenerative response (Lenkowski and Raymond 2014). Despite the wide divergence in the regenerative capacity between species, Müller glia are the cellular source for regenerated neurons in the fish, chick, and mouse retina (Fausett and Goldman 2006; Fischer and Reh 2001; Ueki et al. 2015). In uninjured retina, Müller glia perform a wide variety of support functions to retinal neurons (Reichenbach and Bringmann 2013). However, even in healthy retinas, Müller glia express genes commonly associated with progenitor cells (Blackshaw et al. 2004; Roesch et al. 2008; Ueno et al. 2017). This unique genomic profile may underlie the ability of Müller glia to reprogram into proliferating progenitors. Understanding the signaling pathways responsible for regulating the regenerative potential of MGPCs is important for developing novel therapies to treat sight-threatening diseases of the retina.

A large network of cell-signaling pathways is known to regulate the reprogramming of Müller glia into MGPCs (reviewed by (Gallina et al. 2014a; Goldman 2014; Hamon et al. 2016; Lenkowski and Raymond 2014). MAPK, Jak/Stat, Wnt/β-catenin, PI3K/Akt/mTOR, Hedgehog, BMP/TGFβ/Smad and Notch signaling have been shown to be involved in the reprogramming of Müller glia in both the fish and the chick retina (Conner et al. 2014; Fischer et al. 2009a; Fischer et al. 2009b; Gallina et al. 2015; Ghai et al. 2010; Kassen et al. 2009; Meyers et al. 2012; Nelson et al. 2012; Todd and Fischer 2015; Todd et al. 2017; Todd et al. 2016; Wan et al. 2014; Zelinka et al. 2016; Zhao et al. 2014). Relatively little is known about the signaling pathways that drive the reprogramming of Müller glia in the mammalian retina. MAPK- and Wnt-signaling can stimulate, to a small extent, the proliferation of Müller glia in damaged rodent retinas (Karl et al. 2008; Liu et al. 2012). Virus-mediated gene transfer of β-catenin- to Müller glia has been reported to stimulate the formation of MGPCs in undamaged rodent retinas (Yao et al. 2016). Additionally, forced expression of the proneural bHLH transcription factor Ascl1 has been shown to stimulate neuronal regeneration from Müller glia in the rodent retina (Jorstad et al. 2017; Pollak et al. 2013; Ueki et al. 2015). Ascl1 is required for regeneration of the fish retina (Fausett et al. 2008) and is known to be up-regulated in MGPCs in the retinas of both fish and chicks (Fausett et al. 2008; Fischer and Reh 2001; Hayes et al. 2007).

The retinoic acid (RA)-signaling may play important roles in retinal regeneration since this cell-signaling pathways is known to be essential in neuronal differentiation and patterning during development (Maden 2007). RA-signaling promotes neural differentiation in the developing zebrafish, chick, and rodent retina (Hyatt et al. 1996; Kelley et al. 1994; Kelley et al. 1999; Stenkamp et al. 1993; Valdivia et al. 2016). During embryonic development in the chick retina, interference of RA-signaling through forced expression of a dominant-negative RA receptor resulted in reduced proliferation of progenitors and disruption of dorsal-ventral patterning (Sen et al. 2005). In the context of retinal regeneration in rodents, exogenous RA has been shown to promote the differentiation of bipolar neurons from MGPCs (Ooto et al. 2004), whereas other studies failed to replicate these results (Karl et al. 2008). The purpose of this study was to investigate how RA-signaling impacts the

reprogramming of Müller glia into proliferating neurogenic MGPCs in the chick retina *in vivo*.

Methods and Materials

Animals

The use of animals was according to the guidelines established by the National Institutes of Health and the Ohio State University. Newly hatched chickens (*Gallus gallus domesticus;* white leghorn strain) were obtained from Meyer Hatchery (Polk, Ohio). Chicks were housed in a stainless steel brooder at 25°C, received water and Purina^{tm} chick starter *ad libitum,* and kept on 12:12 hour light:dark cycle (lights on at 8 am).

Intraocular injections

Chickens were anesthetized and intraocular injections performed as described previously (Fischer et al., 1999). In short, anesthesia was achieved via inhalation of 2.5% isoflurane in oxygen (Fischer et al., 1999). The right eyes were injected with the "test" compound and the contra-lateral left eyes were injected with vehicle (control). Compounds were injected in 20 µl sterile saline with 0.05 mg/ml bovine serum albumin added as a carrier. Compounds used in these studies included N-methyl-D-aspartate (NMDA; 38.5 or 154 µg/dose), FGF2 (250 ng/dose; R&D systems), liarozole dihydrochloride (4µg/dose; 5-[(3-Chlorophenyl)-1*H*-imidazol-1-ylmethyl]-1*H*-benzimidazole dihydrochloride; Tocris), TTNBP (4µg/dose; 4-[(*E*)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid), Retinoic Acid (RA; 5µg/dose; Sigma-Aldrich), BMS493 (4-[(1*E*)-2-[5,6-Dihydro-5,5-dimethyl-8-(2-phenylethynyl)-2-naphthalenyl]ethenyl]benzoic acid: 2µg/dose; Tocris), IGF1 (400ng/dose; R&D Systems). A two µg dose of EdU (5-ethynyl-2′-deoxyuridine) was injected to label proliferating cells. Injection paradigms are included in each figure.

Fixation, sectioning and immunocytochemistry

Tissues were fixed, sectioned and immunolabeled as described previously (Fischer et al. 2008b; Fischer et al. 2009b). The working dilutions and sources of antibodies are listed in table 1. None of the fluorescence resulted from auto-fluorescence or non-specific binding of secondary antibodies; sections labeled with secondary antibodies alone contain no significant fluorescence. Secondary antibodies included donkey-anti-goat-Alexa488/568, goat-anti-rabbit-Alexa488/568/647, goat-anti-mouse-Alexa488/568/647, goat anti-rat-Alexa488 (Life Technologies) diluted to 1:1000 in PBS plus 0.2% Triton X-100.

Labeling for EdU

Following immunolabeling procedures, sections were fixed in 4% formaldehyde in PBS for 5 minutes at room temperature, and washed twice for 5 minutes in PBS. Sections were incubated for 30 minutes at room temperature in 2M Tris, 50 mM $CuSO_4$, Alexa Fluor 568 Azide (Thermo Fisher Scientific), and 0.5M ascorbic acid in dH_2O. Finally, sections were washed in PBS for 5 minutes and coverglass mounted 80% glyercol in water, as described previously (Todd, et al. 2017).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Dying cells were identified by labeling for fragmented DNA by using the TUNEL method. We used the *In Situ* Cell Death Kit (TMR red; Roche Applied Science), according to the manufacturer's instructions.

Photography, measurements, cell counts and statistics

Wide-field photomicrographs were obtained using a Leica DM5000B microscope and Leica DC500 digital camera. Confocal photomicrographs were obtained using a Leica SP8 imaging system at the Hunt-Curtis Imaging Facility in the Department of Neuroscience at The Ohio State University. Images were adjusted and figures constructed by using Adobe Photoshop.

Cell counts were performed on images that were sampled from different regions of the retina. To avoid region-specific differences within the retina, cell counts were consistently made from 5,400 μm^2 from central or peripheral regions of retina for each data set. Central retina was defined as the region within a 3mm radius of the posterior pole of the eye, and peripheral retina was defined as an annular region between 3mm and 0.5mm from the far peripheral edge of the retina. The cell-type identity of EdU-labeled cells was determined based on findings that 100% of the proliferating cells in the chick retina are comprised of Sox2/9$^+$ Müller glia in the INL/ONL, Nkx2.2$^+$ Non-astrocytic Inner Retinal Glial (NIRG) cells in the IPL, GCL and NFL, and CD45$^+$ microglia (Fischer et al. 2010; Zelinka et al. 2012). Sox2/9$^+$ nuclei in the INL/ONL were identified as Müller glia based on their relatively large size and fusiform shape.

Similar to previous reports (Fischer et al. 2009a; Fischer et al. 2009b; Fischer et al. 2010; Ghai et al. 2009), immunofluorescence was quantified by using ImagePro6.2 (Media Cybernetics, Bethesda, MD, USA). Images to be using for quantification were obtained using identical illumination, microscope, and camera settings. Retinal areas were randomly sampled over the INL and ONL. Measurement for content in the nuclei of Müller glia/MGPCs were made by selecting the total area of pixels with values ≥70 (0 = black and 255 = saturated) for Sox2 or Sox9 (in the red channel), and copying Klf4 or Pax6 (in the green channel). These copied data were pasted into a separate file for quantification or onto 70% grayscale background to produce figures. The density sum was calculated as the sum of values for all pixels within thresholded regions. These calculations were determined for at least 5 different retinas for each experimental condition.

GraphPad Prism 6 was used for statistical analyses. A two-tailed, paired t-test was used to determine significance of difference between two treatment groups accounting for inter-individual variability (means of treated-control values). A two-tailed, unpaired t-test was used to determine significance of difference between two treatment groups.

Results

Up-regulation of CRABP in Müller glia after NMDA damage

RA binds to cellular RA-binding proteins (CRABP1/2) which allows RA to shuttle through the cytoplasm and enter the nucleus (Cvekl and Wang 2009). In the nucleus, RA dissociates from CRABP and interacts with receptors that can bind to RA-target genes and activate transcription (Cvekl and Wang 2009). Accordingly, we probed for the expression of CRABP in NMDA-damaged retinas where proliferating MGPCs are known to form (Fischer and Reh 2001). Consistent with previous reports (Fischer et al. 1999), immunoreactivity for CRABP was detected in presumptive bipolar and amacrine cells (Fig. 1). Many of these cells were destroyed or down-regulated CRABP by 1 day after NMDA-treatment (Fig. 1). At 2 days after NMDA-treatment, when MGPCs are known to re-enter the cell cycle (Fischer and Reh 2001), there was an increase in the levels of CRABP in Müller glia/MGPCs cytoplasm and nuclei (Fig. 1). By 3 days after NMDA-treatment, CRABP was detected in the nuclei of $Sox2^{+}$ MGPCs (Fig. 1). Collectively, these findings suggest that Müller glia and/or MGPCs respond to retinal damage by up-regulating RA-signaling.

RA-signaling stimulates MGPC proliferation after retinal damage

RA has been reported to regulate the proliferation of neural progenitor cells in different regions of the developing CNS (Hyatt et al. 1992; Marsh-Armstrong et al. 1994; Sen et al. 2005). Accordingly, we tested whether application of TTNPB, a potent analog of RA (Astrom et al. 1990), influenced the proliferation of MGPCs following low-levels of NMDA-induced damage where relatively few MGPCs are known to form (Fischer et al. 2004). Treatment of damaged retinas with TTNPB resulted in a significant increase in the numbers of proliferating Müller glia/MGPCs (Figs. 2a–b). By comparison, TTNPB did not affect the proliferation of Non-astrocytic Inner Retinal Glial (NIRG) cells and microglia (Figs. 2c,d). NIRG cells are a distinct type of glial cell that has been described in the retinas of birds (Fischer et al. 2010; Rompani and Cepko 2010) and possibly snakes and turtles (Todd et al. 2015a). Treatment with TTNPB also increased levels of Pax6-expression in Müller glia/MGPCs (Figs. 2e,f). Pax6 has been shown to be required for Müller glia-mediated regeneration in the fish retina (Thummel et al. 2010). By comparison, levels of the stem cell factor Klf4 were unaffected by treatment with TTNPB (not shown). To further examine whether RA-signaling is involved in the formation of MGPCs we examined whether proliferation was influenced by inhibition of RA-degradation. We applied the compound liarozole-dihydrochloride, an inhibitor of cytochrome P450 (Van Wauwe et al. 1992); cytochrome P450 metabolizes RA making it inactive, therefore, the inhibition of cytochrome P450 leads to elevated levels of RA (Thatcher and Isoherranen 2009). We found that intraocular injections of liarozole significantly increased the number of proliferating Müller glia/MGPCs in damaged retinas (Fig. 2g).

Since activation of RA-signaling enhanced the formation of proliferating MGPCs, we tested whether inhibition of RA-signaling suppressed the formation of MGPCs in damaged retinas. We tested whether the small-molecule Retinoic Acid Receptor (RAR) antagonist BMS493 (Germain et al. 2009) influenced the formation of proliferating MGPCs in retinas damaged by a relatively high dose of NMDA. We found that inhibition of RAR significantly

decreased numbers of EdU-labeled MGPCs (Figs. 3a,b). This effect was specific to Müller glia/MGPCs as inhibition of RAR had no effect on the proliferation of NIRG cells or microglia/macrophages (Figs. 3c,d). Collectively, these findings suggest that RA-signaling is mitogenic to Müller glia/MGPCs in damaged retinas.

The formation of proliferating MGPCs is known to be increased with elevated levels of retinal damage (Fischer et al. 2004) and reactive microglia (Fischer et al. 2014b). We found that treatment of damaged retinas with TTNBP, liarozole, or BMS493 had no effect upon numbers of dying TUNEL+ cells (Fig 2h, 3d) or levels of CD45 in microglia (data not shown) which is diagnostic of microglia reactivity (Fischer et al. 2014b; Gallina 2015). Thus, the effects of RA-agonists and –antagonists on the proliferation of MGPCs occurred independent of levels of retinal damage or microglial reactivity.

Activation of RA-signaling in FGF2-treated retinas stimulates MGPC-formation in the absence of retinal damage

We tested whether activation of RA-signaling in undamaged retinas stimulated the formation of proliferating MGPCs. Four consecutive daily intraocular injections of TTNBP or exogenous RA had no influence on the proliferation of Müller glia in the absence of damage (not shown). By comparison, treatment with four consecutive daily doses of FGF2 is sufficient to result in the formation of numerous proliferating MGPCs in healthy retinas (Fischer et al. 2014b). However, three doses of FGF2 in combination with other mitogens such as Sonic Hedgehog (Todd and Fischer 2015), insulin, IGF1 (Fischer et al. 2002; Fischer and Reh 2000), CNTF (Todd et al. 2016), or in combination with inhibitors of glucocorticoid- (Gallina et al. 2014b) or TGFβ/Smad2-signaling (Todd et al. 2017) have been shown to potentiate the formation of MGPCs. We found that three consecutive daily doses of FGF2 in combination with RA or TTNPB stimulated the formation of proliferating MGPCs (Figs. 4a–c). The potentiating effects of RA and TTNPB were specific to Müller glia as microglia and NIRG cell proliferation was unaffected (Figs. 4c,d,f,g). TTNPB increased levels of cFos-expression in the nuclei of FGF2-treated Müller glia/MGPCs (Figs. 4h,i). RA combined with FGF2 also stimulated cFos expression in Sox2+ Müller glia/MGPCs (not shown). We have previously found that expression levels of cFos are correlated to the proliferation of MGPCs (Fischer et al. 2009b; Todd and Fischer 2015). Consistent with the notion that activation of RA-signaling promotes the reprogramming of Müller glia into MGPCs, we found increased expression of the stem cell-associated transcription factors Pax6 and Klf4 in Müller glia treated with FGF2 and TTNBP compared to levels seen in Müller glia treated with FGF2 alone (Figs.4j–m).

In accordance with findings that RA-agonists stimulate the formation of MGPCs, we found that inhibition of RA-signaling in FGF2-treated retinas suppresses the formation of proliferating MGPCs. When BMS483 was combined with three consecutive daily injections of FGF2, we found a significant decrease in the levels of Klf4 that were expressed by Müller glia compared to levels seen in Müller glia treated with FGF2 alone (Figs. 5a,b). These findings suggest that inhibition of RA-signaling in FGF2-treated Müller glia suppresses the acquisition of progenitor phenotype.

When BMS493 was combined with four consecutive daily injections of FGF2, we found a significant decrease in the number of EdU-labeled Müller glia/MGPCs (Figs. 5c,d). In addition, we found that BMS493 inhibited the proliferation of NIRG cells, whereas the proliferation of microglia was not affected (Figs. 5c,d). Inhibition of RA-signaling in FGF2-treated retinas resulted in a significant decrease in levels of Pax6 in the nuclei of Müller glia/MGPCs (Figs. 5e,f), consistent with the notion that RA-signaling promotes the reprogramming of Müller glia into MGPCs.

RA-treatment increases neuronal differentiation from MGPC-progeny

RA-signaling is known to promote neuronal and glial differentiation during neural development (reviewed by (Janesick et al. 2015)). Thus, we tested whether application of RA following the proliferation of MGPCs influence the differentiation of the progeny. We applied RA at 3 and 4 days after NMDA-treatment, starting 24 hours after an application of EdU to label proliferating MGPCs which are known re-enter the cell cycle at 48 hours after NMDA-treatment (Fischer and Reh 2001). We found that RA-treatment increased the percentage of MGPC-progeny that differentiated into HuC/D-expressing neurons by nearly 40% (Figs. 6a,b). In addition, RA-treatment resulted in a decrease in the percentage of MGPC-progeny that differentiated into GS-expressing Müller glia by nearly 20% (Figs. 6c,d). Since exogenous RA may be degraded by endogenous CYP26, we tested whether RA combined with liarozole influenced neurogenesis from MGPCs. No further increase in neurogenesis resulted from the combination of liarozole and RA compared to RA alone (Fig. 6b). This suggests that the effects of exogenous RA were not diminished by degradation. We failed to find evidence of differentiation of MGPC progeny into Lim1/2^{+} horizontal cells or Lim3^{+} bipolar cells/immature photoreceptors (data not shown). In the chick retina, Lim1/2 is expressed by GABAergic horizontal cells (Fischer et al. 2007), and Lim3 is expressed by a subset of mature bipolar cells and is transiently expressed by immature photoreceptors (Fischer et al. 2008a). Since these newly generated Edu+ cells colocalize with HuD, a marker found in amacrine cells and because they are found within the INL we presume that these are regenerated amacrine cells (Fischer and Reh, 2001).

Neuronal differentiation is increased by RA-treatment of retinal progenitors in the CMZ

Progenitor cells are known to be organized into a CMZ at the far peripheral edge of the retina in the eyes of different vertebrate species including chicks (Fischer and Reh 2000; Ghai et al. 2008). The progenitors in the CMZ are relatively quiescent and capable of proliferating at increased rates and differentiating as neurons when treated with exogenous growth factors (Fischer et al. 2002). Accordingly, we tested whether RA influenced the proliferation of CMZ progenitors at posthatch day 1 when the CMZ is most active (Fischer and Reh, 2000. We found that 3 consecutive daily intraocular injections of RA alone had no effect upon the proliferation of CMZ progenitors (not shown). We next tested application of RA in combination with IGF1, a factor known to prime proliferation of CMZ progenitors (Todd & Fischer, 2015). The combination of RA with IGF1 significantly increased the proliferation of retinal progenitors in the CMZ compared to IGF1 alone (Figs. 7a,b.). In addition, the combination of RA and IGF1 resulted in a significant increase in the percentage of cells at the retinal margin that differentiated as neurons. We found that the percentage of cells that differentiated as HuC/D+ neurons was increased nearly 150% (Figs.

7c,e,f), and the percentage of cells that differentiated as Otx2+ neurons was increased by nearly 60% (Figs. 7d,g,h).

Discussion

Our findings implicate RA-signaling as an important player in the complex network of signaling pathways that controls MGPC-formation. Components of the RA-signaling pathway are up-regulated during retinal regeneration in the goldfish (Nagashima et al. 2009) and frog retinas (Duprey-Diaz et al. 2016) in response to optic nerve injury. We found that CRABP is up-regulated in Müller glia in response to retinal damage when MGPCs are forming in the chick retina. This suggests that the involvement of the RA-signaling pathway may be conserved across species. It is likely that RA-signaling is manifested in Müller glia and MGPCs. There are many RA-pathway and RA-target genes expressed by normal Müller glia in the rodent retina (Roesch et al. 2008). Normal Müller glia appear to express very low levels of RARγ, RARα, and RARβ (Roesch et al. 2008) and the levels of expression are increased in response to retinal damage (Roesch et al. 2012). In the frog retina, RALDH and CRABP1 were detected in Müller glia processes (Duprey-Diaz et al. 2016). Müller glia are known to regulate local RA-metabolism with respect to photopigment regeneration. Cone photoreceptors recycle their chromophores via Müller glia. In this pathway, all-trans retinol is transported from cones to Müller glia, retinol is converted into 11-cis retinol by all-trans retinol isomerase and then stored as retinyl esters within Müller glia or transported back to the cones (Wang and Kefalov 2011). Thus, components of the RA-signaling pathway are in place for Müller glia to respond and provide signals.

Glial and neural progenitor cells are known to contribute to RA-signaling in different contexts. Cultured astrocytes express the enzymes for RA biosynthesis and produce active RA and antagonism of RAR prevents glia-induced neuronal differentiation from stem cells (Kornyei et al. 2007). Additionally, during development, radial glia and slowly dividing astrocytes in the postnatal sub-ventricular zone (SVZ) respond to RA-signaling by proliferating at increased rates (Haskell and LaMantia 2005). The cell-signaling and metabolic pathways that are involved in RA-signaling appear to be in place for glia and neural progenitors to respond by proliferating and/or modifying their phenotype. However, further studies are required to better determine the effects of RA on mature glia in the central nervous system.

RA-agonists failed to stimulate the formation of proliferating MGPCs in the absence of damage, suggesting that co-activation of additional signaling pathways or up-regulation of receptors is required to render Müller glia responsive. In support of this notion, RA-agonists stimulated the proliferation of MGPCs in NMDA-damaged retinas or undamaged retinas treated with FGF2. These findings are reminiscent of previous findings wherein activation of Hedgehog-, Smad1/5/8-, mTor or Jak/Stat-signaling in Müller glia is not sufficient to stimulate the formation of proliferating MGPCs in undamaged retinas, whereas activation of these pathways stimulated the proliferation of MGPCs in damaged retinas or undamaged FGF2-treated retinas (Todd and Fischer 2015; Todd et al. 2017; Todd et al. 2016; Zelinka et al. 2016). Interestingly, in undamaged retinas, Müller glia readily activate the second messengers that are part of BMP/Smad, CNTF/Jak/Stat, IGF/PI3K/mTor cell-signaling

pathways (Todd et al. 2017; Todd et al. 2016; Zelinka et al. 2016). However, the mitogenic effects of these pathways manifest only when combined with neuronal damage or FGF/MAPK-signaling and in the presence of reactive microglia (Fischer et al. 2014b). Alternatively, FGF/MAPK-signaling and NMDA-mediated damage may lead to changes in pathways, such as the Notch- and β-catenin pathways, that enable the reprogramming of Müller glia into proliferating MGPCs (Gallina et al. 2015; Ghai et al. 2010; Hayes et al. 2007).

Consistent with our findings, RA-signaling has been found to be mitogenic to neural progenitors in different regions of the developing nervous system. In the fish retina, exogenous addition of RA during the optic primordia stage causes proliferation in ventral retina and a duplication of the entire retina (Hyatt et al. 1992), while inhibition of RA-signaling leads to reduced proliferation of progenitors and retinas that lack a ventral region (Marsh-Armstrong et al. 1994). In the embryonic chick retina, inhibition of RA-signaling by a dominant-negative RA receptor resulted in reduced proliferation of retinal progenitors (Sen et al. 2005). Similarly, disulfram, an inhibitor of RA synthesis attenuates the proliferation of SVZ progenitors *in vivo* (Wang et al. 2005). However, other reports have found that RA has no effect or suppresses the proliferation of neural progenitor cells (Hyatt et al. 1996; Jacobs et al. 2006; Valdivia et al. 2016), possibly due to the pro-differentiation effects of RA (Janesick et al. 2015). Taken together, these findings suggest that the effects of RA-signaling on proliferation and neuronal differentiation are context dependent.

We find that RA-signaling is recruited into the network of pathways that regulate the formation of proliferating MGPCs. This is evident by the findings that inhibition of RAR in FGF2-treated retinas reduced numbers of proliferating MGPCs. In the undamaged avian retina, consecutive daily application of FGF2 is sufficient to stimulate the formation of proliferating MGPCs by activating a network of cell signaling pathways that includes MAPK-, glucocorticoid-, Hedgehog-, Wnt/β-catenin-, Hedgehog, Jak/Stat- and BMP/Smad-signaling (Fischer et al. 2009b; Gallina et al. 2015; Gallina et al. 2014b; Todd and Fischer 2015; Todd et al. 2017; Todd et al. 2016). A similar network of signaling pathways regulates the regenerative potential of MGPCs in the zebrafish retina (reviewed by (Goldman 2014; Lenkowski and Raymond 2014). Relatively little is known about the cell signaling events underlying MGPC formation in the mammalian retina. However, common between fish, bird, and rodent model systems, activation of MAPK-, Hedgehog- and Wnt/β-catenin-signaling in damaged retinas promotes the formation of MGPCs (reviewed by (Hamon et al. 2016)). Interactions between FGF/MAPK and RA-signaling are known to occur during neural development where these signaling pathways drive neural patterning and proliferation of progenitor cells (Diez del Corral et al. 2003; Liu et al. 2001). Furthermore, RA can activate MAPK effectors in hippocampal neurons and neuroblastoma cells (Chen et al. 2008; Masia et al. 2007). The precise mechanisms by which RA-signaling interacts with MAPK-signaling during the formation of MGPCs requires further investigation. It is possible that crosstalk between MAPK and RA-signaling occurs at the level GATA transcription factors which can be phosphorylated by MAPKs and then form complexes with ligand-bound RARα (Tsuzuki et al. 2004).

The potential of MGPCs to produce neurons in the retinas of birds and mammals is very limited. Thus, to harness the regenerative potential of MGPCs methods to enhance the neuronal differentiation of the progeny of MGPCs is required. Activation of RA-signaling increased the percentage of neuronal progeny from MGPCs in damaged retinas. This data adds RA-signaling to the small list of pathways, including Notch-, glucocorticoid-, and gp130/Jak/Stat-signaling, that are known to influence neuronal differentiation from MGPCs in the higher vertebrate (Gallina et al. 2014b; Hayes et al. 2007; Todd et al. 2016). Previously, RA was reported to increase the amount of bipolar cell differentiation from MGPCs in the rat retina (Ooto et al. 2004), however another study in the mouse failed to replicate this result (Karl et al. 2008). RA is a key determinant of neuronal differentiation in a variety of systems (reviewed by (Janesick et al. 2015). During development, RA-signaling promotes photoreceptor differentiation in zebrafish, chick and rodent retinas (Hyatt et al. 1996; Kelley et al. 1999; Stenkamp et al. 1993). RA-signaling has recently been implicated in the regulation of photoreceptor patterning in the high-acuity area of the chick retina and potentially to human fovea patterning (da Silva and Cepko 2017). In rodent, monkey, and human, exogenous RA promotes rod photoreceptor differentiation from embryonic stem cells (Osakada et al. 2008). In adult mammals, RA-signaling promotes neurogenesis from stem cells. Mice fed a diet that is retinoid-depleted have decreased neurogenesis in the dentate gyrus (Jacobs et al. 2006). By comparison, exogenous RA increases neuronal differentiation in explant cultures of SVZ progenitors (Jacobs et al. 2006; Wang et al. 2005). RA-signaling may promote neuronal differentiation through interactions with Notch-signaling and Ascl1-mediated transcription (Jacob et al. 2013; Johnson et al. 1992). Interestingly, forced expression of Ascl1 is sufficient to reprogram Müller glia into neurogenic MGPCs in the mammalian retina (Jorstad et al. 2017; Ueki et al. 2015). Taken together, these data suggest RA-signaling is important for adult neurogenesis and is active in adult stem cells niches that support neurogenesis.

In the retina, a discrete stem cell niche is found within the CMZ. In juvenile and adult fish, retinal growth occurs by the addition of concentric rings of cells produced by CMZ progenitors (Fischer et al. 2014a). CMZ progenitors have also been described in the embryonic and posthatch chick retina, where these progenitors proliferate and express a variety of progenitor-associated markers (Fischer and Reh 2000; Ghai et al. 2008). Recent reports have described a population of CMZ progenitors that give rise to neurons in the mouse retina (Belanger et al. 2017; Marcucci et al. 2016). IGF1 is known to stimulate the proliferation of CMZ progenitors in the chick (Fischer and Reh 2000). Here we report that exogenous RA combined with IGF1 increases the neurogenic capacity of CMZ progenitors in the chick retina. Interestingly, IGF1 is also known to prime CMZ progenitors and non-pigmented epithlial cells, adjacent to the CMZ, to become receptive or respond differentially to factors such FGF2, EGF, HB-EGF and Sonic Hedghog (Fischer et al. 2002; Fischer and Reh 2003; Ritchey et al. 2012; Todd and Fischer 2015; Todd et al. 2015b). We provide novel data that CMZ progenitors in the chick retina are capable of producing Otx2+ neurons. Presumably, these $Otx2^{+}/EdU^{+}$ cells are newly born bipolar cells or photoreceptors (Nishida et al. 2003). We failed to find newly born $Otx2^{+}$ neurons derived from RA-treated MGPCs, suggesting that the CMZ progenitors have a broader neurogenic potential than MGPCs, and/or the neurogenic micro-environment is more permissive near the CMZ compared to

more central regions of the retina. By comparison, elevated RA-signaling increases the neurogenesis from CMZ progenitors in the zebrafish (Valdivia et al. 2016). Collectively, these findings implicate RA-signaling in the regulation of CMZ progenitor function and neurogenesis.

Conclusions

We conclude that activation of RA-signaling stimulates both the proliferation and neurogenic potential of MGPCs in the avian retina. We find that RA-signaling is included in the network of cell-signaling pathways that are activated in response to neuronal damage and FGF2-mediated stimulation. Although activators and inhibitors of RA-signaling had little effect upon Müller glia in normal retinas, RA-signaling promoted progenitor phenotype and proliferation of MGPCs in damaged and FGF2-treated retinas. Importantly, activation of RA-signaling following proliferation of MGPCs enhanced neuronal differentiation at the expense of glial differentiation. We conclude that RA-signaling is a promising target to enhance the formation of neurogenic MGPCs.

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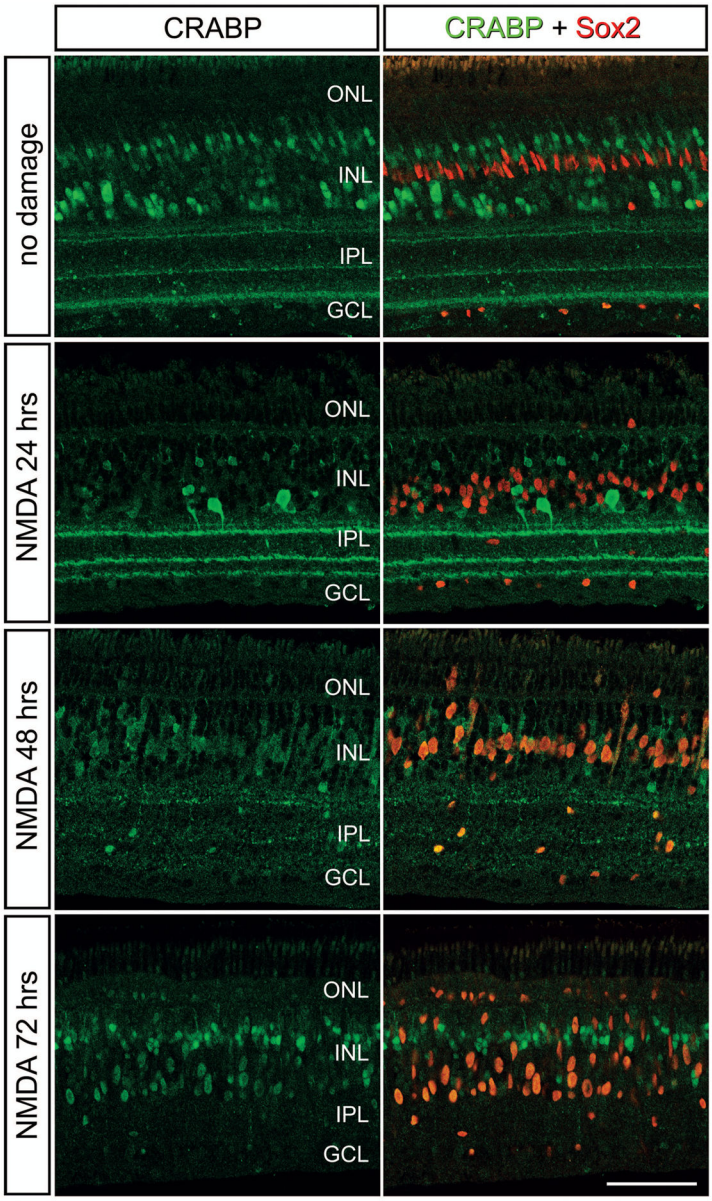

Figure 1.

Patterns of expression of CRABP in damaged retinas. Retinas were obtained from eyes that were injected with saline at P7 or 1 μmol of NMDA, and tissues harvested at 1, 2, and 3 days later. Sections of the retina were labeled with antibodies to CRABP (green) and Sox2 (red). The calibration bar (50 μm) in the bottom right panel applies to all panels. Abbreviations: INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer, ONL – outer nuclear layer.

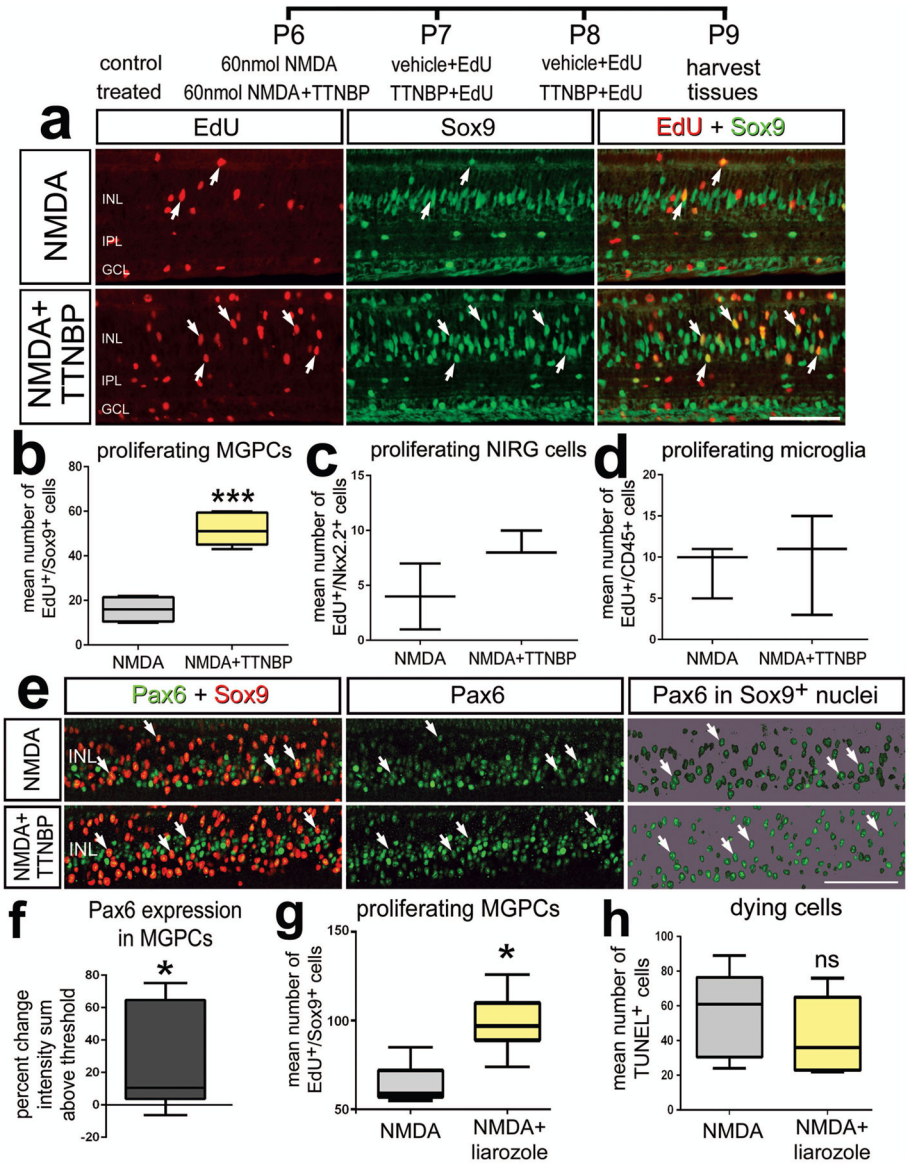

Figure 2.

Activation of RA-signaling stimulates the proliferation of MGPCs in damaged retinas. Eyes were injected with a relatively low dose (60 nmol) of NMDA (control) or NMDA + RAR agonist (TTNBP; treated) at P6, vehicle + EdU (control) or TTNBP + EdU (treated) at P7 and P8, and tissue harvested at P9. Sections of the retina were labeled for EdU-incorporation (red) and antibodies to Sox9 (green; **a**), or Pax6 (green) and Sox9 (red; **c**). Arrows indicate the nuclei of Müller glia/MGPCs. The box plots in illustrate the mean, upper extreme, lower extreme, upper quartile and lower quartile (n≥6 animals). Significance of difference (***p<0.0001) was determined by using a t-test (**b**,**c**,**d**,**g**,**h**) or (*p<0.05) was determined by using a Mann-Whitney U test (**f**). Arrows indicate the nuclei of MGPCs. The calibration bar panels **a** and **e** represents 50 μm. Abbreviations: INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer.

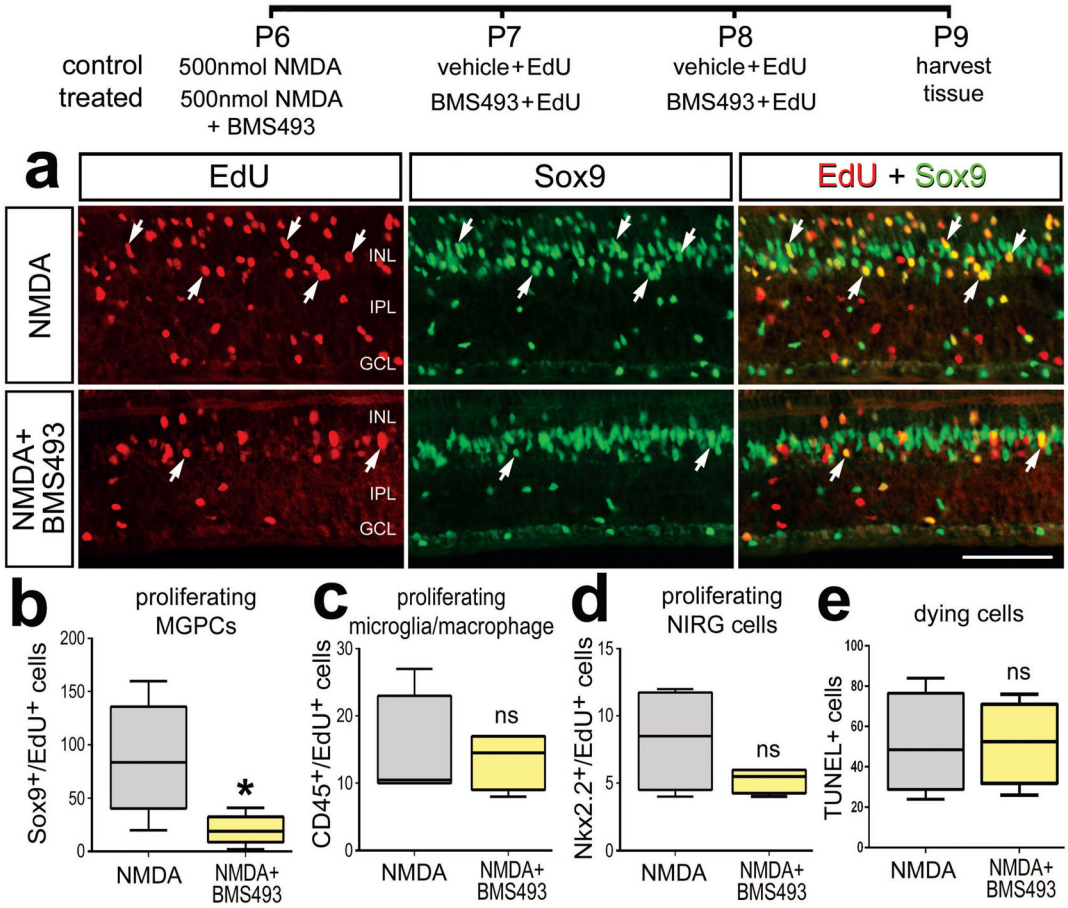

Figure 3.

Inhibition of RA-signaling suppresses the proliferation of MGPCs in damaged retinas. Eyes were injected with a relatively high dose (500 nmol) of NMDA alone (control) or NMDA +RAR antagonist (BMS493) at P7, vehicle+EdU or RAR antagonist + EdU at P8 and P9, and tissues harvested at P10. Sections of the retina were labeled for EdU-incorporation and antibodies to Sox9 (green; a). The box plots illustrate the mean, upper extreme, lower extreme, upper quartile and lower quartile (n=7 animals). Significance of difference (*p<0.05) was determined by using a *t*-test. Arrows indicate the nuclei of MGPCs. The calibration bar (50 μm) in panel **a** applies to **a** alone. Abbreviations: INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer, ns – not significant.

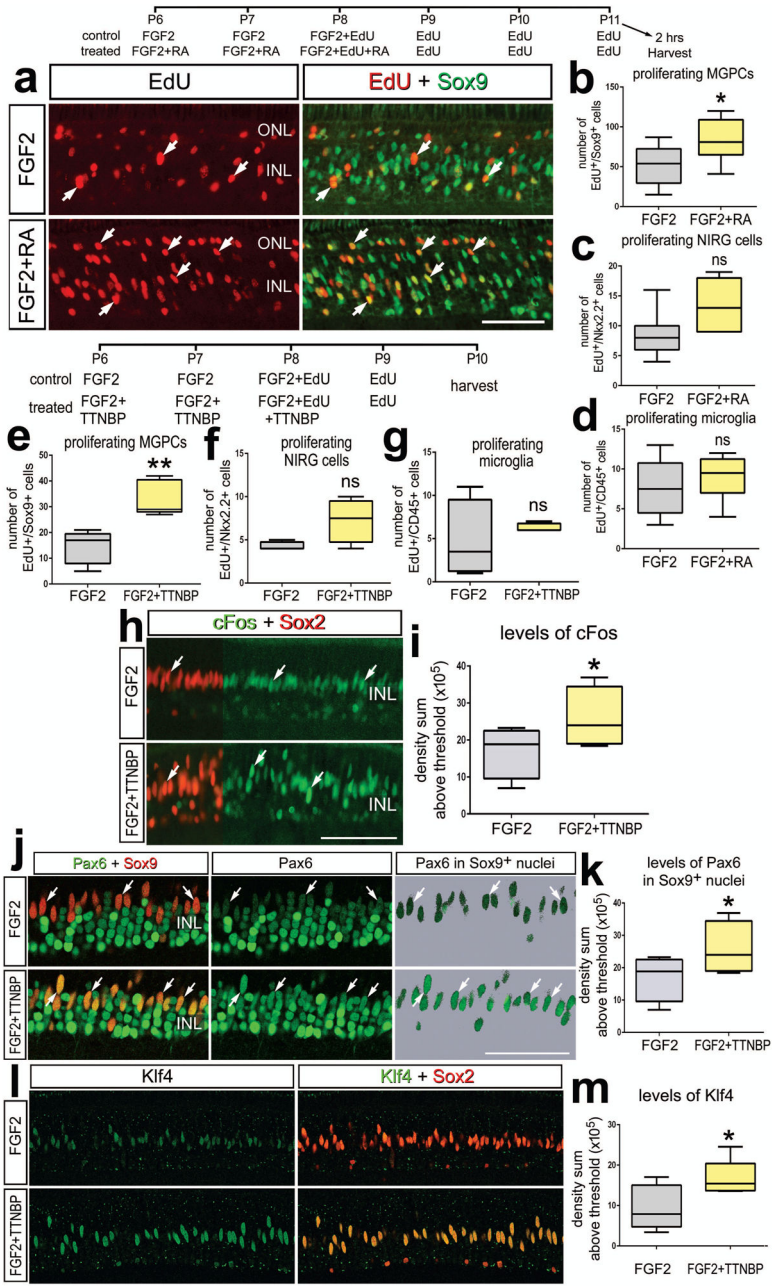

Figure 4.

In the absence of retinal damage, activation of RA-signaling stimulates the formation of proliferating MGPCs in FGF2-treated retinas. (**a**–**d**) Eyes were injected with FGF2 alone (control) or FGF2+RA (treated) at P6 and P7, FGF2+EdU or FGF2+EdU+RA at P8, EdU alone at P9, P10 and P11, and tissues harvested 2 hrs after the last injection. (**e**–**m**) Eye were injections with FGF2 alone (control) or FGF2+TTNBP at P6 and P7, FGF2+EdU or FGF2+EdU+TTNBP at P8, EdU alone at P9 and tissues harvested at P10. Sections of the retina were labeled for EdU-incorporation and antibodies to Sox9 (green; **a**), cFos (green) and Sox2 (red; **h**), Pax6 (green) and Sox9 (red; **j**), or Klf4 (green) and Sox2 (red; **l**). Arrows

indicate the nuclei of MGPCs. (**b**–**g**,**I**,**k**,**m**) The box plots illustrate the mean, upper extreme, lower extreme, upper quartile and lower quartile (n≥6 animals). Significance of difference (*p<0.05) was determined by using a *t*-test. The calibration bar in panels **a**,**h**,**j** and **l** represents 50 μm. Abbreviations: INL – inner nuclear layer, IPL – inner plexiform layer, ONL – outer nuclear layer, ns – not significant.

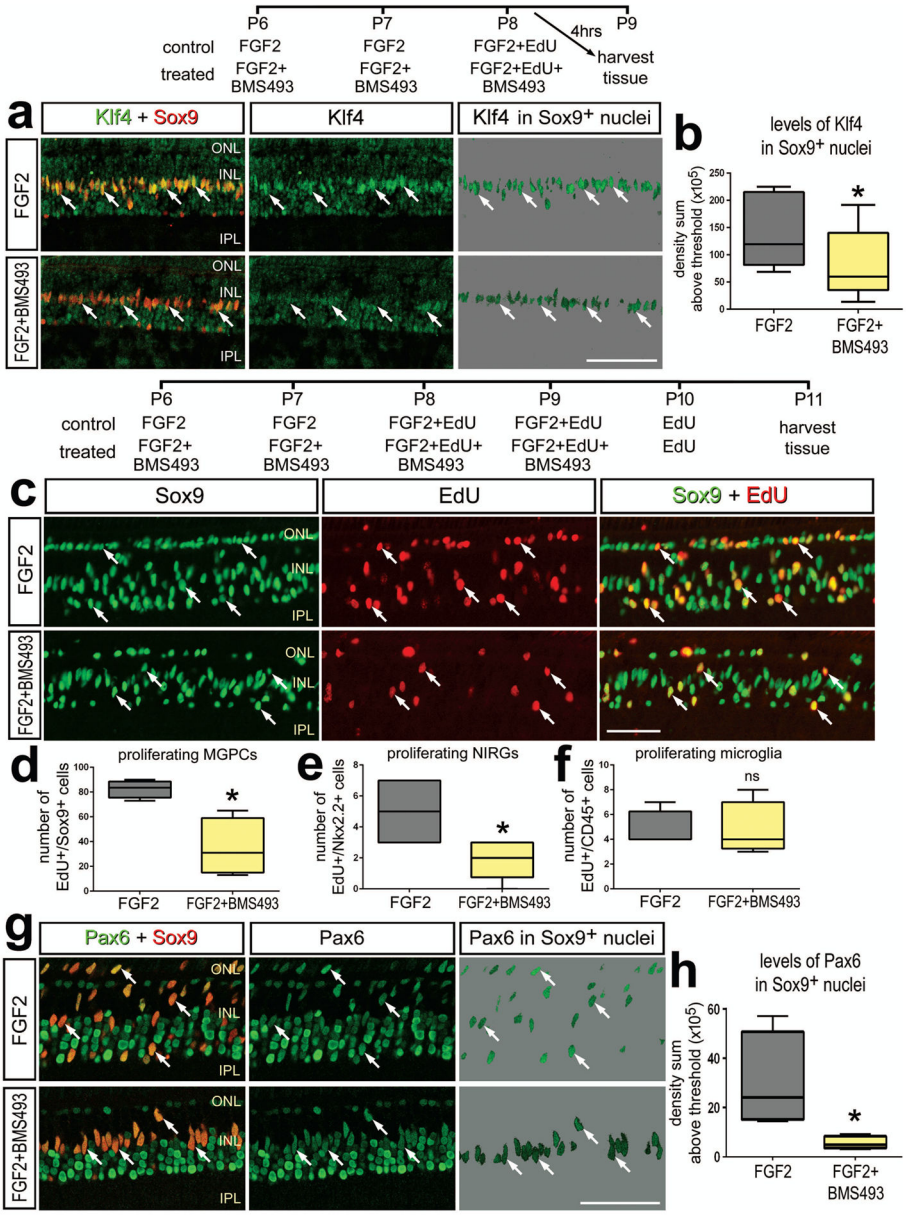

Figure 5.

In the absence of retinal damage, inhibition of RA-signaling with BMS493 suppresses the proliferation of MGPCs in FGF2-treated retinas. **a**,**b**: Eyes were injected with FGF2 alone (control) or FGF2+BMS493 (treated) at P6, P7 and P8, and tissues harvested at 4 hrs after the last injection. **c**–**h**: Eye were injected with FGF2 alone (control) or FGF2+BMS493 (treated) at P6 and P7, FGF2+EdU or FGF2+BMS439+EdU at P8 and P9, EdU alone at P10, and tissues harvested at P11. Sections of the retina were labeled for Sox9 (red) and Klf4 (green;**a**); EdU-incorporation (red) and Sox9 (green; **c**), or Pax6 (green) and Sox9 (red; **g**). Arrows indicate the nuclei of MGPCs (**b**, **d**–**f**, **h**). The box plots illustrate the mean, upper extreme, lower extreme, upper quartile and lower quartile (n=7 animals). Significance of difference (*p<0.05) was determined by using a t-test. The calibration bar in panels **a, c** and

g represent 50 μm. Abbreviations: INL – inner nuclear layer, IPL – inner plexiform layer, ONL – outer nuclear layer.

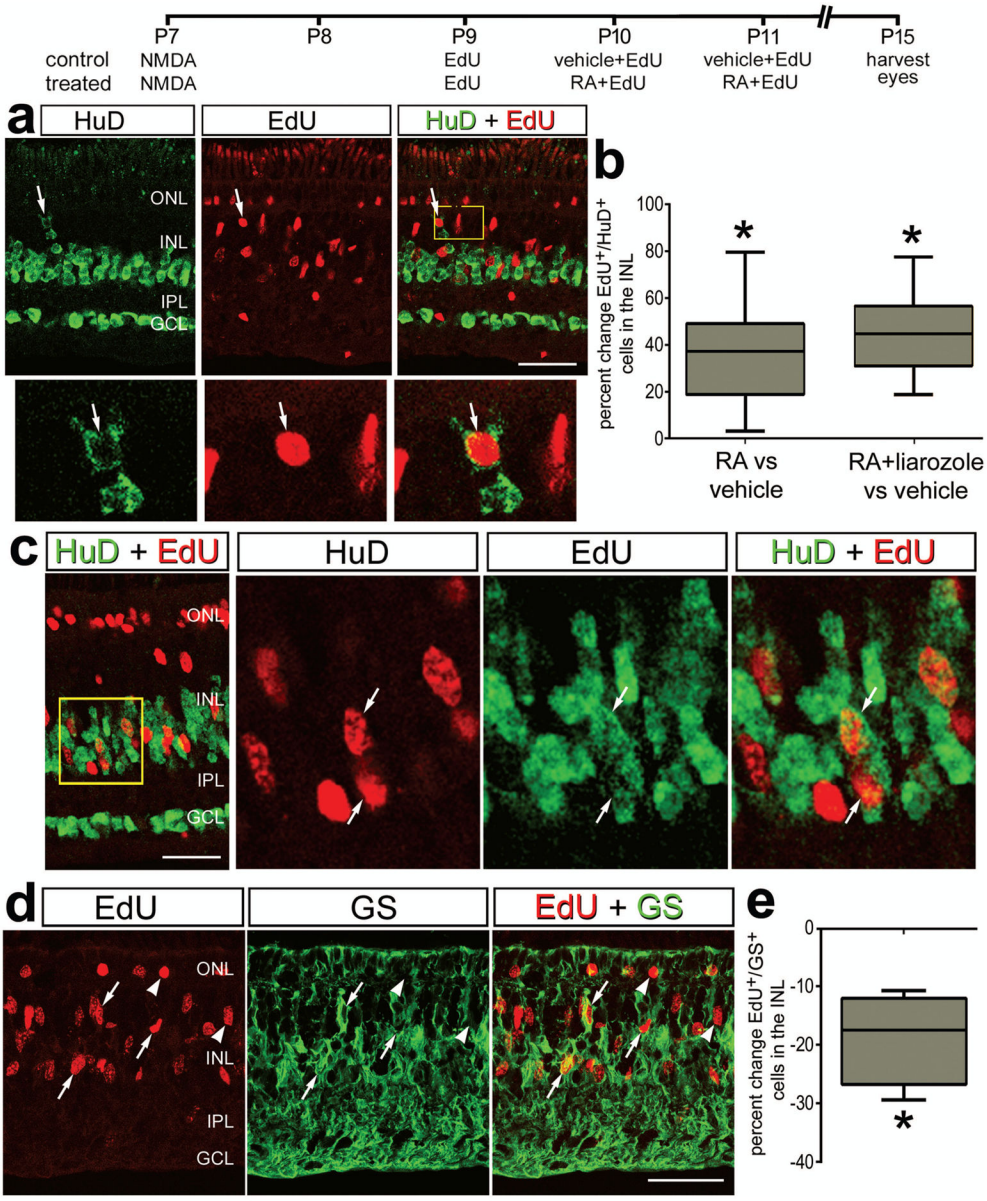

Figure 6.

Activation of RA-signaling in damaged retinas stimulated the neuronal differentiation and suppressed the glial differentiation of MGPC-progeny. Eyes were injected with 500 nmol NMDA at P7, EdU at P9, vehicle+EdU (control) or RA+EdU or RA+liarozole+EdU (treated) at P10 and P11, and tissues harvested at P15.

Sections of the retina were labeled for EdU-incorporation (red) and antibodies to HuC/D (green) or GS (green). Arrows indicate the nuclei of EdU-labeled neurons or glia.

The box plots illustrate the mean, upper extreme, lower extreme, upper quartile and lower quartile (n=6 animals). Significance of difference (*p<0.05) was determined by using a Mann-Whitney U test. Arrows indicate the nuclei of MGPCs. The calibration bar in panels **b, c,d** represents 50 μm. Abbreviations: INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer, ONL – outer nuclear layer.

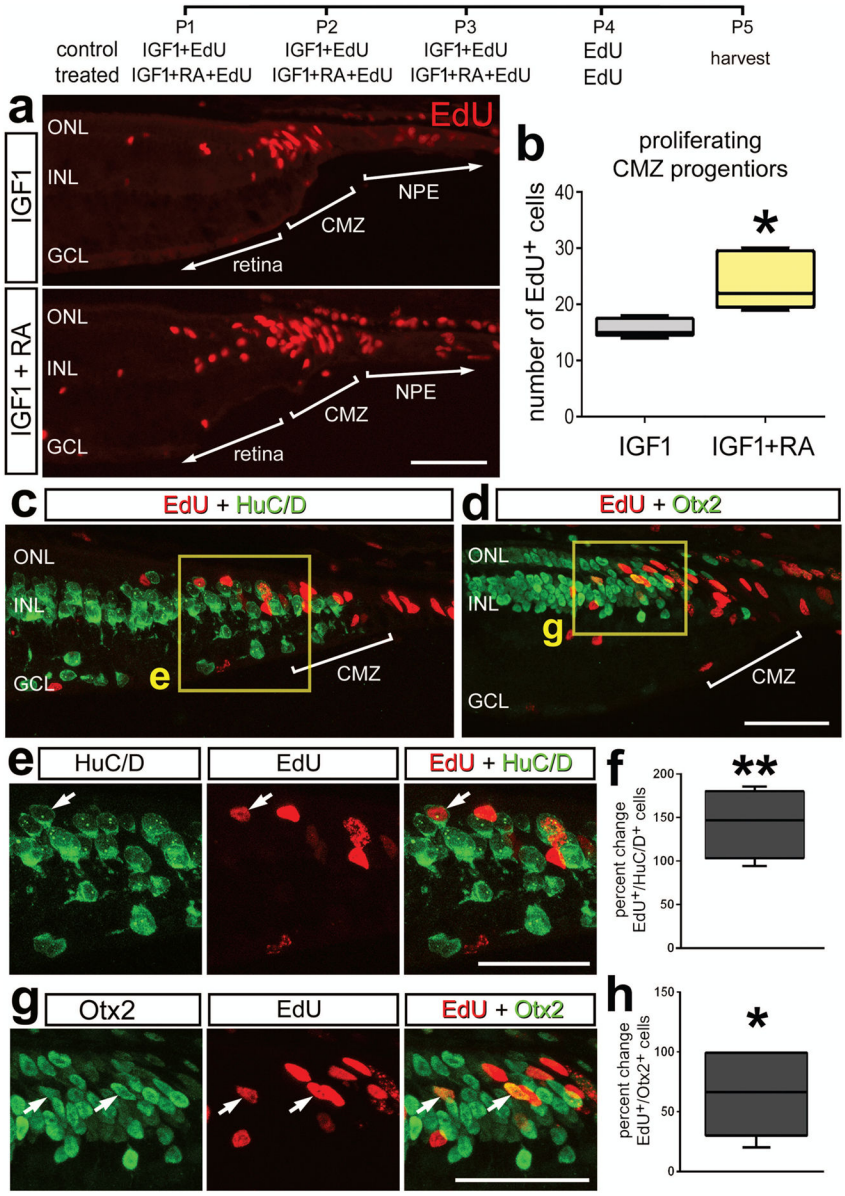

Figure 7.

Activation of RA-signaling stimulates the neuronal differentiation of progeny of CMZ progenitor cells. Eyes were injected with IGF1+EdU (control) or IGF1+EdU+RA (treated) at P1, P2 and P3, EdU alone at P4, and tissues harvested at P5. Sections of the peripheral retina and CMZ were labeled for EdU-incorporation (red; **a**,**c**,**d**,**e**,**g**) and antibodies to HuC/D (green; **c** and **e**) or Otx2 (green; **d** and **g**). Arrows indicate the nuclei of EdU-labeled neurons. The areas boxed-out in yellow in panels c and d are enlarged 1.5-fold and split into red and green channels in panels e and g. The box plots illustrate the mean, upper extreme, lower extreme, upper quartile and lower quartile (n≥4 animals). Significance of difference (*p<0.05) was determined by using a *t*-test (b) or (*p<0.05, **p<0.01) by using a Mann Whitney U test. Arrows indicate the nuclei of newly generated cells. The calibration bar (50 μm) in panel **a** applies to **a** alone, and the bar in **d** applies to **d**, **e** and **g**. Abbreviations: INL

– inner nuclear layer, GCL – ganglion cell layer, ONL – outer nuclear layer, CMZ – circumferential marginal zone, NPE – non-pigmented epithelium.

Table 1

Antibodies, sources and working dilutions. Patterns of labeling and stimulus-dependent changes in levels of immunolabeling using these antibodies are consistent with previous reports (Fischer and Omar, 2005; Fischer et al., 2009a, 2009b; Fischer et al., 2014; Todd and Fischer, 2015).

Antigen	Working dilution	Host	Clone or catalog number	Source
Sox2	1:1000	goat	Y-17	Santa Cruz Immunochemicals
Sox9	1:2000	mouse	AB5535	Chemicon
Pax6	1:1000	rabbit	PRB-278P	Covance
Klf4	1:50	rabbit	ARP38430	Aviva Systems Biology
cFos	1:400	rabbit	K-25	Santa Cruz Immunochemicals
Glutamine Synthetase	1:2000	mouse	ab125724	Abcam
CD45	1:200	Mouse	HIS-C7	Cedi Diagnostic
Nkx2.2	1:80	Mouse	74.5A5	DSHB
CRABP	1:1000	Mouse	C1	Dr. J Saari, University of Washington
Lim 1/2	1:50	Mouse	4F2	DSHB
Lim 3	1:100	Mouse	67.4E12	DSHB
Otx2	1:1000	Goat	AF1979	R&D Systems
HuD/HuC	1:600	Mouse	A21271	Invitrogen