The Signaling Pathways that Regulate the Proliferative and Neurogenic Capacity of Müller glia

DISSERTATION

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Abstract

Eye diseases that result in blindness are often caused by the death of retinal neurons. Müller glia are the major glial support cells in the retina and possess the potential to reprogram into neurogenic progenitor cells. In the fish, Müller glia are able to regenerate a fully functional retina following severe retinal damage. In avian and mammalian retinas, Müller glia retain their regenerative potential but to an insufficient extent to restore lost vision. A better understanding of the mechanisms that govern the reprogramming of Müller glia into neurogenic Müller glia-derived progenitor cells (MGPCs) may allow us to harness these cells in a therapeutically useful context. This dissertation examines the cell signaling mechanisms that regulate Müller glia homeostasis, de-differentiation, proliferation, and neurogenesis.

The first chapter focuses on how Hedgehog signaling stimulates Müller glia to reprogram into proliferating progenitors in the chick retina. We show that the Hedgehog-pathway components are up-regulated in damaged retinas when MGPCs are known to form. Furthermore, we find that the Shh-ligand is potentially released by retinal ganglion cells and received by proliferating Müller glia. We report that activation of the Hedgehog pathway increases Müller glia proliferation in damaged and FGF2-stimulated retinas. Consistent with these findings, inhibition of Hedgehog-signaling at the level of the ligand, receptor, and transcription factors attenuate MGPC formation. Activation of

Hedgehog signaling in the absence of damage or FGF2-application has no effect on Müller glia. We propose a model wherein retinal damage or FGF2-stimulation renders Müller glia responsive to the mitogenic effects of Hedgehog-signaling.

The second chapter examines how Jak/Stat signaling impacts the regenerative capacity of the avian retina. We find that Jak/Stat signaling is rapidly activated in Müller glia in response to retinal damage. We also show that inhibition of the Jak/Stat pathway at the level of the gp130-receptor, Jak2 kinase, and Stat3 transcription factor each diminish the ability of Müller glia to reprogram in to proliferating progenitors. Importantly, we find that inhibition of Jak/Stat signaling increases the neurogenic capacity of MGPCs after damage. This chapter implicates Jak/Stat signaling as a key component of MGPC-mediated retinal regeneration and an attractive target for boosting the neurogenic potential of Müller glia.

The third chapter analyzes how Retinoic acid (RA)-signaling regulates the proliferative and neurogenic potential of avian Müller glia. We find that the RA-component CRABP is specifically up-regulated by Müller glia in response to retinal injury. We also show that RA-activation increases MGPC formation in damaged and FGF2-treated retinas. This chapter also provides novel data that RA-activation can increase neural differentiation from MGPC progeny. Taken together, these data implicate RA-signaling has an important pathway in the regulation of Müller glia-mediated retinal regeneration.

Collectively, the work described in this dissertation represents significant advances in understanding the mechanisms underlying retinal regeneration in the avian

retina. Each chapter describes novel data regarding the signaling pathways that regulate the de-differentiation, proliferation, and neural differentiation of MGPCs. This work provides rationale for novel lines of inquiry into stimulating Müller glia-mediated regeneration in the mammalian retina.

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Fields of Study

Major Field: Neuroscience

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List of Abbreviations

bHLH	basic helix loop helix
BMP	Bone morphogenetic protein
CMZ	Circumferential marginal zone
CNTF	Ciliary neurotrophic factor
CRABP	Cellular retinoic acid binding protein
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
EFTF	Eye field transcription factors
FGF	Fibroblast growth factor
GCL	Ganglion cell layer
GFP	Green fluorescent protein
GLAST	Glutamate-aspartate transporter
HB-EGF	Heparin-binding EGF-like growth factor
IGF	Insulin-like growth factor
IL-6	Interleukin-6
INL	Inner nuclear layer
IPL	Inner Plexiform layer
MAPK	Mitogen-activated protein kinase
MGPC	Müller glia-derived progenitor cell

mTOR	Mechanistic target of rapamycin
NMDA	N-methyl-D-aspartate
NIRG	Non-astrocytic inner retinal glia
OLM	Outer limiting membrane
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PCR	Polymerase Chain Reaction
RA	Retinoic Acid
RPE	Retinal pigmented epithelium
Shh	Sonic Hedgehog
TGFB	Transforming growth factor beta
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

CHAPTER 1

General Introduction

The retina is a narrow neural tissue lining the back of the eye. This delicate structure is responsible for transmitting light from the environment into electrochemical signals to the brain for visual perception. Vision is generally regarded as the most precious of the human senses and loss of vision causes a severe impact on an individual's quality of life. Diseases leading to visual loss result in an economic burden of over 50 billion dollars per year in the United States. The work presented in this dissertation is focused on harnessing the regenerative potential of Müller glia. Müller glia are the primary support cell of the retina and are able to reprogram into neurogenic progenitor cells. Understanding the mechanisms that underlie Müller glia reprogramming will help guide future therapeutic interventions for sight-threatening diseases.

Overview of the Retina

Vertebrate retinas consist of seven cell types organized into three distinct layers of cell bodies and two layers of synapses. The cell bodies of the photoreceptor cells, the rods and cones, reside in the outer nuclear layer (ONL). Photoreceptor cells are the predominant responders to light in the retina and they synapse with horizontal cells and bipolar cells in the outer plexiform layer (OPL). The inner nuclear layer (INL) is the next

layer of cell bodies and it consists of horizontal cells, bipolar cells, and amacrine cells. Bipolar cells transmit signals from photoreceptors to the ganglion cells in the inner plexiform layer (IPL). Ganglion cells are the projection neurons of the retina and their nuclei reside in the ganglion cell layer (GCL). The axons of the ganglion cells converge at the optic nerve head to transmit the computational output of the retina to the brain for visual interpretation. Four glial types can be found in vertebrate retinas, although some discrepancies exist between species. Müller glia are the predominant glial cell of the retina. Their nuclei reside in the INL and their processes span all retinal layers. Astrocytes are typically associated with retinal vasculature and can be found in the GCL and IPL. Interestingly, some species contain avascular retinas and have a distinct glial cell type, the Non-astrocytic inner retinal glia (NIRG) (Fischer et al. 2010). The presence of oligodendrocytes in the retina varies between species, avascular retinas often contain oligodendrocytes that myelinate ganglion cell axons (Kohsaka et al. 1980; Narang 1977; Wyse and Spira 1981). Microglia, the innate immune cells of the nervous system are present in all vertebrate retinas.

Retinal Development – a general summary

The retina is bonafide brain tissue that derives from an outgrowth of the diencephalon during development (Wilson and Houart 2004). Eye development begins when a region of the anterior neural plate is specified as the "eye field". The eye field is characterized by a highly conserved combination of transcription factors termed the eye field transcription factors (EFTFs). The primary transcription factors that make up the

EFTFs are Rx, Pax6, Six3, Six6, Tbx3, Tll and Lhx2 (Fuhrmann et al. 2014; Zuber et al. 2003). Following the induction of the EFTFs, the lateral walls of the anterior neural ectoderm evaginate to form optic vesicles. Multipotent retinal progenitor cells (RPCs) reside in the optic vesicles that give rise to all neuronal cell types found in the mature retina (Turner and Cepko 1987; Wetts and Fraser 1988). The birth order of retinal neurons is highly conserved across species (Cepko 2014). The ganglion cells are the first cell type generated by RPCs followed by horizontal cells and cone photoreceptors. The latter born cell types consist of amacrine cells, rod photoreceptors, bipolar cells, and lastly Müller glia (Livesey and Cepko 2001). Temporally regulated mechanisms involving intrinsic changes in retinal progenitors combined with extrinsic changes in the signaling environment are responsible for the strict birth order of retinal genesis (Cepko 2014). Retinal astrocytes are not derived from retinal progenitor cells during development; instead they migrate into the retina through the optic nerve (Watanabe and Raff 1988). Oligodendrocytes also migrate into the retina and are derived from a common progenitor shared with retinal astrocytes (Rompani and Cepko 2010). Microglia are derived from myeloid precursors in the yolk sac and invade the retina during development via the optic nerve and vitreous (Ginhoux et al. 2010; Santos et al. 2008).

Müller glia – functions in the healthy retina

Müller glia are the predominant glial cell type of the vertebrate retina and their processes span the entire width of the retina and contact all neuronal cell types. Müller glia are functionally similar to the astrocytes in the brain and perform a wide variety of

support functions. Müller glia facilitate neurotransmission of retinal neurons by harboring transporter and exchange machinery for a variety of neurotransmitters (Bringmann et al. 2009). Glutamate is the principle neurotransmitter in the retina and Müller glia express the glutamate-aspartate transporter (GLAST) (Derouiche and Rauen 1995). Disruption of GLAST function in Müller glia leads to abnormal electroretinograms in mice, suggesting that Müller glia-mediated transport of glutamate is essential for normal retinal function (Barnett and Pow 2000). Müller glia exclusively express glumatine synthetase (GS) in the retina, which allows glutamate transported from the extracellular space to be converted into glutamine, which can then be provided to neurons for glutamate and GABA synthesis (Pow and Crook 1996). Pharmacological inhibition of GS in Müller glia leads to a severe loss of glutamate content in bipolar and ganglion cells and quickly results in functional blindness (Barnett and Pow 2000). In addition to facilitating neurotransmission, Müller glia release "gliotransmitters" that influence synaptic activity. The primary gliotransmitters can have both excitatory (glutamate, ATP) and inhibitory (adenosine) effects on neurons (Newman 2004). Müller glia are also able to release D-serine, GABA, and nitric oxide, all of which impact neural signaling (Bringmann et al. 2009). In addition to influencing synaptic transmission, Müller glia perform key homeostatic functions by regulating ion and water homeostasis (Bringmann et al. 2006). Neural activity results in increases in extracellular potassium, which can evoke hyperexcitation and glutamate toxicity. To prevent this from happening, Müller glia perform a function termed "potassium siphoning", which is achieved by their expression of the inward rectifier potassium channel Kir4.1 that can counteract fast increases in

extracellular potassium (Newman et al. 1984). Changes in ionic gradients associated with neuronal transmission cause osmotic imbalances that need to be actively regulated at the synapse. Müller glia express the glial specific water channel aquaporin-4 (AQP4) that allows water control at retinal synapses (Nagelhus et al. 1999). Interestingly, Kir4.1 is often co-localized with AQP4 in distinct membrane domains that allow Müller glia to couple potassium buffering with water control (Nagelhus et al. 2004). Müller glia are also responsible for protecting the retina from the byproducts of photoreceptor activity. Photoreceptors have one the highest rates of oxidative metabolism in the nervous system (Wong-Riley 2010). This level of activity leads to high demands on glucose metabolism which generates excess levels of carbon dioxide in the retina (Newman 1994). Müller glia perform "carbon dioxide siphoning" by the expression of carbonic anhydrase II, which allows carbon dioxide to be hydrated into bicarbonate and protons (Oakley and Wen 1989). This regulation of carbon dioxide allows Müller glia to also regulate extracellular pH levels (Newman 1996). Because of the level of photoreceptor activity and the constant exposure to light, the retina has a high requirement for antioxidant protection. Müller glia provide antioxidant support by their production of glutathione, from the neuronal glutamate they uptake (Pow and Crook 1995). It is also thought that Müller glia release an array of trophic factors that help protect retinal neurons (Bringmann et al. 2009).

Rudolf Virchow originally described glial cells as "Nervenkitt" or nerve-putty, predicting that these cells simply provide a passive structural framework for the nervous system (Parpura and Verkhratsky 2012). As described in the previous paragraphs, Müller

glia perform an astonishing amount of indispensable functions. Additionaly, they appear to provide structural support to the retina. After ablation of Müller glia, the retina becomes susceptible to tensile forces and tears apart (MacDonald et al. 2015). This suggests that Müller glia act as a structural substrate in the retina that provides resistance to deformation from mechanical stress. This may be achieved by inner filaments such as Vimentin and GFAP which are highly expressed by Müller glia (Kinouchi et al. 2003).

Müller glia – response to retinal injury and disease

Glia across the nervous system perform critical roles in response to injury and disease (Sofroniew 2005). The activation of glia in response to injury is typically referred to as "gliosis" and includes a cellular response to tissue damage that can be both protective and damaging (Sofroniew 2014). Müller glia become "gliotic" in response to a wide variety of retinal injuries including but not limited to, retinal ischemia, retinal detachment, photic damage, and age related macular degeneration (Bringmann and Reichenbach 2001). Müller glia can beneficially respond to pathology by release of neurotrophic factors, uptake of excess glutamate and potassium and the release of antioxidants (Bringmann et al. 2009). However, Müller glia activation can also be detrimental to retinal tissue by producing proinflammatory cytokines, forming glial scar tissue, or by proliferating to form an epiretinal membrane characteristic of proliferative vitreoretinopathy (Bringmann et al. 2009; Sethi et al. 2005). Müller glia are also known to dedifferentiate in response to injury, which may impair their typical functions such as glycolysis, carbon dioxide siphoning, potassium syphoning, water clearance and

neurotransmitter recycling (Bringmann et al. 2009). Clearly, the Müller glial response to retinal injury is a "double edge sword". By understanding the mechanistic underpinnings of the glial response to damage, we may be able to therapeutically promote a protective phenotype to help limit retinal damage. One of the most remarkable features of Müller glia is the primary topic of this dissertation. Müller glia possess a capacity to respond to injury by reprogramming into neurogenic progenitor cells that can replace lost neurons.

Müller glia-mediated retinal regeneration: a survey across species.

Fully functional regeneration in the teleost fish retina

Retinal regeneration in the teleost fish was reported nearly 50 years ago (Lenkowski and Raymond 2014). Regeneration of retinal neurons and photoreceptors was first observed in a model of surgical transection. Regeneration after surgical transection has been observed in goldfish, zebrafish, trout, and white flounder (Cameron 2000; Faillace et al. 2002; Hitchcock 1997; Mader and Cameron 2004). Subsequently, more selective damage models have been developed. Retinal regeneration can occur in the fish in response to selective death of photoreceptors, dopaminergic amacrine cells, and retinal stab wounds (Lenkowski& Raymond, 2014). Astonishingly, functional regeneration can occur in response to oubain mediated destruction of the entire retina (Sherpa et al. 2008). For decades, the cellular origin of retinal regeneration in the fish was unknown. Two studies converged to provide evidence that Müller glia are the source of regenerated neurons (Bernardos et al. 2007; Fausett and Goldman 2006). The first

employed a transgenic line that specifically induced GFP in Müller glia from the a-1 tubulin (tuba1a) promoter element (Fausett and Goldman 2006). This study found that in response to a retinal stab lesion, GFP co-localized with Müller glia, GFP+ Müller glia re-entered the cell cycle and expressed retinal progenitor markers. Seven days after the lesion injury GFP+ cells co-localized with HuC/D, a marker of ganglion and amacrine cells. The second study, used a transgenic zebrafish wherein the glial-specific promoter *gfap* drives expression of GFP (Bernardos et al. 2007). In these experiments, acute light lesions led to GFP+ Müller glia re-entering the cell cycle, expressing progenitor markers, and producing GFP+ cone photoreceptors (Bernardos et al. 2007). Research investigating the mechanisms of MGPC formation in the fish has significantly expanded in the decade since these studies and will be summarized in the section below.

An intermediate capacity for retinal regeneration in the bird retina

Retinal regeneration has been investigated in the embryonic chick for over 50 years (Coulombre and Coulombre 1965). Retinal pigmented epithelial cells of the embryonic chick can transdifferentiate to produce all layers of the neural retina (Coulombre and Coulombre 1965). The regenerative capacity of the avian retina is limited to early stages of embryonic development and does not occur past embryonic day 5 (Park and Hollenberg 1989). While studying how different retinal toxins affected ocular growth in the posthatch chick, the authors noticed an accumulation of proliferating cells in the INL (Fischer 2011). These cells were later discovered to be Müller glia, which in response to NMDA-induced excitotoxic damage, de-differentiate, re-enter the cell cycle,

and express transcription factors associated with retinal progenitors (Fischer and Reh 2001). Using BrdU to track the proliferating Müller glia and their progeny, the authors found that about 80% of cells that accumulated BrdU remain undifferentiated, although a small percentage of cells differentiated as BrdU+ amacrine and bipolar cells (Fischer and Reh 2001). Follow up studies using colchicine to selectively kill ganglion cells suggested that Müller glia can regenerate ganglion-like cells (Fischer and Reh 2002). Over the past decade many advances have been made in understanding the cell-signaling events underlying MGPC formation in the chick retina and this will be further described in the subsequent section.

Mammalian retinal regeneration

In contrast to the fish and chick, the mammalian retina primarily reacts to injury by undergoing reactive gliosis (Bringmann et al. 2009). When proliferation does occur in response to injury it is typically associated with upregulation of gliosis-associated genes and retinal dysplasia (Bringmann et al. 2009; Dyer and Cepko 2000). Guided by studies in the chick retina, investigations have observed that mammalian Müller glia have the capacity to respond to injury by undergoing MGPC formation, particularly when damage is coupled with growth factor stimulation (Karl et al. 2008; Ooto et al. 2004). Interestingly, this effect varies according to the strain of mice (Suga et al. 2014). However, in all contexts observed the extent of MGPC formation in the mammalian retina is significantly lower than what occurs in the chick and fish. Recently, strategies to transgenically overexpress the proneural transcription factor *ascl1* in Müller glia have

significantly increased the capacity of mammalian MGPC formation (Jorstad et al. 2017).

Müller glia in the human retina

Müller glia extracted from human retinas and cultivated *in vitro* can undergo spontaneous immortalization (Lawrence et al. 2007). These immortalized cell lines have been reported to possess the capacity to differentiate into neuron-like cells and some investigators have attempted to transplant these Müller glia-derived neurons into diseased retinas (Jayaram et al. 2014; Singhal et al. 2012). It remains to be determined whether the benefit derived from these transplanted cells results from functional integration or trophic support to host neurons. However, these data suggests that human Müller glia may possess the plasticity that will allow them to be attractive targets for retinal regeneration in the future.

Cell-signaling pathways underlying Müller glia reprogramming

Despite the vast differences in the regenerative capacity across species, Müller glia are the cellular source of regeneration in fish, chick, and mammalian retina. Therefore, understanding the mechanisms underlying Müller glia reprogramming in lower vertebrates will help guide effective strategies for stimulating regeneration in mammalian retinas. Müller glia are the last cell type derived from retinal progenitor cells during development and maintain a significant transcriptome overlap with retinal progenitors (Blackshaw et al. 2004; Roesch et al. 2008; Ueno et al. 2017). Analysis in the zebrafish retina revealed that progenitor genes known to underlie MGPC formation are

hypomethylated even in the healthy retina (Powell et al. 2013). Surprisingly, Müller glia in healthy mouse retina also contained low methylation levels of genes associated with pluripotency (Powell et al. 2013). These reports suggest that the unique genomic architecture of Müller glia primes them to reprogram into retinal progenitors. This section will summarize the data regarding the cell signaling pathways known to influence Müller glia reprogramming.

Mitogen-Activated Protein Kinase-Signaling

The importance of the MAPK-signaling pathway in MGPC formation is apparent in fish, chick, and rodent studies. In the zebrafish retina, the MAPK effector pERK is up-regulated in Müller glia as rapidly as one hour after stab injury (Wan et al. 2014). The growth factor HB-EGF is necessary and sufficient for retinal regeneration in the fish and HB-EGF exerts its effects through the MAPK pathway (Wan et al. 2012). In addition to HB-EGF, FGF2+IGF1 can stimulate MGPC formation in the uninjured zebrafish retina and this effect depends on pERK activity (Wan et al. 2014). Similar to the fish, retinal damage in the chick transiently activates the MAPK effectors, pERK and p38 MAPK as well as downstream MAPK target genes Egr1, cFos and pCREB (Fischer et al. 2009b). Inhibition of MAPK-signaling in damaged chick retinas with small molecule inhibitors to MEK or the FGF-receptor suppresses MGPC formation (Fischer et al. 2009b). FGF2 is the only factor known to be able to stimulate the formation of proliferating MGPCs in the absence of damage in the chick retina (Fischer et al. 2014). FGF2 exerts its effects primarily through MAPK-signaling in Müller glia (Fischer et al. 2009a). In the rodent

retina, NMDA damage results in a Müller-glia specific accumulation of pERK and cFOS (Nakazawa et al. 2008). Application of the exogenous growth factors FGF, EGF, and HB-EGF have been shown to be mitogenic to Müller glia following retinal injury, ostensibly through activation of MAPK-signaling (Karl et al. 2008; Todd et al. 2015).Taken together, these data suggests that MAPK-activation is a conserved response to retinal damage across vertebrate species. Furthermore, stimulation of MAPK-signaling is a proven target to boosting MGPC formation in the fish, chick, and mouse retina.

Wnt/β-catenin-Signaling

Similar to the MAPK-pathway, activation of Wnt-signaling is a common requirement for MGPC formation across species. In damaged fish retina, Wnt-signaling has been shown to enhance the formation of MGPCs (Meyers et al. 2012; Ramachandran et al. 2011). Furthermore, activation of Wnt-signaling is sufficient to stimulate MGPCs in the uninjured zebrafish (Ramachandran et al. 2011). In the chick, activation of Wnt/B-catenin is not sufficient to stimulate MGPC formation in the absence of damage (Gallina et al. 2015). However, after NMDA damage when MGPCs are known to form in the chick retina, Müller glia up-regulate the Wnt pathway component β-catenin (Gallina et al. 2015). Furthermore, activation of Wnt-signaling with GSK3β-catenin -inhibitors increases MGPC formation, whereas inhibition of Wnt-signaling suppresses MGPC formation in the injured chick retina (Gallina et al. 2015). Wnt-signaling has also been implicated in boosting MGPC formation in the mammalian retina. In rodent retinal explants, the exogenous Wnt-ligand Wnt3a increased the proliferation of Müller glia

(Osakada et al. 2007). In axin2 null mice, where a gain of Wnt-signaling occurs via the loss of a negative regulator, Müller glia responded to light injury by proliferating at elevated rates (Liu et al. 2012). Recently, virus mediated gene transfer of β-catenin specifically to Müller glia has been shown to be sufficient to stimulate MGPCs in the uninjured mouse retina (Yao et al. 2016).

PI3K/Akt/mTOR-Signaling

Müller glia in the fish retina rapidly activate pAkt in response to injury and small molecule PI3K inhibitors suppress the ability of Müller glia to proliferate in response to damage or growth factor stimulation (Wan et al. 2014). In the avian retina, mTOR-signaling is rapidly activated in Müller glia in response to damage and mTOR-activation is required for MGPC formation in both damaged and FGF2-treated retinas (Zelinka et al. 2016). In mouse retinal explants, the PI3K/AKT pathway has been shown to be required for EGF-induced proliferation of Müller glia (Ueki and Reh 2013). Whether PI3K/AKT-signaling impacts MGPC formation in the *in vivo* mouse retina remains to be explored.

BMP/TGFB-SMAD signaling

The SMAD transcription factors play a critical role in neural stem cell function during development and in mature neurogenic niches (Aigner and Bogdahn 2008; Bond et al. 2012). How BMP-signaling impacts retinal regeneration in the teleost fish has yet to be examined. However, TGFB-SMAD-signaling has been demonstrated to negatively regulate MGPC formation in response to photoreceptor injury in the fish (Lenkowski et

al. 2013). In the avian retina, BMP/Smad1/5/8 and TGFB/Smad2/3-signaling pathways are coordinated to influence MGPC reprogramming (Todd et al. 2017). BMP signaling-promotes the formation of MGPCs, whereas TGFB-signaling suppresses MGPC proliferation in the chick retina (Todd, et al. 2017). In the mouse retina, addition of BMP4 promotes the proliferation of Müller glia in retinal explants through activation of Smad1/5/8 (Ueki and Reh 2013). Similar to the fish and chick retinas, TGFB-signaling has been found to suppress proliferation of MGPCs in the rat retina (Close et al. 2005). Considered together, these reports suggest that promotion of BMP-signaling while simultaneously inhibiting the TGFB-pathway is an attractive strategy for promoting retinal regeneration.

Notch-signaling

During early stages of retinal development, Notch-signaling keeps retinal progenitor cells undifferentiated and proliferating (Furukawa et al. 2000; Jadhav et al. 2006). In later stages of development, Notch activity promotes Müller glia differentiation (Furukawa et al. 2000; Nelson et al. 2011). Surprisingly, inhibition of Notch-signaling enhances Müller glia proliferation at the site of injury in the fish retina (Wan et al. 2012). Subsequent studies discovered that Notch inhibition combined with Tnfα application stimulated MGPC formation in the uninjured fish retina (Conner et al. 2014). The pro-proliferative effects of Notch-inhibition contrasted with previous data showing Notch activity stimulated proliferation of retinal progenitors during development (Bernardos et al. 2005). Consistent with the role of Notch in development, some reports have found that

Notch-activation stimulates Müller glia proliferation during photoreceptor regeneration in zebrafish (Taylor et al. 2015). In the bird retina, Notch-signaling components are upregulated in Müller glia when they are reprogramming in damaged retinas (Ghai et al. 2010). Inhibition of Notch-signaling with the gamma-secretase inhibitor DAPT suppresses MGPC formation in the chick (Ghai et al. 2010; Hayes et al. 2007). Although Notch signaling inhibits MGPC formation acutely after injury, at later time points Notch inhibition increases neural differentiation of MGPC-progeny (Hayes et al. 2007). How Notch signaling impacts MGPC formation in the mouse retina has yet to be investigated. However, when Müller glia are coaxed to proliferate in the mouse retina by NMDA+EGF, elevated expression of Notch signaling components were observed via RT-PCR (Karl et al. 2008). Remarkably, Ascl1 overexpression in Müller glia has recently been shown to lead to functional neural regeneration in the mouse (Jorstad et al. 2017). In previous studies, viral transduction of Ascl1 in cultured Müller glia lead to upregulation of neural progenitor genes that included Notch-pathway components (Pollak et al. 2013). Considering the conflicting results regarding Notch signaling and MGPCs, whether Notch is an obstacle or a promoter of Müller glia reprogramming may depend on the context of injury and species being investigated.

Hedgehog- signaling

Hedgehog (HH)-signaling is known to play crucial roles in retinal development, including stimulating progenitor proliferation (Wallace, et al. 2008). In the damaged zebrafish retina, the Sonic Hedgehog (Shh) ligand derived from surviving retinal neurons

has been suggested to facilitate regeneration (Sherpa, et al. 2014). However, how HH-signaling is manifested in fish Müller glia/MGPCs has yet to be elucidated. In the rodent retina, intraocular injections of exogenous Shh in the methyl N-nitrosourea (MNU)-damaged retina increased the capacity of Müller glia to de-differentiate and express progenitor markers (Wan, et al. 2007). A thorough study into how HH-signaling impacts the regenerative capacity of the mammalian retina remains to be investigated. Chapter 2 of this thesis will describe data regarding how HH-signaling impacts MGPC formation in the chick retina.

Jak/Stat-Signaling

The Jak/Stat-pathway is widely known to play important roles in glial development, stem cell maintenance, and neuroprotection (Bonni, 1997; Niwa, 1998; Rhee, et al. 2013). In response to retinal injury in the zebrafish, Stat3 is rapidly activated in Müller glia (Nelson, et al. 2012; Kassen et al. 2007). CNTF and Leptin can both stimulate Müller glia proliferation in the absence of injury in the fish retina and this effect is manifested through Jak/Stat-signaling (Nelson, et al. 2012; Zhao, et al. 2014). Importantly, Stat3-activation in zebrafish represents a crucial convergence point for a wide network of signaling pathways and is necessary for MGPC formation to occur in response to retinal injury or growth factor activation (Wan, et al. 2014). How Jak/Stat-signaling impacts MGPC formation in the mammalian retina remains to be investigated. It has been shown that Müller glia in the mouse retina upregulate pStat3 in response to the mitogenic combination of NMDA+HB-EGF (Todd, et al. 2015). This suggests that

Jak/Stat signaling activation may be associated with MGPC formation in the rodent retina. Chapter 3 of this thesis will examine how Jak/Stat signaling impacts the regenerative capacity of Müller glia in the avian retina.

Cross talk between signaling pathways

A significant body of data has accumulated demonstrating that the cell-signaling pathways that control MGPC formation form a complex and interacting network. The Notch, Wnt, Pi3k/Akt , Jak/Stat and MAPK-pathways display significant cross talk in orchestrating MGPC formation in the zebrafish (reviewed by: Wan and Goldman, 2017). Despite a significant amount of upstream crosstalk, this diverse array of signaling pathways converges on β-catenin and Stat3 in the fish retina (Wan, et al. 2014). Similar to the fish retina, an interacting network of pathways has been uncovered to drive MPGC formation in the chick retina (reviewed by: Gallina, et al. 2013). These pathways include Notch, Hedgehog, Wnt, Akt/mTor, Jak/Stat, Mapk, Bmp/SMAD and glucocorticoid-signaling (Ghai, et al. 2010; Todd & Fischer, 2015; Gallina, et al. 2014; Zelinka, et al. 2016; Todd, et al. 2016; Fischer, et al. 2009; Todd, et al. 2017). In the bird retina, MAPK-signaling may represent the most crucial hub in this network of pathways. FGF2 is the only known factor that can stimulate MGPCs in the uninjured chick retina and it recruits and depends on the signaling pathways Notch, Hedgehog, Wnt, Jak/Stat, mTOR and glucocorticoid-signaling to exert this effect (Ghai, et al. 2010; Todd & Fischer, 2015; Gallina, et al. 2014; Zelinka, et al. 2016; Todd, et al. 2016; Todd, et al. 2017). Relatively little is known about how much signaling cross talk occurs in the mammalian

retina during MGPC formation. Current data does suggest that application of the MAPK agonist HB-EGF in damaged mouse retina also activates Jak/Stat and mTor pathways specifically in Müller glia (Todd, et al. 2015). The three subsequent chapters in this dissertation will address attempts to further delineate this network of signaling pathways that controls Müller glia reprogramming in the chick retina. Additionally, Chapter 4 provides novel data that retinoic acid (RA) signaling promotes the regenerative capacity of Müller glia in the chick retina. The effects of RA-signaling on MGPC formation has yet to be investigated in other species.

Neural stem cells at the retinal margin

A population of retinal stem cells organized into a circumferential ring in the peripheral retina exists in lower vertebrates (reviewed by Fischer, et al. 2014a). This region of stem cells is termed the circumferential marginal zone (CMZ) and is responsible for the production of new cells that accompany ocular growth in the fish, amphibian, and possibly reptilian retinas (Hitchcock & Raymond, 1992; Reh and Levine, 1998, Todd, et al. 2016). A residual CMZ has also been described in the retina of birds (Fischer and Reh, 2000). The CMZ-progenitors in the chick express retinal stem cell markers and accumulate BrdU in the mature retina, suggesting the existence of a zone of cycling progenitors (Fischer and Reh, 2000; Kubota, et al. 2002). The mammalian retina was thought to lack a CMZ (Kubota, et al. 2002), however recent reports have described strategies to stimulate a population of CMZ progenitors in the mouse retina (Belanger, et al. 2017; Marcucci, et al. 2016). Since the CMZ may provide an additional source for

regenerated neurons, future investigations into the mechanisms that control CMZ-progenitors are important. The following chapters in this dissertation contain novel data describing the signaling pathways that influence the proliferative and neurogenic capacity of CMZ-progenitors in the chick retina.

Chapter 2

Hedgehog Signaling Stimulates the Formation of Proliferating Müller-glia Derived Progenitor Cells in the Chick Retina

Abstract

Müller glia can be stimulated to de-differentiate and become proliferating progenitor cells that regenerate neurons in the retina. The signaling pathways that regulate the formation of proliferating Müller glia-derived progenitor cells (MGPCs) are beginning to be revealed. The purpose of this study was to investigate whether Hedgehog (Hh) -signaling influences the formation of MGPCs in the chick retina. We find that Hh-signaling is increased in damaged retinas where MGPCs are known to form. Sonic Hedgehog (Shh) is normally present in the axons of ganglion cells, but becomes associated with Müller glia and MGPCs following retinal damage. Activation of Hh-signaling with recombinant human Shh or Smoothened agonist (SAG) increased levels of ptch, gli1, gli2, gli3, hes1 and hes5, and stimulated the formation of proliferating MGPCs in damaged retinas. In undamaged retinas, SAG or rhShh had no apparent effect upon the Müller glia. However, SAG combined with FGF2 potentiated the formation of MGPCs, whereas SAG combined with IGF1 stimulated the nuclear migration of Müller glia, but not the formation of MGPCs. Conversely, inhibition of Hh-signaling with KAAD-cyclopamine, Gli antagonists or antibody to Shh reduced numbers

of proliferating MGPCs in damaged and FGF2-treated retinas. Hh-signaling potentiates Pax6, Klf4 and cFos expression in Müller glia during the formation of MGPCs. We find that FGF2/MAPK-signaling recruits Hh-signaling into the signaling network that drives the formation of proliferating MGPCs. Our findings implicate Hh-signaling as a key component of the network of signaling pathways that promote the de-differentiation of Müller glia and proliferation of MGPCs.

Introduction

Müller glia are the predominant type of glial cell in the retina and normally provide structural, synaptic and metabolic support to neurons. However, Müller glia can also act as a source of proliferating progenitors to regenerate neurons. Müller glia are distinctly different from progenitor cells based on functions, yet share a significant overlap of transcriptomes with retinal progenitors (Blackshaw et al., 2004). Müller glia are capable of de-differentiating, acquiring a progenitor-phenotype and proliferating in response to damage or exogenous growth factors (reviewed by (Fischer and Bongini, 2010; Gallina et al., 2014a). Müller glia have been identified as the cellular source of retinal regeneration in birds (Fischer et al., 2002b; Fischer and Reh, 2001), zebrafish (Bernardos et al., 2007; Fausett and Goldman, 2006) and rodents (Karl et al., 2008; Ooto et al., 2004). Similar to the glia in mammalian retina, the Müller glia in the chick retina have a limited capacity to generate new neurons. By comparison, the Müller glia in the fish retina have an extraordinary capacity to regenerate retinal neurons (reviewed by (Gallina et al., 2014a).

Proliferation is an integral step in the formation of Müller glia-derived progenitor cells (MGPCs; (Fischer, 2005; Fischer and Reh, 2003b; Reh and Fischer, 2001). The identification of the signaling pathways that influence de-differentiation, proliferation and neurogenesis is key to harnessing the regenerative potential of MGPCs. The Hh-signaling pathway may be important to the formation of MGPCs. Hh-signaling is known to have many important pleiotropic actions during early eye and retinal development(Dakubo et al., 2003; Levine et al., 1997; Perron et al., 2003; Wang et al., 2002; Zhang and Yang, 2001b). In developing retina, Sonic Hedgehog (Shh) is expressed by the ganglion cells, is exported from the eye via ganglion cell axons, and stimulates the formation of glia in the optic nerve and chiasm (Gao and Miller, 2006; Wallace and Raff, 1999). In the rodent retina, patched (*ptch*; integral membrane receptor for Hh and antagonist of *smo*) and smoothened (*smo*; co-receptor and activator of intracellular signaling) are expressed by mature Müller glia (Moshiri and Reh, 2004; Nelson et al., 2011; Roesch et al., 2008; Roesch et al., 2012). Studies in the embryonic chick have demonstrated regeneration in peripheral regions of prospective retina, and this regeneration requires coordination of Hh and FGF/MAPK-signaling (Spence et al., 2007a; Spence et al., 2004). In the rodent, it has been proposed that MGPCs regenerate photoreceptors in response to Hh (Wan et al., 2007). However, this study was performed in rats with retinal damage elicited via systemic N-nitroso-methyl-urea on or before P7, with Shh applied before P21, when the proliferation of late-stage retinal progenitors progressively declines and Müller glia have not fully differentiated (Close et al., 2005; Nelson et al., 2011). Thus, it remains unknown whether Hh-signaling influences mature

Müller glia in the rodent retina. In the zebrafish, Shh produced by retinal neurons facilitates regeneration from MGPCs (Sherpa et al., 2014). Here we investigate Hh-signaling after retinal injury or in response to FGF2-treatment, and how Hh-signaling impacts the formation of MGPCs in the chick retina *in vivo*.

Materials and Methods

Animals:

The use of animals in these experiments was in accordance with the guidelines established by the National Institutes of Health and the Ohio State University. Newly hatched male and female wild type leghorn chickens (*Gallus gallusdomesticus*) were obtained from Meyer Hatchery (Polk, Ohio). Chicks were kept on a cycle of 12 hours light, 12 hours dark (lights on at 8:00 AM). Chicks were housed in a stainless steel brooder at about 25^{o}C and received water and Purina^{tm} chick starter *ad libitum*. All of chicks used in these studies were between 6 and 28 days of age.

Intraocular injections:

Chickens were anesthetized and intraocular injections were performed as described previously (Fischer et al., 2008; Fischer et al., 2009a). Injected compounds included NMDA (6.4 or 154 μg/dose; Sigma-Aldrich), FGF2 (250 ng/dose; Sigma-Aldrich), IGF1 (400 ng/dose; R&D Systems), rhShh (500 ng/dose; R&D Systems), KAAD-cyclopamine (1 μg/dose; Selleck Chemicals; CAS 306387-90-6), 5E1 anti-Shh (20μl/dose; Developmental Studies Hybridoma Bank; DSHB), Gli antagonist (GANT58)

(1 μg/dose; Selleck Chemicals), Gli antagonist (GANT 61) (1 μg/dose; Tocris Bioscience) smoothened agonist (SAG) (500 ng/dose; EMD Millipore; CAS 364590-63-6), DAPT ((*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester) (865 ng/dose; Sigma-Aldrich), UO126 (1.7 μg/dose; Calbiochem) and BrdU (2 μg/dose; Sigma-Aldrich). Hydrophobic compounds were diluted and injected in 30% DMSO in saline. Injection paradigms are included in the figures and legends.

PCR:

Tissue dissections, RNA isolation, reverse transcriptase reactions and PCR reactions were performed as described previously (Fischer et al., 2004a; Fischer et al., 2010; Ghai et al., 2010). PCR primers were designed by using the Primer-BLAST primer design tool at NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are included in Table 2.1. qPCR reactions were performed using SYBR^{tm} Green Master Mix and StepOnePlus Real-Time system (Applied BioSystems). Samples were run in triplicate on a minimum of 4 individuals. Ct values obtained from real-time PCR were normalized to GAPDH for each sample and the fold change between control and treated samples was determined using the 2-ΔΔCt method (=Fold Change $2^{(-\Delta\Delta Ct)}$) and represented as a percentage change from the control which was assigned a value of 100. Significance of difference for percent change was determined by using a non-parametric Mann-Whitney U test.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL):

To identify dying cells that contained fragmented DNA, the TUNEL method was used. We used an *In Situ* Cell Death Kit (TMR red; Roche Applied Science), as per the manufacturer's instructions.

In situ hybridization:

The plasmid carrying a cDNA fragment of *ptch1* was provided by Dr. Clifford Tabin. The riboprobe to *ptch1* covered nucleotides 900 to 3.4kb. Riboprobe to *shh* was generated from PCR product using primers that included RNA polymerase initiation sites, as described elsewhere (Stanke et al., 2010). The riboprobe to *shh* covered nucleotides 332 to 1415.Digoxigenin-labeled riboprobes were synthesized by using a kit provided by Roche, and stored at -80^{o}C until use. Eyes were dissected, fixed, sectioned and processed for *in situ* hybridization as described previously (Fischer et al., 2002a; Fischer et al., 2004a; Ghai et al., 2009; Ghai et al., 2010).

Fixation, sectioning and immunocytochemistry

Tissues were fixed, sectioned and immunolabeled as described previously (Fischer and Reh, 2000; Fischer and Reh, 2002; Fischer et al., 1998). Primary and secondary antibodies are listed in Table 2.2.

Photography, cell counts and statistics:

Digital photomicroscopy was performed as described in previous studies (Fischer et al., 2008; Fischer et al., 2010; Ghai et al., 2009; Ghai et al., 2010). 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 CMZ. The identity of BrdU-labeled cells was determined based on previous findings that 100% of the proliferating cells in the chick retina are comprised of Sox2/9^{+} Müller glia in the INL/ONL, Sox2/9/Nkx2.2^{+} NIRG cells in the IPL, GCL and NFL (the NIRG cells do not migrate distally into the retina), and CD45^{+} (Sox2/9^{-}) microglia (Fischer et al., 2010; Zelinka et al., 2012). Sox2^{+} nuclei in the INL were identified as Müller glia based on their large size and fusiform shape which was distinctly different from the Sox2+ nuclei of cholinergic amacrine cells which are small and round (Fischer et al., 2010). None of the proliferating Sox2/Sox9-positive cells within peripheral regions of the retina were derived from CMZ progenitors; cells produced by CMZ progenitors do not migrate laterally into the retina (Fischer and Reh, 2003a). Similar to previous studies (Fischer et al., 2009a; Fischer et al., 2009b; Fischer et al., 2010; Ghai et al., 2009), immunofluorescence was quantified by using ImagePro 6.2 (Media Cybernetics, Bethesda, MD, USA). The mean area, intensity, and density sum was calculated for the pixels within threshold regions from ≥5 retinas for each experimental condition. GraphPad Prism 6 was used for statistical analyses.

Results

Hedgehog-signaling in normal and damaged retinas:

Shh binds to the transmembrane receptor patched (Ptch) to relieve repression of the transmembrane protein smoothened (Smo) to permit activation of Gli's (Ruiz i Altaba

et al., 2002). Activated Smo liberates Gli second messengers from a cytoplasmic retention complex permitting translocation of Gli's to the nucleus to influence transcription (Hooper and Scott, 2005). Levels of Ptch (Goodrich et al., 1996; Marigo and Tabin, 1996) and Glis (Bai et al., 2002; Litingtung and Chiang, 2000; Marigo et al., 1996) are up-regulated in response to Hh-signaling. We analyzed whether components of the Hh-pathway are expressed in the chick retina by using RT-PCR. In normal undamaged retina, we detected mRNA for *shh*, *gli1*, *gli2*, *gli3*, *ptch1*, *smo* and *sufu* (Fig 1a), suggesting that Hh-signaling is active in the avian retina under normal conditions. Similar to a previous report (Dakubo, et al. 2003), we failed to detect mRNA for the *desert hedgehog* and *indian hedgehog* in normal and NMDA-damaged retinas or in retinal pigmented epithelium (not shown).

We used quantitative RT-PCR to assay for components of the Hh-pathway following NMDA-treatment where MGPCs are known to form. *Shh* was rapidly up-regulated within 4 hrs after NMDA-treatment, down-regulated at 1 and 2 days after treatment, and returned to control levels by 3 days after treatment (Fig.2.1b). By comparison, *gli1* and *gli2* were up-regulated from 4hrs through 3 days after treatment, and *gli3* was up-regulated from 1 through 3 days after treatment (Fig.2.1b). Levels of *ptch1* were increased after NMDA-treatment with levels peaking at 1 day after treatment, and levels of *smo* were elevated from 4hrs through 3 days after treatment (Fig.2.1b). These data suggest that Hh-signaling is rapidly up-regulated after damage, with signaling peaking at 24 hrs after treatment. This peak in Hh-signaling coincides with when Müller

glia are known to de-differentiate and is prior to entry into the cell cycle following NMDA-treatment (Fischer and Reh, 2001).

We next examined patterns of *ptch1* expression within normal and damaged retinas. *ptch* is expressed at low levels by mature Müller glia and some cells (possibly astrocytes) in the ganglion cell layer (GCL) in the rodent retina (Moshiri and Reh, 2004; Wang et al., 2002). In addition, microarray data from single or sorted Müller glia indicate that low levels of expression of *ptch, smo* and *gli*s in mature mouse retina (Roesch et al., 2008; Roesch et al., 2012). Although *ptch* and *smo* are expressed by retinal progenitors in the embryonic chick (Zhang and Yang, 2001a), the identity of the cells that are receptive to Hh in the mature chick retina remains uncertain. In undamaged retina, weak signal for *ptch1* was observed in the proximal inner nuclear layer (INL) and GCL (Fig.2.1c). By comparison, 24hrs after NMDA-induced damage, there was a robust induction of *ptch1* (Fig.2.1d), consistent with data from qRT-PCR analysis. We found *ptch1* in the GCL, cells scattered in the inner plexiform layer (IPL), and in the inner half of the INL (Fig.2.1d), suggesting that *ptch1* may be up-regulated by ganglion cells, amacrine cells, NIRG cells in the IPL, and Müller glia. The NIRG (Non-astrocytic Inner Retinal Glia) cells have been characterized as a unique type of glial cell that reside in the inner retina (Fischer, et al. 2010). At 72 hrs after NMDA-treatment, *ptch1* remained prevalent in the GCL and INL, and in cells scattered across the IPL and nerve fiber layer (NFL; Fig.2.1e). In addition to diffuse labeling across inner retinal layers, *ptch1* appeared colocalized to $PCNA^{+}$ cells in the IPL/GCL and INL (Fig.2.1e), suggesting that *ptch1* is expressed by proliferating NIRG cells and MGPCs.

We next characterized patterns of expression for Shh. Shh-immunofluorescence is normally present in the axons of ganglion cells in the NFL (Fig.2.2a), consistent with the notion that Shh is normally expressed by ganglion cells and exported out of the eye (Dakubo et al., 2003; Traiffort et al., 2001; Wallace and Raff, 1999). At 2 days after NMDA-treatment, Shh-immunoreactivity remains prominent in the NFL and appears as distinct puncta in the inner nuclear layer (INL; Fig.2.2b). By 3 days after treatment, Shh-immunofluoresence is diminished in the NFL, further accumulates as puncta in the INL, and accumulates in the OPL (Fig.2.2c). By 5 days after treatment, Shh is no longer present in the NFL, whereas Shh appears in the OPL and in puncta scattered across the INL and ONL (Fig.2.2d). The Shh^{+} puncta were associated with Pax6-expressing MGPCs that accumulate in the distal INL and ONL at 3 days after NMDA-treatment (Figs.2,2e-i). The Shh-immunoreactivity that accumulates in the OPL overlaps, in part, with the axon terminals of $calbindin^{+}$ cone photoreceptors (Fig.2.2j-m). It remains uncertain whether the Shh accumulates within or at the surface of photoreceptor terminals. To further investigate the expression patterns of shh we performed *in situ* hybridization. We detect *shh*in the GCL of control retinas (Fig.2.2n), and this pattern of expression was prominent at 4hrs after NMDA-treatment (Fig. 2.2o). However, signal for *shh*was diminished at 3 days after NMDA-treatment (Fig. 2.2n), consistent with data from qRT-PCR and immunolabeling experiments.

Hh-signaling stimulates the proliferation of MGPCs in damaged retinas:

We next examined whether Hh-signaling influenced the formation of MGPCs. Four consecutive daily injections of rhShh failed to stimulate the formation of MGPCs in undamaged retinas (not shown). Thus, we investigated whether activation of Hh-signaling influenced the formation of MGPCs following retinal injury where components of the Hh-pathway are up-regulated. A low dose of NMDA failed to stimulate the accumulation of large numbers of $BrdU^+$ MGPCs (Figs.2.3a,e). Application of rhShh following a low dose of NMDA significantly increased numbers of proliferating MGPCs in central and peripheral regions of the retina (Figs.2.3b,e). Numbers of proliferating NIRG cells and microglia/macrophages were unaffected by rhShh (Fig.2.3c-e). Hh-signaling has been shown to coordinate with Notch-signaling effectors in retinal progenitor cells (Wall et al., 2009), and Notch-signaling stimulates the formation of MGPCs (Ghai et al., 2010; Hayes et al., 2007). Thus, we tested whether Notch influenced the formation of MGPCs in retinas treated with NMDA and rhShh. We found that inhibition of Notch with DAPT reduced the number of proliferating MGPCs in retinas treated with NMDA and rhShh (Fig.3f). We next tested whether Smoothened agonist (SAG), which bypasses Ptch1 and activates Smo (Chen et al., 2002), influences the formation of MGPCs. Similar to rhShh, four consecutive daily intraocular injections of SAG failed to stimulate the formation of MGPCs in undamaged retinas (not shown). Using antibodies to pERK, p38 MAPK, cFos, pS6, vimentin, GFAP and transitin, we failed to find activation of different signaling pathways or reactivity in Müller glia treated with rhShh or SAG (data not shown), suggesting that Müller glia are normally not receptive to rhShh or SAG. However, following a low dose of NMDA, SAG stimulated

the accumulation of $BrdU^+$ MGPCs in central and peripheral regions of retina (Fig.3g). Similar to rhShh, SAG had no influence upon the proliferation of NIRG cells or microglia/macrophages in NMDA-damaged retinas (not shown). In older animals (≥P27), we found that numbers of proliferating MGPCs were significantly increased by SAG following NMDA-treatment (Fig.S2), suggesting that Hh-signaling retains the capacity to stimulate Müller glia to become MGPCs in older damaged retinas.

Hh-mediated regeneration of embryonic chick retina is known to require MAPK-signaling (Spence et al., 2007b; Spence et al., 2004). Accordingly, we tested whether MAPK-signaling influenced the ability of SAG to stimulate the proliferation of MGPCs. We found that inhibition of MAPK-signaling with U0126 reduced the number of BrdU-labeled MGPCs in retinas treated with NMDA+SAG (Fig.2.3h). U0126 also reduced the amount of Sox2+/PCNA+ cells after NMDA+SAG (treated 82.2±21.1 vs control 155 ±30.2, n=5, p=0.002). Consistent with this finding, we found that level of *ptc, gli1, and gli2* were decreased in NMDA-damaged retinas treated with U0126 (Fig 2.3i), suggesting that MAPK-signaling is required to enable Hh-signaling during the reprogramming of Müller glia into MGPCs.

To better understand how rhShh stimulates the formation of MGPCs in damaged retinas, we used qRT-PCR to assess changes in expression of components of the Hh-pathway and genes that are known to be up-regulated in MGPCs. In NMDA-damaged retinas treated with rhShh, we found significant increases in mRNAs of components in the Hh-pathway (Fig.2.3j). We found that injections of rhShh following NMDA-treatment significantly increased levels of *ptch*, *gli1*, *gli2* and *gli3* (Fig.2.3j). In addition,

rhShh increased levels of transcription factors that are associated with progenitors including *pax2*, *hes1,hes5* and *yap,* whereas levels of *notch1,ascl1a* and *lin28* were unaffected (Fig.2.3j).

To further investigate how activation of Hh-signaling in damaged retinas stimulates the proliferation of MGPCs we probed for expression of components of the complement system. In embryonic chick, the complement peptide C3a and the C3a Receptor (C3aR) stimulate retinal regeneration during early stages of development (Haynes et al., 2013). In post-hatch chicks, levels of *c3* and *c3aR* are elevated in NMDA-damaged retinas, but are significantly reduced when the microglia were ablated wherein MGPCs fail to form (Fischer et al., 2014). We found that levels of *c3* were not affected by rhShh, whereas levels of *c3aR* were significantly increased (Fig.2.3j).

Levels of cell death and damage can impact the formation of MGPCs (Fischer and Reh, 2001; Fischer et al., 2004b). Accordingly, we tested whether rhShh influenced cell death when applied after NMDA. The TUNEL assay revealed no difference in the numbers of dying cells in retinas treated with rhShh compared to numbers of dying cells in retinas treated with vehicle (treated 46.3 ±11.1 vs control 43.1 ±11.6, n=6, p=0.64).

Inhibition of Hh-signaling reduces the proliferation of MGPCs:

We next tested whether KAAD-cyclopamine, which directly binds to and inhibits Smo, influenced the formation of MGPCs. KAAD-cyclopamine significantly reduced numbers of proliferating MGPCs in retinas damaged by a high dose of NMDA (Figs.2.4a-c).This inhibition of proliferation was specific to MGPCs as the proliferation

of $CD45^+$ microglia and $Nkx2.2^+$ NIRG cells were unaffected by KAAD-cyclopamine (Fig.2.4c). Inhibition of Hh-signaling with KAAD-cyclopamine after NMDA resulted in significant decreases in retinal levels of *ptch1* and *gli1*, whereas levels of *hes1* were unaffected (Fig.2.4d). In older animals (≥P27), we found that numbers of proliferating MGPCs were significantly decreased by KAAD-cyclopamine following NMDA-treatment (data not shown), suggesting that Hh-signaling retains the capacity to stimulate Müller glia to become proliferating MGPCs in older damaged retinas.

Cyclopamine can have off-target effects (Yauch et al., 2008), by acting independent of Smo function and activating neutral sphingomyelinase 2 and ceramide production (Ponnusamy et al., 2010). Accordingly, we tested whether inhibition of Hh-signaling with drugs that target different components of the Hh-pathway influence the formation of MGPCs. We inhibited the Gli's with the small molecule inhibitors GANT58, which interferes with Gli1-DNA binding, and GANT61, which interferes with Gli1/2-DNA binding (Lauth et al., 2007). Similar to treatment with KAAD-cyclopamine, GANT58 or GANT 61 suppressed the formation of proliferating MGPCs in NMDA-damaged retinas (Figs.2.4e and 2.4f), whereas the proliferation of microglia and NIRG cells was not affected (not shown). To block Shh-ligand we injected the 5E1 monoclonal antibody into eyes with damaged retinas. Application of the 5E1 antibody after NMDA-treatment caused a significant decrease in the number of proliferating MPGC's (Fig.2.4g), whereas the proliferation of microglia and NIRG cells was unaffected (not shown). The decrease in proliferation of MGPCs with 5E1-treatment was not as pronounced as that observed with KAAD-cyclopamine or GANT-treatment, and only

became statistically significant when accounting for inter-individual variability (Fig.4g). TUNEL was performed at 3 days after NMDA-treatment, 24hrs after the last dose BrdU ± Hh-inhibitor, to assess whether decreases in cell death might underlie decreases in proliferation of MGPCs. We found that the Hh-inhibitors did not influence cell death in NMDA-damaged retinas (KAAD-cyclopamine – control 49.25 ± 34.2 versus treated 64.5 ± 31.8, p=0.45; GANT58 – control 39.0 ± 40.1 versus treated 71 ± 36.4, p=0.27).

The Hh-pathway synergizes with FGF/MAPK-signaling to influence MGPCs:

Previous work in regeneration of prospective retina in the embryonic chick has implicated a requirement and interdependence of Hh- and FGF/MAPK-signaling (Spence et al., 2007b; Spence et al., 2004). How these pathways interact in the context of MGPCs remains undetermined. Recently, it has been shown that sustained FGF/MAPK-signaling is sufficient to stimulate the formation of MGPCs in the absence of damage (Fischer et al., 2014). Further, FGF2 is known to activate and up-regulate components of the Notch-pathway including *notch1, dll4, jagged, hes5, hey1* and *hey2* (Ghai et al., 2010), and activation of this pathway is required for the formation of MGPCs (Ghai et al., 2010; Hayes et al., 2007).We sought to investigate whether Hh-signaling activated by FGF/MAPK-signaling and whether the Hh-pathway contributes to the formation of MGPCs in FGF2-treated retinas. We began by probing for changes in expression of components of the Hh-pathway in response to Smo-agonist (SAG), FGF2 or IGF1. Consistent with findings that intraocular injections of SAG fail to stimulate the formation of MGPCs in undamaged retinas, treatment with SAG failed to increase retinal levels of

smo, *ptch,* or *gli2*, whereas levels of *gli1* were increased (Fig.2.5a). We found that FGF2-treatment, which selectively activates MAPK-signaling in Müller glia (Fischer et al., 2009a), up-regulated A levels of *smo* and *gli2*, but not *ptch* or *gli1* (Fig.2.5b). By comparison, we detected no significant increases in components of the Hh-pathway in response to IGF1 (data not shown).

We found that 3 consecutive daily injections of the combination of FGF2 and SAG stimulated increased numbers of proliferating MGPCs compared to FGF2 alone (Figs.2.5c,d). To better understand the mechanism of the synergistic effects of FGF2 and SAG we assayed for cell-level changes in Müller glia following an acute dose of these factors. FGF2 is known to stimulate MAPK-signaling in Müller glia (Fischer et al., 2009a). Thus, we probed for changes in expression levels of the MAPK effectors pERK, p38 MAPK, pCREB, Egr1, and cFos. Müller glia treated with FGF2 and SAG had elevated levels of the immediate early gene cFos compared to levels seen in Müller glia treated with FGF2 alone (Figs.5e,f), whereas levels of pERK, Egr1, p38 MAPK, pS6 or pCREB (data now shown) in Müller glia were unaffected.

Since Hh-agonist stimulated the formation of MGPCs in FGF2-treated retinas whereas Hh-agonist alone had no effect, we tested whether Hh-signaling was part of signaling network activated by FGF2. Four consecutive daily doses of FGF2 are known to stimulate the formation of MGPCs in the absence of damage (Fischer et al., 2014). Co-application of KAAD-cyclopamine with FGF2 significantly reduced numbers of proliferating MGPCs compared to numbers seen in retinas treated with FGF2 alone (Figs.2.5h,i). To better understand how KAAD-cyclopamine attenuates the formation of

MGPCs, we investigated cell-level expression of signaling components in Müller glia following FGF/KAAD-treatment. One day after 2 consecutive daily injections of FGF2 all of the $Sox2^{+}$ Müller glia (320 of 320 cells, n=4) were positive for cFos (Fig.2.5j). KAAD-cyclopamine significantly reduced FGF-induced cFos in Müller glia (Figs.2.5j,k). In contrast to the embryonic chick (Spence et al., 2007a), we observed no differences between FGF-induced pERK in Müller glia following KAAD-cyclopamine treatment (data not shown). Further, we observed no KAAD-cyclopamine-induced differences in p38 MAPK, Egr1 and pCREB (not shown). Collectively, these data suggest that; (i) Hh-signaling amplifies the ability of FGF2/MAPK to stimulate the formation of MGPCs, (ii) FGF2/MAPK-signaling renders Müller glia receptive to Hh-signaling, and (iii) inhibition of Hh-signaling disrupts FGF2/MAPK-mediated formation of MGPCs at the level of downstream of MAPK-signaling, possibly at the level of cFos expression.

Hedgehog and IGF1-signaling:

It has recently been shown that Shh- and PI3K/mTOR-pathways synergizes to promote proliferation of human glioblastoma cells (Gruber Filbin et al., 2013). Accordingly, we tested whether co-activation of PI3K/mTor- and Hh-pathways stimulates the proliferation of MGPCs in the uninjured retina. We applied 4 consecutive daily doses of SAG, IGF1 or SAG and IGF1. There was no nuclear migration of Müller glia in retinas treated with SAG alone, whereas there was some nuclear migration in retinas treated with IGF alone (Figs.2.6a,b), consistent with a previous report (Fischer et al., 2010). By comparison, treatment with SAG+IGF1 caused widespread migration of

Müller glia nuclei (Figs.2.6a,b). The nuclei that migrated were ovoid shape in retinas treated with IGF1+SAG, compared to the more fusiform nuclei of Müller glia treated with SAG or IGF1 that did not undergo widespread nuclear migration (Figs.2.6a,b). Proliferating MGPCs are known to undergo nuclear migration, reminiscent of proliferating embryonic retinal progenitors (Fischer et al., 2002b; Fischer and Reh, 2001). Despite widespread nuclear migration, Müller glia failed to incorporate BrdU or express significant levels of PCNA, GFAP or vimentin (not shown). Few $BrdU^+$ MGPCs were found in far peripheral regions of the retina (Fig.2.6c,d). Although numbers of $Sox2^+$ Müller glia appeared increased in retinas treated with IGF1+SAG, cell counts revealed no significant difference in total numbers of Müller glia in retinas treated with IGF1 vs IGF1+SAG (Fig.2.6e), consistent with the notion that treatment with IGF1+SAG fails to stimulate the formation of proliferating MGPCs.

To further examine the effects of IGF1 and SAG on proliferation, we tested whether the proliferation of progenitors in the circumferential marginal zone (CMZ) was affected. Intraocular injections of IGF1 or SAG stimulated the CMZ progenitors and the addition of new cells to the far peripheral edge of the retina by stimulating the proliferation of retinal progenitors (Figs.2.6f,g), consistent with previous reports (Fischer and Reh, 2000; Moshiri et al., 2005). By comparison, the combination of IGF1 and SAG further stimulated the proliferation of retinal progenitors in the CMZ (Figs.2.6f,g). These findings indicate that the doses IGF1 and SAG were sufficient to stimulate the proliferation of progenitors that were capable of responding, unlike the Müller glia.

Most, if not all, proliferating MGPCs undergo nuclear migration that is accompanied by proliferation (Fischer and Reh, 2001). Thus, we investigated whether delaminated Müller glia de-differentiated to up-regulate Pax6 in response to IGF1 and SAG. Müller glia express little or no Pax6 (Fischer et al., 2014), whereas MGPCs up-regulate a number of different transcription factors, including Pax6, that are characteristic of retinal progenitors (reviewed by Gallina et al, 2014, Fischer, et al. 2010). In Müller glia treated with 4 consecutive daily doses of SAG, levels of Pax6 remain low and nuclei remain laminated in the middle of the INL (Fig.2.7a), consistent with the notion that Hh-signaling does not influence Müller glia. By comparison, nuclei migrated away from the middle of the INL and levels of Pax6 remained relatively low in Müller glia treated with 4 consecutive daily doses of SAG and IGF1 (Fig.2.7a). Levels of Pax6 appeared elevated with relatively little nuclear migration in Müller glia treated with 3 consecutive daily doses of FGF2 (Fig.2.7a), where relatively few MGPCs proliferate (see Fig.2.5). Levels of Pax6 were increased most and nuclear migration was widespread in Müller glia treated with 3 doses of FGF2+SAG or 4 doses of FGF2 alone (Fig.2.7a), coincident with significant numbers of proliferating MGPCs (see Fig.5 and (Fischer et al., 2014). By comparison, levels of Pax6 were reduced and nuclear migration remained prominent in Müller glia treated with 4 doses of FGF2+KAAD-cyclopmine (Fig.2.7a), suggesting that inhibition of Hh-signaling may, in part, suppress the proliferation of MGPCs by inhibiting Pax6 expression.

To better understand how different combinations of growth factors influence the de-differentiation of Müller glia into progenitor cells, we probed for the expression of the

Klf4. Klf4 is one of the key transcription factors, when combined with Sox2, Myc and Oct4, that is capable of reprogramming cells into iPSCs (Takahashi and Yamanaka, 2006). We found that Klf4 is not expressed at detectable levels in normal Müller glia (Fig.2.7b). By comparison, treatment with 3 consecutive daily intraocular injections of FGF2, IGF1 or the combination of IGF1+SAG induced robust expression of Klf4 in Müller glia (Fig.2.7b). The up-regulation of Klf4 in Müller glia appeared uniform across central and peripheral regions of retinas treated with FGF2, IGF1 or IGF1+SAG (data not shown). In central regions of retinas treated with FGF2 + KAAD-cyclopamine, levels of Klf4 in Müller glia appeared diminished, and the nuclei remained laminated near the middle of the INL (Fig.2.7b). By comparison, in peripheral regions of retinas treated with FGF2 + KAAD-cyclopamine, levels of Klf4 in the Müller glia appeared reduced compared to levels those seen in glia treated with FGF2 alone, and the nuclei migrated away from the middle of the INL (Fig.2.7b).

Discussion:

Collectively, our findings indicate that the Hh-pathway plays an important role in the signaling network that underlies the formation of MGPCs. We found that components of the Hh-signaling pathway are present in normal retina. It is possible that Hh-signaling remains active in mature glia to maintain homeostasis (Alvarez, et al. 2011). Deletion of *smo* in adult astrocytes results in reactive gliosis, suggesting that baseline Hh-signaling maintains homeostasis or glial quiescence (Garcia et al., 2010). In response to NMDA-induced damage there was a rapid and sustained increase in retinal levels of Hh-signaling

components. Similarly, injury to the cerebral cortex induces Hh-signaling (Amankulor et al., 2009; Sirko et al., 2013). Our data suggests that in NMDA-treated retinas, neuron-to-glia signaling mechanisms may exist to direct Shh from ganglion cells to Müller glia and/or MGPCs. Re-distribution of Shh from the axons of ganglion cells to retinal cells following damage is similar to the re-distribution of Shh observed in zebrafish retina in response to injury (Sherpa et al., 2014). Additionally, our data are consistent with other reports in the nervous system demonstrating that neurons release Hh, whereas glia are receptive to Hh (Garcia et al., 2010; Sirko et al., 2013; Traiffort, et al.1999).

Although Hh-agonists failed to influence Müller glia in normal retinas, Hh-agonists stimulated the proliferation of MGPCs in damaged retinas and in retinas treated with FGF2 in the absence of damage. Our findings suggest damage or FGF2-treatment renders Müller glia responsive to Hh-signaling and, thereafter, activation of Hh-signaling stimulates the proliferation of MGPCs. We find that Hh-signaling in Müller glia is downstream of MAPK in retinas damaged by NMDA; inhibition of MAPK-signaling down-regulated components of the Hh-pathway and inhibited the ability of Hh-agonists to stimulate the proliferation of MGPCs. In undamaged retinas, by comparison, activation of FGF2/MAPK-signaling in Müller glia up-regulated components of the Hh-pathway and enabled Hh-agonists/antagonists to influence the formation of MGPCs. Our findings suggest that rhShh activates a proliferative program through Gli effectors and/or Notch-signaling and the Notch-effectors Hes1 and Hes5 (Fig.8). Additionally, inhibition of Notch-signaling attenuated the ability of rhShh to stimulate the proliferation of MGPCs. Hh-signaling is known to stimulate proliferation of neuronal precursors via

activation of D-type cyclins, and Gli2 is able to directly regulate these cyclins (Kenney and Rowitch, 2000; Mill et al., 2003). In addition, Hh-signaling is known to stimulate the expression of Hes1 and increase the proliferation of retinal progenitor cells (Wall et al., 2009) and in SVZ neural stem cells (Ferent et al., 2014). We failed to observe up-regulation of *ascl1*, a factor known to stimulate the formation of MGPCs in zebrafish and mouse retinas (Pollak et al., 2013; Ramachandran et al., 2010). It is possible that we failed to detect increases in *ascl1a* due to the time point of our analysis; following NMDA damage *ascl1a* up-regulation is maximal at 4 days after damage (Hayes, et al. 2007), whereas our analysis took place 3 days after damage. We observed increases in Pax2 after treatment with rhShh in the damaged retina. During development, the expression of Pax2 is stimulated in optic nerve glia by Shh (Dakubo et al., 2003; Take-uchi et al., 2003; Wallace and Raff, 1999). By comparison, Pax2 is expressed by Müller glia in central regions of the chick and zebrafish retina (Boije et al., 2010; Stanke et al., 2010). Interestingly, we observed Hh-induced up-regulation of C3aR, the receptor recently discovered to mediate the regeneration of embryonic chick retina in response to complement factor C3 (Haynes, et al. 2013). These data suggest that C3aR-mediated signaling could play a role in Hh-stimulated regeneration of mature retina.

We found that inhibition of Hh-signaling by targeting the Hh-ligand, receptor, or transcriptional effectors significantly reduced numbers of proliferating MGPCs in damage retinas. Blocking Hh-signaling pharmacologically in retinal progenitors is known to suppress progression through the cell cycle (Locker et al., 2006). Similarly, the FGF2-mediated regeneration of prospective retina in the embryonic chick fails when Hh-

signaling is inhibited (Spence, et al. 2007a). In mature Müller glia *in vivo*, FGF2 selectively activates MAPK-signaling (Fischer et al., 2009a), and stimulates the formation of proliferating MGPCs in the absence of damage (Fischer et al., 2014). Collectively, these data suggest that FGF2/MAPK- signaling "kick-starts" a network of inter-dependant pathways to stimulate the formation of proliferating MGPCs. These pathways are known to include up-regulation of Notch-signaling (Ghai et al., 2010), up-regulation of Hh-signaling (current study), and down-regulation of glucocorticoid signaling (Gallina et al., 2014b). Our current findings suggest that Notch-signaling is downstream of both MAPK- and Hh-pathways (Fig.8).

In the chick eye, CMZ progenitors normally respond to Hh-signaling (see Fig.6) and (Moshiri et al., 2005), whereas Müller glia must be "primed" by damage or FGF2/MAPK-signaling to become responsive to Hh. Our data indicate that IGF1 is not sufficient to stimulate Müller to respond to the mitogenic effects of Hh-agonists. In response to the combination of IGF1 and Hh-agonist, we observed widespread nuclear migration of Müller glia nuclei and changes in nuclear morphology, but no proliferation. These data suggest that nuclear migration of Müller glia can occur independent of cell cycle re-entry. By comparison, proliferation of MGPCs may require nuclear migration away from the center of the INL. For example, the vast majority of pHisH3^{+} mitotic figures for MGPCs are observed in the distal INL or ONL (Fischer and Reh, 2001 and unpublished observation). In retinal development, nuclear migration of progenitor cells is coordinated with cell cycle regulation. However, nuclear migration can be uncoupled from cell cycle re-entry (reviewed by Baye and Link, 2008).

Stimulation of Müller glia with IGF1 and Hh-agonist is not sufficient to up-regulate Pax6 to levels seen in proliferating MGPCs. In normal Müller glia, Pax6 is expressed at very low levels but is rapidly induced during the formation of proliferating MGPCs (Fischer and Reh, 2001). Up-regulation of Pax6 is symptomatic of Müller glial de-differentiation. In addition, Pax6 plays a key role in the proliferation and multipotency of retinal progenitor cells in development (reviewed by Ashery-Padan and Gruss, 2001) and is required for Müller glia-mediated retinal regeneration in zebrafish (Thummel et al., 2010). Consistent with the notion that up-regulation of Pax6 is an important step in the formation of MGPCs, inhibition of Hh-signaling suppressed the formation of MGPCs and prevented the up-regulation Pax6, in addition to Klf4, in Müller glia treated with FGF2.

Our data suggest that in response to IGF1 and Hh-agonist Müller glia make initial steps to becoming progenitor-like by migrating away from the INL and up-regulating low levels of Pax6 and Klf4, but fail to fully de-differentiate and re-enter the cell cycle as MGPCs. Alternatively, Müller glia that respond to IGF1 and Hh-agonist may migrate as part of a gliotic response. Inhibition of Hh-signaling in FGF2-treated retinas suppressed Pax6 expression in Müller glia, but does not suppress nuclear migration, suggesting that Hh- and FGF2/MAPK-signaling synergize to stimulate Pax6 expression during the transition in phenotype from Müller glia to MGPC. Müller glia are known to undergo nuclear migrate in response to various retinopathies (reviewed by Bringmann et al., 2006; Gruber Filbin et al., 2013). However, we failed to detect markers of glial reactivity, such as elevated vimentin or GFAP, in retinas treated with IGF1 and Hh-agonist.

We cannot exclude the possibility that the Hh-agonists and -antagonists act through amacrine or ganglion cells to influence the proliferation of MGPCs in damaged retinas. We find that *ptch* is expressed by neurons in addition to Müller glia and/or MPGCs in damaged retinas. Thus, it is possible that Hh-signaling through inner retinal neurons impacts the formation of proliferating MGPCs. Consistent with this hypothesis, a recent study in zebrafish retina demonstrated that regeneration from MGPCs is delayed with a partial loss-of-function mutation in Shh, which is produced by inner retinal neurons (Sherpa et al., 2014). In FGF2-treated retinas in the absence of damage, where Müller glia are known to be selectively activated (Fischer et al., 2009a), we find that activation or inhibition of Hh-signaling influences the expression of cFos and the proliferation of MGPCs. It remains uncertain whether the influences of Hh-signaling on FGF2-induced MGPCs are manifested directly through the Müller glia or through inner retinal neurons

Conclusions

We conclude that Hh-signaling influences the proliferation of MGPCs in NMDA-damaged retinas and in FGF2-treated retinas in the absence of damage. In damaged retinas, Shh-signaling is dynamically regulated and is correlated with a redistribution of Shh from ganglion cell axons to MGPCs. In addition, we find that FGF2/MAPK-signaling stimulates Müller glia to become responsive to Hh-signaling, and that Hh-signaling contributes to the network of signaling pathways, and Pax6-expression to stimulate the de-differentiation and proliferation of MGPCs. We find that the combined

activation of IGF1- and Hh-signaling is sufficient to stimulate some aspects of de-differentiation such as nuclear migration and induction of low levels of Pax6, but fails to fully promote the de-differentiation of Müller glia into proliferating MGPCs.

Figure 2.1. Components of the Hh-pathway in normal and damaged retinas. RT-PCR (**a**) and qRT-PCR (**b**) were used to probe for *gapdh*, *shh*, *gli1*, *gli2*, *gli3*, *ptch1*, *smo* and *sufu*. Beginning at P7, RNA was extracted from normal retinas (**a**) or from retinas treated with saline or 2 µmol NMDA at 4, 24, 48 and 72 hours after treatment (**b**). Significance of difference (*p<0.05, **p<0.01, ***p<0.005) was determined by using a two-tailed Mann-Whitney U test**.** *In situ* hybridization was used to detect *ptch1* in control (**c**) and NMDA-damaged retinas at 24 (**d**) and 72hrs after treatment (**e**). Immunolabeling for PCNA (red) was included to indicate proliferating cells (**e**). Arrows indicate presumptive Müller glia and small double-arrows indicate presumptive NIRG cells. Abbreviations: ONL- outer nuclear layer, INL- inner nuclear layer, IPL- inner plexiform layer, GCL- ganglion cell layer. The calibration bar (50 µm) in **d** applies to **c** and **d**, and the bar in **e** applies to **e** alone.

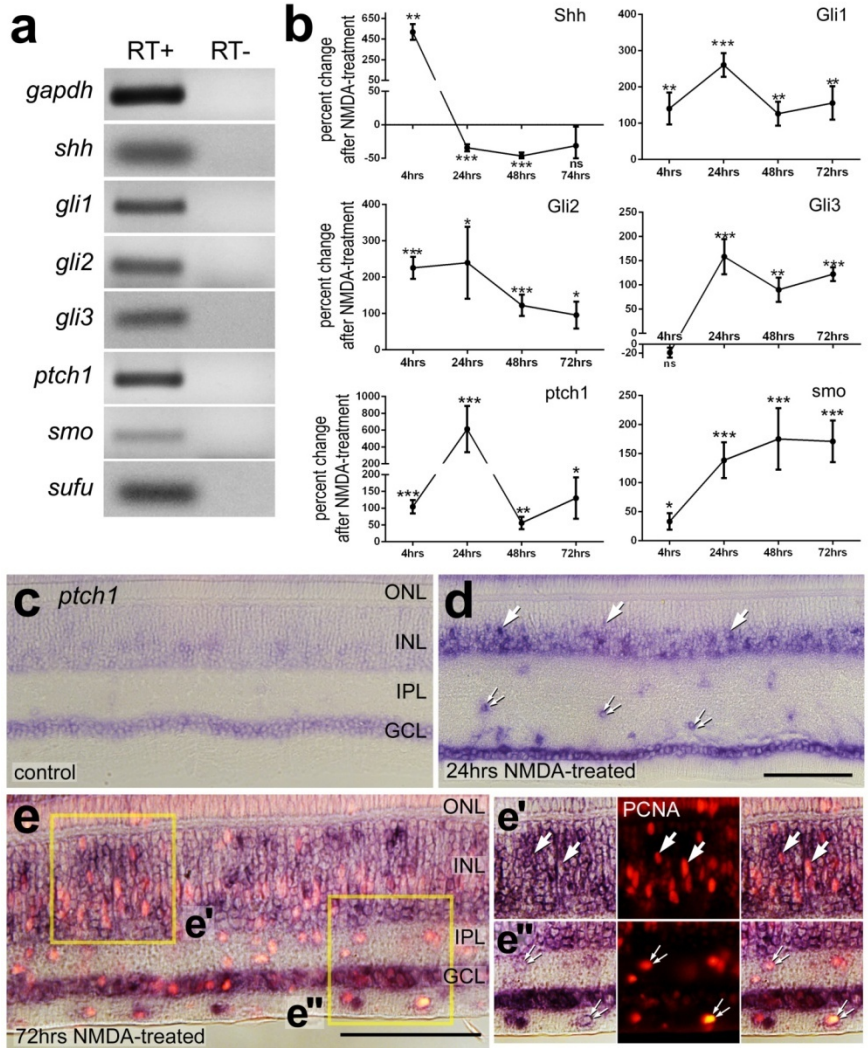

Figure 2.2. Shh-expression in normal and NMDA-damaged retinas. Retinas were obtained from eyes that were injected with saline (control; **a**,**e**,**n**) or 2 µmol NMDA and harvested 4hrs (**o**), 2 days (**b**), 3 days (**c**, **f-i**, **p**) or 5 days (**d,j-m**) later. Retinal sections were labeled with antibodies to Shh (red; **a-m**), Pax6 (green; **e-i**), and calbindin (green; **j-m**). ISH for *shh* was performed on control retina (**n**) and NMDA-treated retinas (**o**,**p**). Arrows indicate Müller glia labeled for Pax6 and Shh-puncta. The calibration bar (50 µm) in **d** applies to **a-d**, the bar in **f** applies to **e** and **f**, the bar in **j** applies to **j** alone, and the bar in **p** applies to **n-p**.

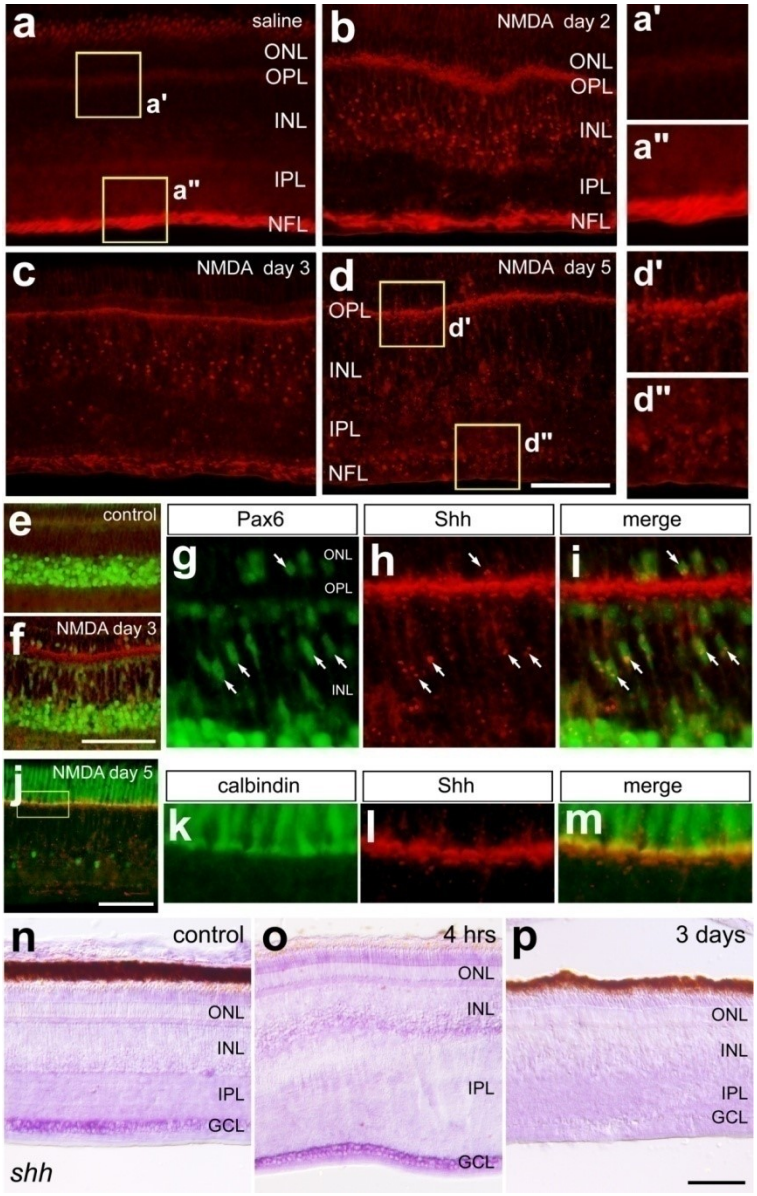

Figure 2.3. Activation of Hh-signaling following retinal injury stimulates the formation of proliferating MGPCs. Eyes were injected with 60 nmol NMDA, followed by vehicle (control), rhShh or SAG (treated) at 4 and 24 hrs later, BrdU at 48 hrs, and retinas harvested 24 after injection of BrdU. Sections of the retinas were immunolabeled for Sox2 (red), BrdU (green) and PCNA (magenta; **a** and**b**), BrdU (green) and Nkx2.2 (red; **c**), or BrdU (green) and CD45 (red; **d**). Arrows indicate proliferating MGPCs, small double-arrows indicate proliferating microglia, and arrow-heads indicate proliferating NIRG cells. The calibration bar (50 μm) in **b** applies to **a** and **b**, and the bar in **d** applies to **c** and **d**. **e** (rhShh-treated), **f** (rhShh±DAPT-treated), **g** (SAG-treated), **h** (SAG±U0126-treated); histograms illustrate the mean number (±SD) of proliferating MGPCs (BrdU/$Sox2^{+}$ $Nkx2.2^{-}$) in central and peripheral retina. Significance of difference (*$p<0.05$, **$p<0.01$) was determined by using a two-tailed***t* **test**. **i**; qRT-PCR was used to measure relative levels of *ptch, gli1, gli2,* in retinas at 2 days after treatment with NDMA ± U0126. **j**qRT-PCR was used to measure relative levels of *ptch, gli1, gli2, gli3, pax2, hes1, hes5, notch1, ascl1a, lin28, yap, c3aR* and *c3* in retinas at 3 days after treatment with NMDA ± rhShh. The mean (±SD) percentage change in mRNA levels was determined, and the significance of difference (*$p<0.05$) between NMDA/vehicle and NMDA/U0126 or rhShh-treated samples was determined by using a Mann-Whitney U-test.

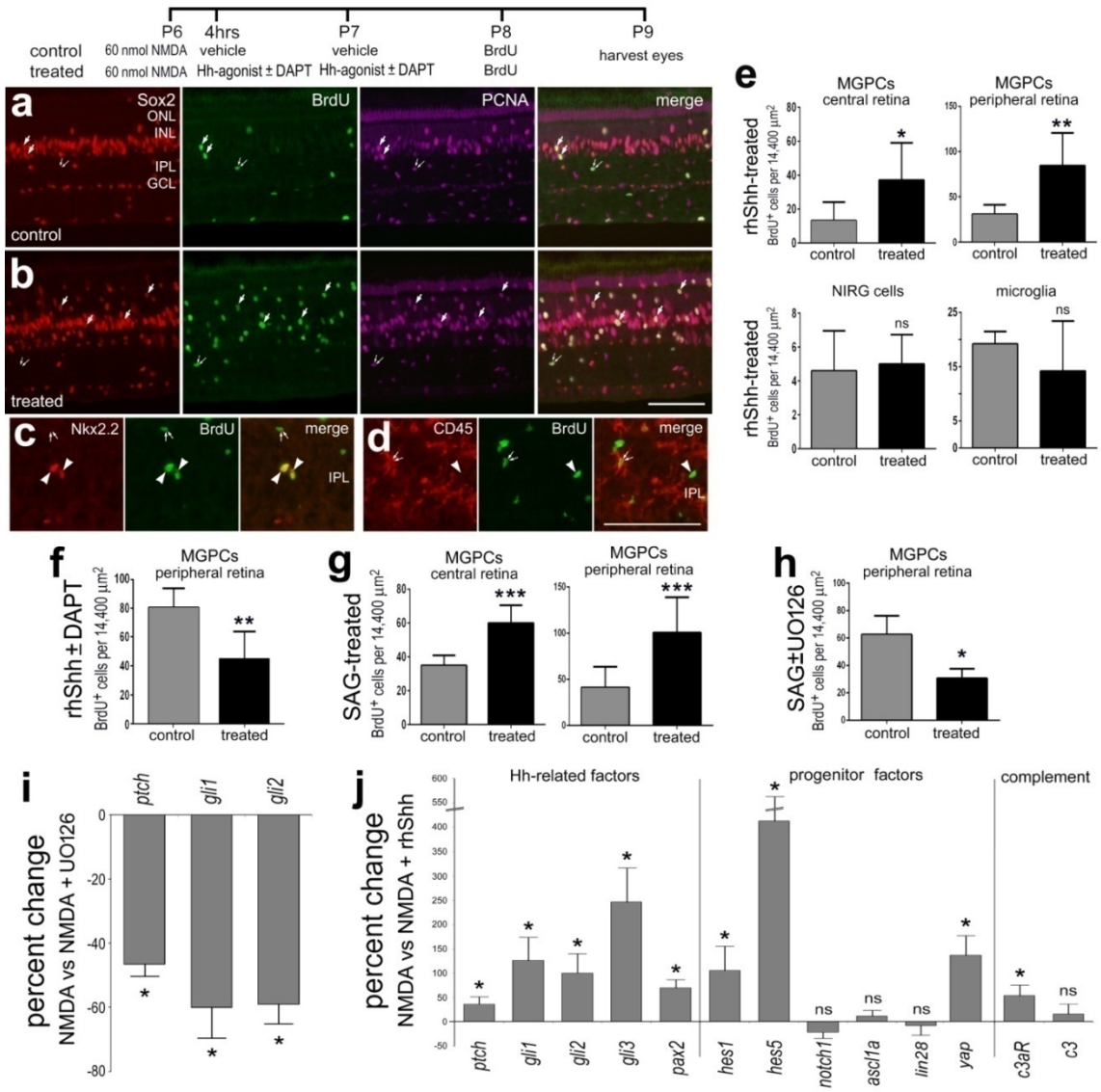
P6
4hrs
P7
P8
P9
control
60 nmol NMDA
vehicle
vehicle
BrdU
harvest eyes
treated
60 nmol NMDA
Hh-agonist ± DAPT
Hh-agonist ± DAPT
BrdU
a
Sox2
ONL
INL
IPL
GCL
BrdU
PCNA
merge
control
b
treated
c
Nkx2.2
BrdU
merge
IPL
d
CD45
BrdU
merge
IPL
e
MGPCs
central retina
MGPCs
peripheral retina
rhShh-treated
BrdU+ cells per 14,400 µm2
control
treated
NIRG cells
microglia
ns
f
MGPCs
peripheral retina
rhShh ± DAPT
g
MGPCs
central retina
MGPCs
peripheral retina
SAG-treated
h
MGPCs
peripheral retina
SAG±UO126
i
percent change
NMDA vs NMDA + UO126
ptch
gli1
gli2
j
percent change
NMDA vs NMDA + rhShh
Hh-related factors
progenitor factors
complement
ptch
gli1
gli2
gli3
pax2
hes1
hes5
notch1
ascl1a
lin28
yap
c3aR
c3

Figure 2.4. Inhibition of Hh-signaling suppresses the formation of MGPCs. Eyes were injected with 500 nmol NMDA, followed by injections of vehicle (control) or KAAD-cyclopamine, GANT58, GANT61 or anti-Shh (treated) at 4 and 24 hrs later, and BrdU at 48 hrs later. Retinas were harvested 24 after the last injection of BrdU. Retinal sections were immunolabeled for Sox2 (red), BrdU (green) and PCNA (magenta; **a** and**b**). Arrows indicate proliferating MGPCs and small double arrow-heads indicate proliferating NIRG cells. The calibration bar (50 μm) in **b** applies to **a** and **b**. **c**, **e, f** and **g**; histograms illustrate the mean number (±SD) of proliferating MGPCs (BrdU/$Sox2^{+}$ $Nkx2.2^{-}$) in central and peripheral retina, NIRG cells (BrdU/Sox2/$Nkx2.2^{+}$) and microglia (BrdU/$CD45^{+}$). **g**; the effects of 5E1 monoclonal were relatively small and significant differences were found only when accounting for inter-individual differences (treated-control). Significance of difference (*$p<0.05$, **$p<0.01$) was determined by using a two-tailed***t* **test**. **d**: qRT-PCR for *ptch1*, *gli1* and *hes1* in retinas at 3 days after treatment with NMDA ± KAAD-cyclopamine. The mean (±SD) percentage change in mRNA levels was determined and the significance of difference (*$p<0.05$) was determined by using a Mann-Whitney U-test.

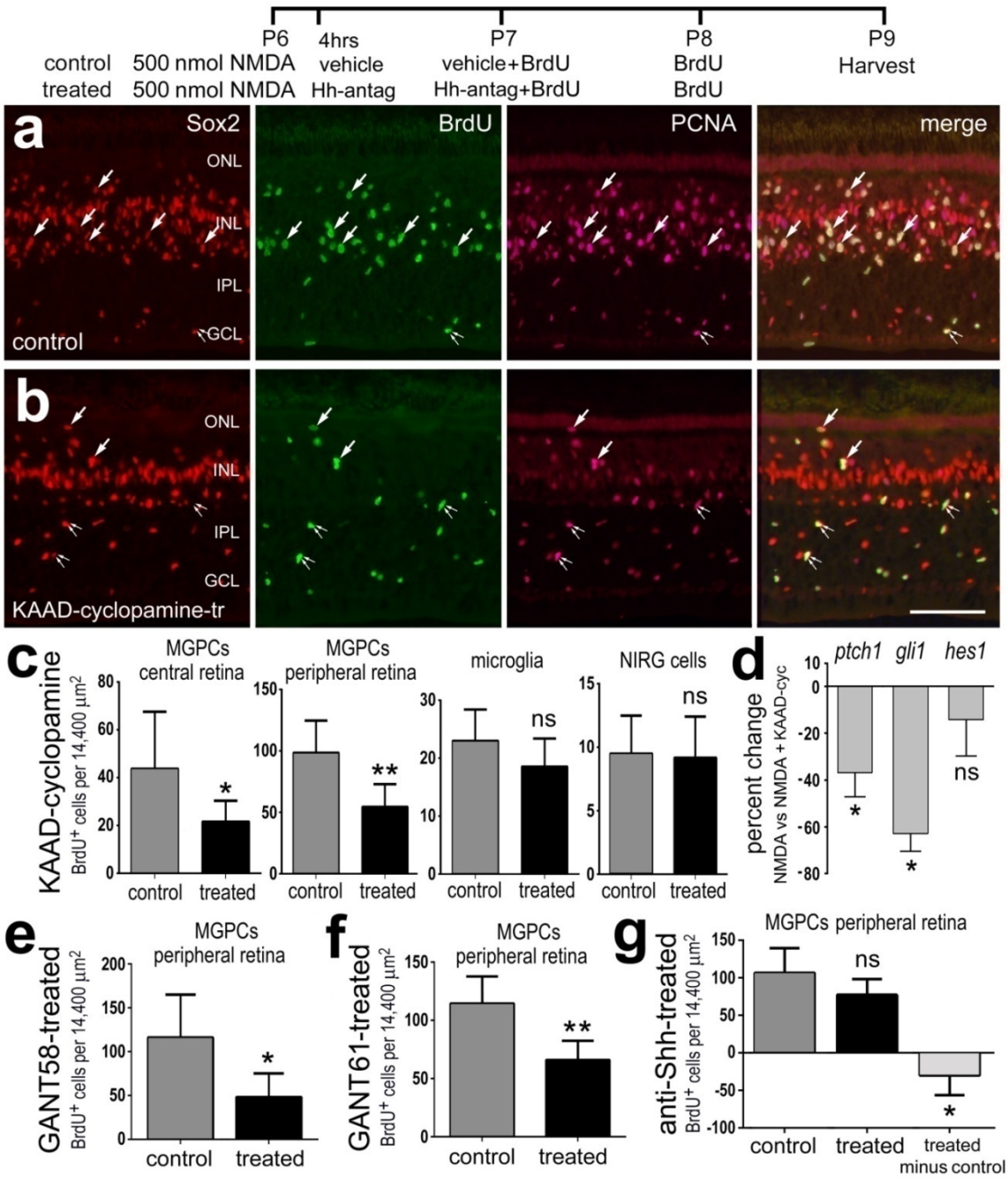
P6
4hrs
P7
P8
P9
control
500 nmol NMDA
vehicle
vehicle+BrdU
BrdU
Harvest
treated
500 nmol NMDA
Hh-antag
Hh-antag+BrdU
BrdU
a
Sox2
BrdU
PCNA
merge
ONL
INL
IPL
GCL
control
b
KAAD-cyclopamine-tr
c
KAAD-cyclopamine
BrdU+ cells per 14,400 µm2
MGPCs central retina
MGPCs peripheral retina
microglia
NIRG cells
control
treated
ns
d
percent change
NMDA vs NMDA + KAAD-cyc
ptch1
gli1
hes1
e
GANT58-treated
f
GANT61-treated
g
anti-Shh-treated
treated minus control

Figure 2.5. Hh-signaling influences the formation of proliferating MGPCs in FGF2-treated retinas in the absence of damage. Eyes were treated with different regimens involving FGF2/vehicle (control), FGF2/SAG (treated) or FGF2/KAAD-cyclopamine (treated). The injection paradigms for each data-set are indicated by lettering. **a, b**; qRT-PCR was used to measure retinal levels of components of the Hh-pathway. Histograms illustrate the mean (±SD; n≥4) percent change in levels for *smo*, *ptch*, *gli1* and *gli2* in retinas treated with SAG or FGF2. Significance of difference (*p<0.05) was determined by using a Mann-Whitney U test. **c-j**; Retinal section were immunolabeled for BrdU (green) and Sox2 (red; **c** and **g**), cFos (green) and Sox2 (red; **e** and **i**). **e, i**;Sox2 (red) is included as a partial-field overlay to indicate nuclear co-localization with cFos (green). The histograms in **d** and **h** illustrate the mean number (±SD; n= 6) of proliferating MGPCs (BrdU/Sox2^{+} Nkx2.2^{-}) in peripheral retina. **f**and **j**; histograms illustrate the mean (±SD; n=6) density sum for cFos immunofluorescence in Sox2^{+} Müller glia. Arrows indicate the nuclei of Müller glia and/or MGPCs. The calibration bar (50 µm) in **c** applies to **c** alone, the bar in **g** applies to **g** alone, and the bar in **i** applies to **e** and**i**. Significance of difference (*p<0.05, **p<0.01) was determined by using a two-tailed *t* test**.**

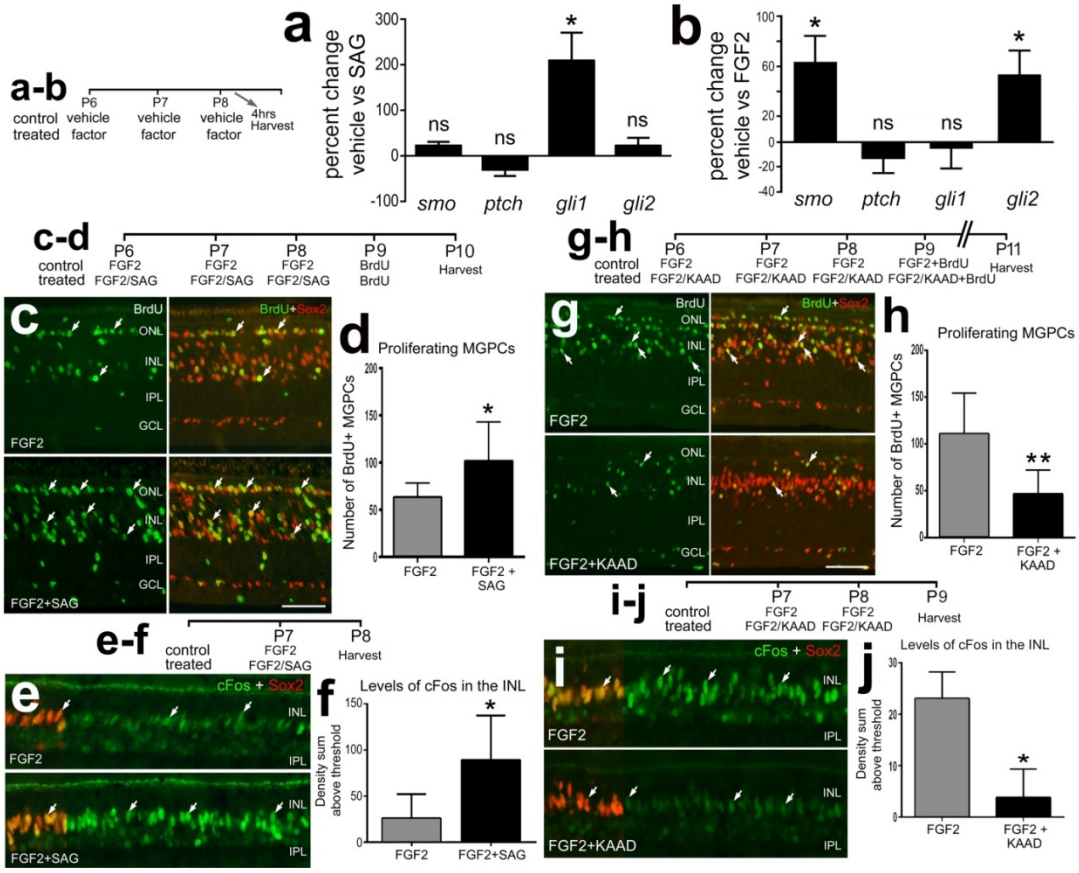

Figure 2.6. The combination of IGF1 and Hh-agonist (SAG) stimulate nuclear migration of Müller glia and proliferation of CMZ progenitors, but fails to stimulate the formation of MGPCs. Retinal sections were immunolabeled for Sox2 (red; **a**-**d**) and BrdU (green; **c,d,g**). Arrows indicate the nuclei of Müller glia/MGPCs, and small double arrows indicate BrdU/Sox2^{+} NIRG cells. **e**; illustrates the mean (±SD) number of Sox2+ Müller glia in the INL of retinas treated with IGF1 or IGF1+SAG. **f;** illustrates the mean (±SD) number of BrdU^{+} cells within the temporal CMZ treated with saline, IGF1, SAG or IGF1+SAG. Significance of difference ($p<0.001$) among the treatment groups was determined by using one-way ANOVA. Significance of difference (*$p<0.05$) between treatment groups was determined by using a two-tailed*t* test with bonferroni correction**.** The calibration bar (50 µm) in **d** applies to **a**,**c**and **d**, and the bar **g** applies **g** alone.

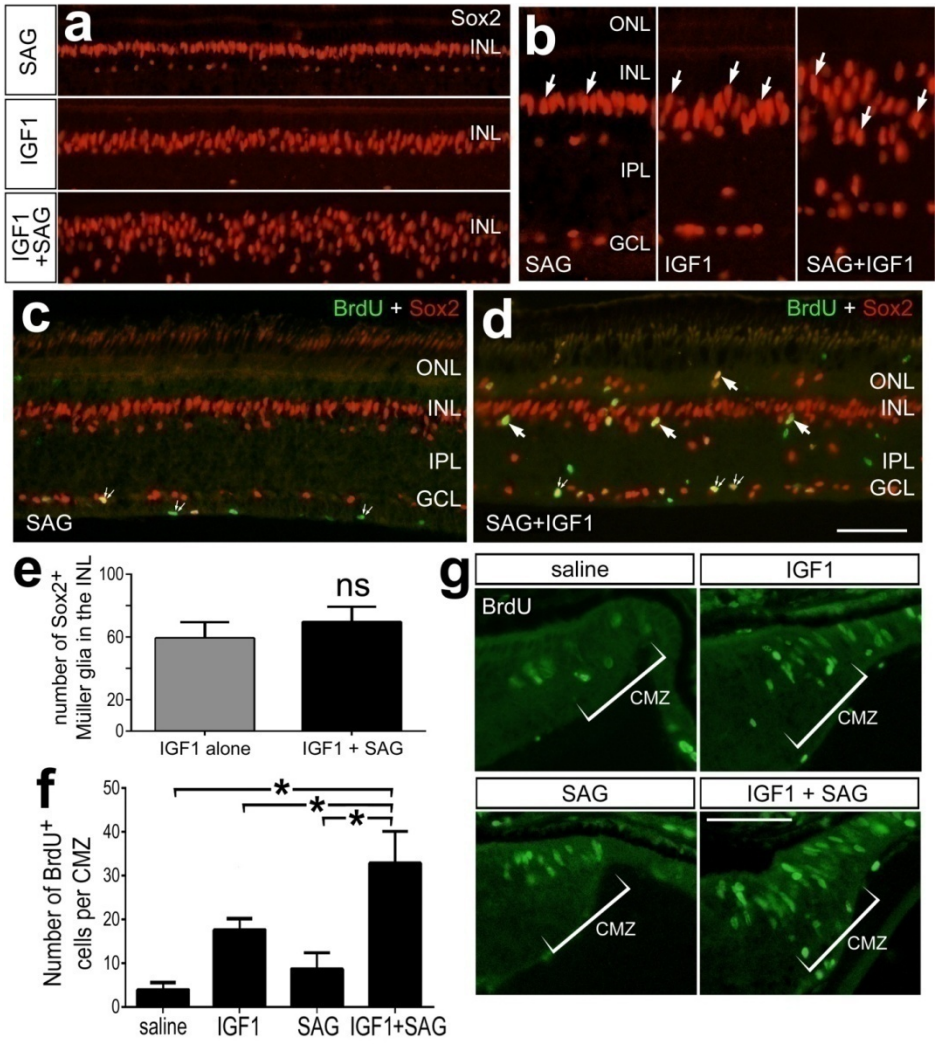

Figure 2.7. Müller glia/MGPCs express Pax6 and Klf4 in response to treatment with different growth factors and/or Hh-agonists/antagonists. Retinas were treated with consecutive daily doses of saline x4, SAG x4, SAG and IGF1 x4, FGF2 x3, FGF2 and SAG x3, FGF2 x4 or FGF2 and KAAD-cyclopamine x4. Retinas were immunolabeled for Pax6 (green) and Sox9 (red; **a**), or Klf4 (green) and TopAP (red; **b**). Arrows indicate the nuclei of Müller glia and/or MGPCs. Images were obtained by using confocal microscopy and projecting 4 optical sections. Therefore, there was some overlap of adjacent amacrine and glial nuclei through the Z-axis, indicated by small double-arrows. The calibration bar represents 50 µm.

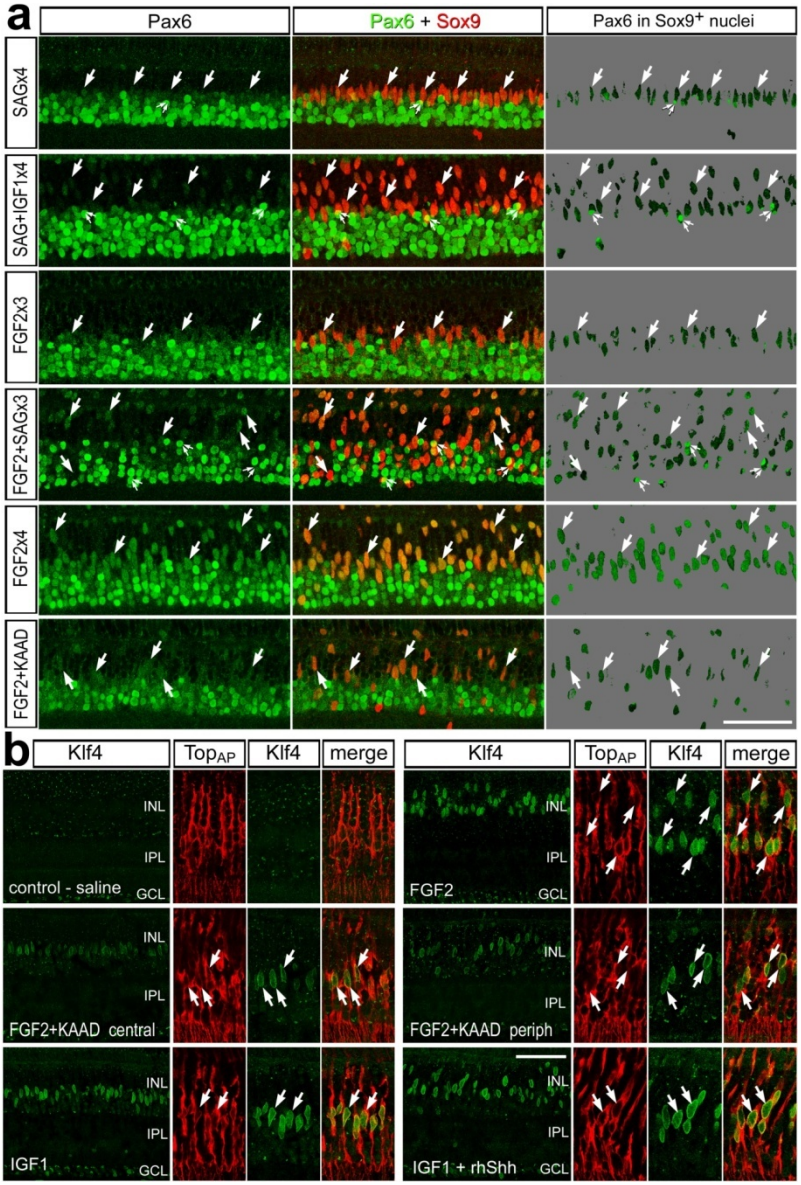

Figure 2.8.Schematic diagram illustrating the induction of MGPCs by NMDA- and FGF2-treatments, activation of MAPK-signaling, subsequent activation of Hh- and Notch-signaling, and sites of action of different compounds used in the study. Agonists (green), antagonists (red).

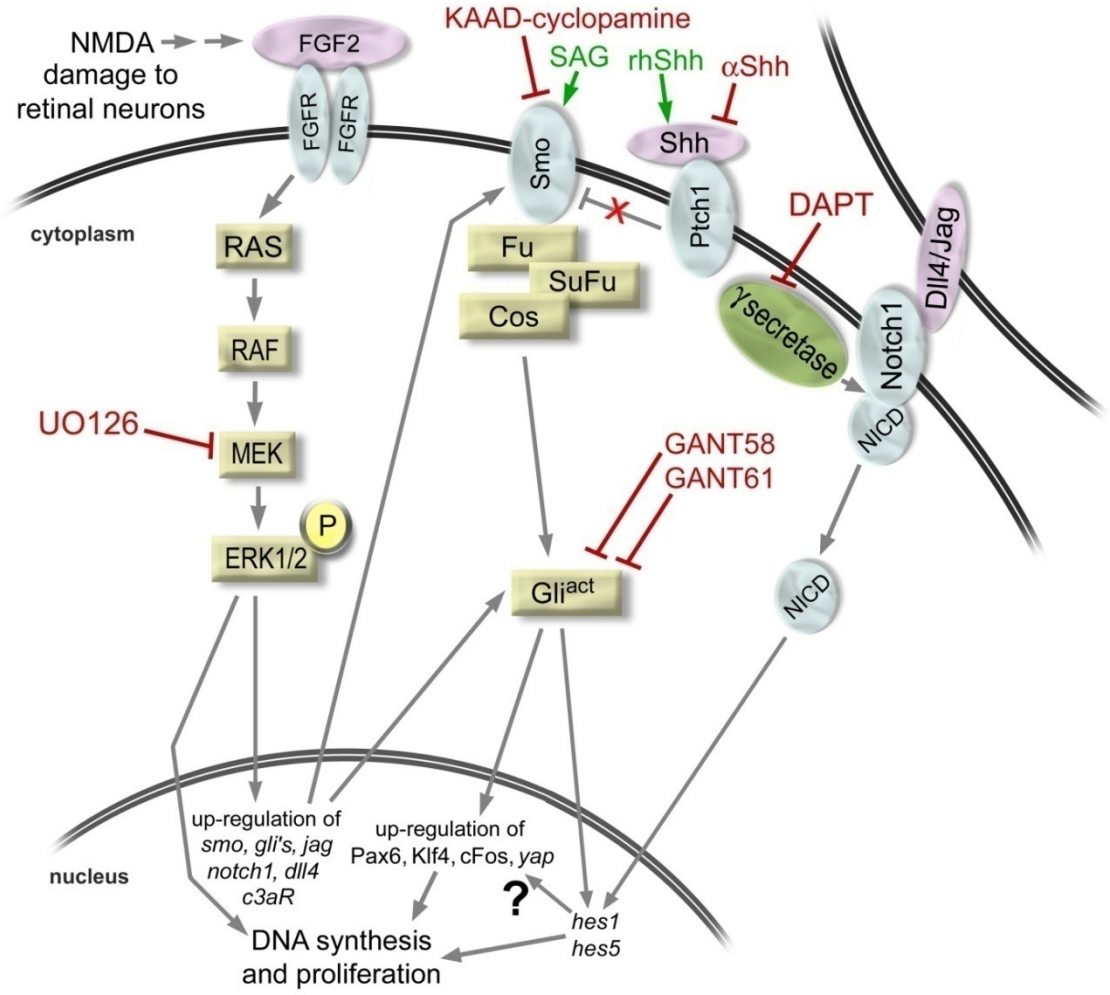

Table 2.1.List of primers and predicted product sizes for RT-PCR and qRT-PCR.

Gene	Forward 5'-3	Reverse 5'-3'	Product size
ascl1	AGGGAACCACGTTTATGCAG	TTATACAGGGCCTGGTGAGC	188
c3	TCCCCCATGAGGAATGGGAT	TAGTCCATGTCCCCAGGCT	73
c3aR	GCCTTTGCTCTGAAGTCCCT	CACTCGCATATGCCAACAGC	74
dhh	GTTTGGCTGAGCTACGGAGG	AGCCTGGAGTACCAATGCAC	153
gapdh	CATCCAAGGAGTGAGCCAAG	TGGAGGAAGAAATTGGAGGA	161
gli1	CTAGCGTTGACCTGCAGACG	ACAGGGTTTCGTGGGAGCTA	127
gli2	CGGTGTAGGCAGAGCTGATG	CCACAAGGCAGAAACACCAA	190
gli3	ATTTTTGGGGCAATGGACAG	TGAATGCCATCTCCAACCAG	209
hes1	CGCTGAAGAAGGATAGTTCG	GTCACTTCGTTCATGCACTC	175
hes5	GGAGAAGGAGTTCCAGAGAC	AATTGCAGAGCTTCTTTGAG	143
ihh	ACTCCCTCTCCAAGTCTCCC	GACAGGGACCGCAACAAGTA	1038
lin28b	GTGGCTAACTGCCCCCATAA	TTCCTCCCCCTTCTCTCAGG	112
notch1	GGCTGGTTATCATGGAGTTA	CATCCACATTGATCTCACAG	154
Shh-T7	AGGAGCCGTGAGTACCAATG	AGCTGACCCCGTTAGCCTAT	1084
pax2	GCCAGGCCTCATTGTAGGTT	CCAACTGGACAAGGCAGCTA	115
ptch1	AACGCATGGGCTAGAAGGAA	ATGCTTGCCTACGCCTGTTT	221
shh	CCACCGATCCCTAGCAAGAC	CTGTCTCCCGACCAAACTCC	86
smo	GGGTGGTTGCTCTTGATGGA	GACTCCGTCAGCGGTATCTG	152
sufu	AGCCCTCTTCGCAAAGTCTC	CTTCACGGAATCTACGGCGA	848
tnfα	AGCAGCGTTTGGGAGTGGC	GCAGATGGGGCAGGAAAGCCA	133
yap	CCCCCGATGACTTCCTGAAC	CCTTCCAGTGTCCCAAGGTC	145
lin28b	TTCCTCCCCCTTCTCTCAGG	GTGGCTAACTGCCCCCATAA	112

Table 2.2 Antibodies, working dilutions, host and source.

Antigen	Working dilution	Host	Clone or catalog number	Source
BrdU	1:200	rat	OBT00030S	AbDSerotec
BrdU	1:100	mouse	G3G4	Developmental Studies Hybridoma Bank (DSHB)
Brn3a (Pou4F1)	1:200	mouse	mab1585	Chemicon
calbindin	1:500	mouse	D-28K	Sigma-Aldrich
CD45	1:200	mouse	HIS-C7	Cedi Diagnostic
cFos	1:400	rabbit	K-25	Santa Cruz Immunochemicals
Egr1	1:1000	goat	AF2818	R&D Systems
GFAP	1:2000	rabbit	N1506	Dako
Klf4	1:50	rabbit	ARP38430	Aviva Systems Biology
neurofilament	1:50	mouse	RT97	DSHB
Nkx2.2	1:80	mouse	74.5A5	DSHB
p38 MAPK	1:400	rabbit	12F8	Cell Signaling Technologies
Pax6	1:50	mouse	PAX6	DSHB
PCNA	1:1000	mouse	M0879	Dako
pCREB	1:500	rabbit	87G3	Cell Signaling Technologies
pERK1/2	1:200	rabbit	137F5	Cell Signaling Technologies
pS6	1:750	rabbit	2211	Cell Signaling Technologies
Shh	1:2000	rabbit		Dr. T. Jessell, Columbia University
Sox2	1:1000	goat	Y-17	Santa Cruz Immunochemicals
Sox9	1:2000	mouse	AB5535	Chemicon
Top_{AP}	1:100	mouse	2M6	Dr. P. Linser University of Florida
transitin	1:80	mouse	EAP3	DSHB
vimentin	1:400	rabbit	H5	DSHB

Chapter 3

Jak/Stat signaling regulates the proliferation and neurogenic potential of Müller glia-derived progenitor cells in the avian retina

Abstract

Müller glia are capable of de-differentiating and proliferating to become Müller glia-derived progenitor cells (MGPCs) with the ability to regenerate retinal neurons. One of the cell-signaling pathways that drives the reprogramming of Müller glia into MGPCs in the zebrafish retina is the Jak/Stat-pathway. However, nothing is known about the influence of Jak/Stat-signaling during the formation of MGPCs in the retinas of warm-blooded vertebrates. Accordingly, we examined whether Jak/Stat-signaling influences the formation of MGPCs and differentiation of progeny in the avian retina. We found that Jak/Stat-signaling is activated in Müller glia in response to NMDA-induced retinal damage or by CNTF or FGF2 in the absence of retinal damage. Inhibition of gp130, Jak2, or Stat3 suppressed the formation of proliferating MGPCs in NMDA-damaged and FGF2-treated retinas. Additionally, CNTF combined with FGF2 enhanced the formation of proliferating MGPCs in the absence of retinal damage. In contrast to the zebrafish model, where activation of gp130/Jak/Stat is sufficient to drive neural regeneration from MGPCs, signaling through gp130 inhibits the neurogenic potential of MGPCs and

promotes glial differentiation. We conclude that gp130/Jak/Stat-signaling plays an important role in the network of pathways that drives the formation of proliferating MGPCs, however this pathway inhibits the neural differentiation of the progeny.

Introduction:

Müller glia in the retina can be reprogrammed into Müller glia-derived progenitor cells (MGPCs) with the potential to regenerate retinal neurons. The ability of Müller glia to produce MGPCs and regenerate neurons varies significantly between vertebrates. The MGPCs in the teleost fish have the ability to regenerate all types of neurons and restore visual function after injury (Lenkowski and Raymond 2014). By comparison, the MGPCs in avian retina have a limited regenerative response; although a large numbers of proliferating MGPCs are formed after damage, the neurogenic capacity of these cells is relatively low (reviewed by (Fischer 2005; Fischer and Bongini 2010)). The Müller glia in mammalian retina predominantly respond to injury by undergoing non-proliferative gliosis (Dyer and Cepko 2000). However, retinal damage followed by treatment with growth factors can stimulate the proliferation of relatively few MGPCs with a very limited neurogenic potential (Karl et al. 2008; Ooto et al. 2004).

The transition of Müller glia into MGPCs involves de-differentiation, acquisition of progenitor phenotype, proliferation, and neuronal differentiation of progeny. A complex network of cell-signaling pathways coordinates Müller glia-mediated retinal regeneration; these pathways are beginning to be uncovered in the zebrafish and avian model systems (Lenkowski et al. 2013; Nelson et al. 2012; Ramachandran et al. 2011;

Wan et al. 2014; Zhao et al. 2014). By comparison, the pathways that drive the formation of neurogenic MGPCs in the mammalian retina are poorly understood. Uncovering the mechanisms that control the formation of MGPCs across vertebrate classes is expected to guide strategies to increase the regenerative potential of MGPCs in higher vertebrates and potentially lead to treatments for diseases of the retina in humans.

In this study, we investigate how cell signaling through glycoprotein 130 (gp130)/Janus kinase/signal transducers (Jak/Stat) impacts the formation, proliferation, and differentiation of MGPCs in the chick retina. In the zebrafish retina, knockdown of Stat3 inhibits MGPC-formation in damaged retinas (Nelson et al. 2012), whereas activation of Jak/Stat-signaling is sufficient to induce Müller glia reprogramming in the absence of retinal damage (Kassen et al. 2009; Zhao et al. 2014). The formation of MGPCs in the zebrafish can be stimulated by insulin, Heparin-binding EGF-like growth factor (HB-EGF), or Insulin growth factor 1 (IGF1)+ Fibroblast growth factor 2 (FGF2), and, conversely, the formation of MGPCs can be suppressed by Jak/Stat pathway-inhibitors (Wan et al. 2014). Collectively, these data suggests that Jak/Stat-signaling represents an important "hub" in the network of signaling pathways that orchestrates the formation of MGPCs in the fish retina. By comparison, nothing is known about the involvement of Jak/Stat-signaling on the formation of MGPCs in the retinas of warm-blooded vertebrates.

Müller glia in the avian retina can undergo wide-spread de-differentiation and proliferation in response to retinal injury or growth factors, namely FGF2 (Fischer 2005; Fischer and Bongini 2010; Gallina et al. 2014a). However, the majority of the MGPCs

appear to remain undifferentiated, and approximately one-fifth of the progeny of MGPCs re-differentiate as glia (Fischer et al. 2002; Fischer and Reh 2001). One of the major obstacles in harnessing the regenerative potential of MGPCs is overcoming limited neuronal differentiation. During neural development, cell-signaling pathways, including Notch-, Bone Morphogenetic Protein (BMP)/Smad-, and Jak/Stat-signaling, are known to inhibit neurogenesis in favor of gliogenesis (Guillemot 2007). For example, Jak/Stat-signaling biases neural progenitor cells to a glial fate (Bonni et al. 1997). Additionally, inhibition of gp130 in the developing cortex increases neuronal differentiation at the expense of gliogenesis (Barnabe-Heider et al. 2005). Therefore, the purpose of this study was to investigate whether gp130/Jak/Stat-signaling influences the acquisition of progenitor phenotype, proliferation, and the neurogenic potential of MGPCs in the avian retina *in vivo*.

Methods and Materials:

Animals:

The use of animals in these experiments was in accordance with the guidelines established by the National Institutes of Health and the Ohio State University. All experiments were approved by the Institutional Animal Care and Use Committee at the Ohio State University. Newly hatched wild type leghorn chickens (*Gallus gallus domesticus*) were obtained from Meyer Hatchery (Polk, Ohio). Chicks were housed in a stainless steel brooder at about 25^{o}C and received water and Purina^{tm} chick starter *ad*

libitum.

Intraocular injections:

Chickens were anesthetized via inhalation of 2.5% isoflurane in oxygen and intraocular injections performed as described previously (Fischer et al. 1998). For all experiments, the right eyes of chicks were injected with the "test" compound and the contra-lateral left eyes were injected with vehicle as a control. Compounds were injected in 20µl sterile saline. Compounds included N-Methyl-D-aspartate (NMDA) (38.5 or 154 µg/dose; Sigma-Aldrich), FGF2 (250 ng/dose; R&D systems), Ciliary neurotrophic factor (CNTF) (300ng/dose; R&D systems), gp130 inhibitor sc144 (1µg/dose; Sigma-Aldrich), pan-Stat inhibitor Stattic (1µg/dose; Sigma-Aldrich), Jak inhibitor JSI-124 (2µg/dose; Sigma-Aldrich), Rapamycin (1µg/dose; Sigma-Aldrich), U0126 (1.7µg/dose; Calbiochem), clodronate-liposomes (500-2000ng; Sigma-Aldrich), Interleukin-6 (200ng/dose; R&D Systems) and insulin (800ng/dose; Sigma-Aldrich). To label proliferating cells, 2 µg of BrdU was added to the injection mixes. The paradigms describing the timeline of injections for control and treated eyes are included in each figure.

Quantitative Reverse transcriptase PCR:

RNA was extracted from retinas using the Trizol protocol (Invitrogen). Genomic DNA was removed from samples by following the DNA Free kit protocol from Ambion. cDNA was synthesized from the extracted mRNA using oligodT primers and

Superscript^{tm} III First Strand Synthesis System (Invitrogen). To insure that primers were not amplifying genomic DNA, identical reactions were ran without including reverse transcriptase. Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primer sequences, and their predicted product sizes and sequence information is included in Table 3.1. Standard protocols were used for PCR reactions, using Platinum^{tm}Taq (Invitrogen) and an Eppendorf thermal cycler. To verify predicted product sizes PCR products were run on an agarose gel. All experiments include sample sizes of $n \geq 4$, per time point, and 3 replicas per sample. Significance of difference (*$p<0.05$, **$p<0.01$) was determined by using a Mann-Whitney U test.

Tissue fixation, sectioning and immunolabeling:

Ocular tissues were fixed, sectioned, and labeled similar to previous descriptions (Fischer et al. 2008; Fischer et al. 2009b). Sources of antibodies and working dilutions are included in Table 3.2. We ruled out non-specific labeling by labeling sections with secondary antibodies alone and observing no fluorescence. Secondary antibodies were diluted 1:1000 in PBS and 0.2% Triton X-100. Secondary antibodies included goat-anti-rabbit-Alexa488/568/647, goat anti-rat-Alexa488, goat-anti-mouse-Alexa488/568/647 and donkey-anti-goat-Alexa488/568 (Invitrogen).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL):

To identify dying cells that had fragmented DNA, we followed the manafacturer's instructions using the *In Situ* Cell Death Kit (TMR red; Roche Applied Science).

Clodronate-Liposomes:

Clodronate-liposomes were made as previously described (Fischer et al. 2014; Van Rooijen 1989; Zelinka et al. 2012). It has been estimated that the liposomes encapsulate roughly 1% of the clodronate, yielding approximately 8mg/mL (Van Rooijen and Sanders 1994). The random nature of the combination of clodronate and liposomes prevent exact quantification. Therefore, we applied doses to a level that ensured over 99% of microglia were ablated.

Microscopy, photography and cell counts:

Cell counts were performed on representative images and consistently made from identical regions of the retina to avoid region-specific effects. Central retina was determined to be 20^{o} of the posterior pole of the eye, with a radius of approximately 2 mm. Peripheral retina was designated as 3 mm annual radius from the peripheral retinal margin. The chick retina is approximately 13 mm across.

Quantification of immunofluorescence:

ImagPro 6.02 (Media Cybernetics) was used to quantify immunofluorescence as previously described (Fischer et al. 2009a; Fischer et al. 2009b; Fischer et al. 2010; Ghai et al. 2009). Microscope illumination and camera settings were held constant to obtain images. Immunofluorescence was measured for the total area of select regions with pixel intensity values 75 or above (0 = minimum and 255 = maximum). The fluorescence

intensity, density sum and mean area were calculated for the pixels within regions above a set threshold.

Since pStat3 was present exclusively in the nuclei of Müller glia (see Fig. 1b), we were able to measure pStat3-immunofluorescence without excluding labeling in retinal neurons. The percentage change in density sum of pStat3 in Figures 2a and 2b was calculated as follows. Fixed areas of the INL were cropped from images of control and treated retinas. By using ImagePro 6.02, immunofluorescence was measured as the summation of pixel values (density sum) above threshold (70 in the green channel) within each cropped area. For each individual the percent change for treated minus control was determined and then averaged across 4 individuals.

Statistics

Significance of difference between two treatment groups accounting for inter-individual variability (means of treated-control values) was determined by using a two-tailed, paired t-test. Significance of difference (*$p<0.05$, **$p<0.01$, ns – not significant) was determined between two treatment groups by using a two-tailed, unpaired t-test or a Mann-Whitney U test. GraphPad Prism 6 was used for statistical analyses. All experiments contained sample sizes between 4 and 8 animals.

Results:

Retinal damage induces Jak/Stat-signaling in Müller glia:

Stat3 is the primary effecter of Jak/Stat-signaling and phosphorylation of Stat3

results in translocation to the nucleus and transcriptional regulation (Li 2008). We used immunofluorescence to assay for phosphorylated Stat3 (pStat3) in retinal sections. Levels of pStat3 were below levels of detection in saline-treated retinas (Fig. 3.1a). We examined whether pStat3 was induced by retinal damage caused by NMDA, which is known to destroy inner retinal neurons and stimulate the generation of MGPCs (Fischer and Reh 2001). In NMDA-damaged retinas there was an accumulation of pStat3 in the INL, which was up-regulated within 2hrs and was sustained through 72hrs after treatment (Fig 3.1a). Immunolabeling for pStat3 and Top_{AP}, a marker specific to avian Müller glia (Ochrietor et al. 2010), revealed that pStat3 is selectively up- regulated in Müller glia (Fig 3.1b). This suggests that Jak/Stat-signaling is active in Müller glia shortly after NMDA-treatment and that Jak/Stat-signaling remains active during the reprogramming of Müller glia into proliferating progenitors.

CNTF activates cell-signaling through the Jak/Stat pathway in the retina (Wen et al. 2012). Activation of this pathway requires that the CNTF-receptor complexes with the gp130 co-receptor (Stahl and Yancopoulos 1994). We used quantitative RT-PCR to measure relative levels of CNTF receptor (*cntfr)* and *gp130* following NMDA-treatment. *cntfr* was rapidly up-regulated at 4hrs after NMDA-treatment, and was down-regulated at 24, 48, and 72hrs after treatment (Fig 3.1c). *gp130*was down-regulated at 4 hours after NMDA treatment, up-regulated at 24hrs, down-regulated at 48hrs, and returned to control levels at 72hrs after NMDA-treatment (Fig 3.1d). These data suggest that Jak/Stat-signaling is dynamically regulated after an acute injury, when the de-differentiation and

proliferation of MGPCs is known to occur (Fischer and Reh 2001).

Inhibition of gp130/Jak/Stat-signaling suppresses the formation of MGPCs in damaged retinas:

We tested whether inhibition of gp130, with the small molecule inhibitor sc144, impacts the formation of MGPCs in NMDA-damaged retinas. sc144 binds to gp130 to induce conformational changes that abrogate initiation of signaling (Xu et al. 2013). Injection of sc144 selectively inhibits the up-regulation of pStat3 in Müller glia that occurs shortly after NMDA-treatment (Fig. 3.2a,b). Following NMDA-treatment, injection of sc144 significantly reduced the number of proliferating MGPCs (Fig 3.2c,d). This effect was specific to Müller glia as the proliferation of CD45-positive microglia/macrophage and non-astrocytic inner retinal glial (NIRG) cells were unaffected by treatment with sc144 (Fig. 3.2c-f). NIRG cells are a unique type of glial cell in the avian retina (Fischer et al. 2010; Rompani and Cepko 2010). Treatment with sc144 did not affect cell death; numbers of TUNEL-positive cells were not significantly different between treated (49.1 ± 24.8) and control (38.9 ± 33.9, n=8, p=0.57) retinas. These results suggest that signaling through gp130 promotes the proliferation of MGPCs in NMDA-damaged retinas.

Since inhibition of gp130 attenuated the formation of MGPCs in damaged retinas, we examined whether inhibition of the downstream kinase (JAK2) and the transcriptional effector (Stat3) influenced the formation of MGPCs. To test this, we utilized JSI-124, which inhibits the phosphorylation of JAK2 (Blaskovich et al. 2003), and Stattic, a small

molecule that targets the SH2 domain of Stat3 to inhibit activation and nuclear translocation (Schust et al. 2006). We found that intraocular application of either JSI-124 or Stattic significantly reduced the number of proliferating MGPCs (Fig 3.2d). Similar to the effects of sc144, JSI-124 and Stattic had no effect on the proliferation of microglia or NIRG cells proliferation (Fig 3.2e,f). JSI-124 had no effect on cell death as assayed by TUNEL (treated 37.3 ±32.4 vs. control 30.5±13.7, n=6, p=0.64). Similarly, Stattic had no effect on cell death (treated 62 .0 ±51.7 vs. control 68.5 ±48.4, n=8, p=0.77).

To better understand the molecular mechanisms underlying the effects of gp130-inhibition on the proliferation of MGPCs, we probed for changes in the expression of genes known to influence retinal progenitors and/or MGPCs. By using qRT-PCR, we found that inhibition of gp130 in the NMDA damaged retina resulted in significant decreases in levels of *notch1* and *dll4*, whereas levels of *hes5*, *ascl1a,* and *gli2* were unaffected (Fig.3.2g). Notch-signaling (and readouts of signaling *notch1, dll4* and *hes5*), Hedgehog-signaling (and a readout of signaling *gli2*), and the transcription factor *ascl1a* are known to be up-regulated and are necessary for the formation of MGPCs in damaged retinas (reviewed by (Gallina et al. 2014a)).

Retinal damage is known to result in reactive gliosis wherein Müller glia become hypertrophic and up-regulate the intermediate filament Glial Fibrillary Acidic Protein (GFAP) (Bringmann et al. 2009). Signaling through gp130/Jak/Stat-signaling is required for gliotic phenotypes in Müller glia in the rodent retina (Kirsch et al. 2010). Inhibition of gp130 after NMDA-treatment suppressed the up-regulation of GFAP in Müller glia (Fig 3.3a), suggesting that signaling through gp130 induces gliotic phenotypes in Müller glia

in the avian retina. By comparison, inhibition of gp130 had no significant effects on the levels of CD45-immunofluorescence, area, and density sum, of microglia/macrophages (Fig. 3.3c and not shown).

Intraocular injections of CNTF are known to induce GFAP-expression in Müller glia in chick and rodent retinas (Fischer et al. 2004a; van Adel et al. 2005; Wang et al. 2011). CNTF activates signaling through CNTFR which complexes with gp130 to initiate signaling (Stahl and Yancopoulos 1994). To confirm the specificity of the gp130 inhibitor, we examined whether GFAP-expression in CNTF-treated Müller glia was influenced by sc144. Indeed, sc144 significantly reduced CNTF-induced GFAP-expression in Müller glia (Fig. 3.3d-e).

Jak/Stat cross-talks with different signaling pathways in Müller glia:

To better understand the network of signaling pathways that influence Müller glial and MGPCs we examined the activation of signaling by growth factors that are known to influence the formation of MGPCs. Injection of CNTF resulted in a rapid (<4 hrs) and robust induction of pStat3 in TOP_{AP}-positive Müller glia (Figs. 3.4a). In addition, we found that CNTF up-regulated pS6, a read-out of Mechanistic Target Of Rapamycin (mTor)-signaling, in Sox2-positive Müller glia (Fig 3.4b). Similarly, we observed a modest increase in pERK-immunoreactivity in Müller glia in CNTF-treated retinas (Fig 3.4c). This is consistent with reports in the rodent retina where CNTF activates Mitogen-Activated Protein Kinase (MAPK)-signaling in the Müller glia (Rhee et al. 2013). Insulin, which is known to primarily activate signaling through the PI3K/Akt

pathway (Duarte et al. 2012) rapidly induced the accumulation of pStat3 in the nuclei of Müller glia (Fig 3.4d). Intraocular injection of FGF2, which is known to activate the MAPK-pathway and stimulate the formation of MGPCs (Fischer et al. 2009b; Fischer et al. 2014) resulted in a rapid induction of pStat3 in the nuclei of Müller glia (Fig. 3.4e). The FGF2-induced up-regulation of pStat3 was blocked by the MEK inhibitor UO126 (Figs.3.4e,f), suggesting cross-talk between MAPK- and Stat-signaling downstream of MEK. The UO126 also blocked FGF2-mediated up-regulation of pERK1/2 in the Müller (not shown). These data suggests that CNTF, FGF2 and insulin activate Jak/Stat-signaling and that this pathway may be coordinated with MAPK- and mTor-pathways in Müller glia.

Activation of Jak/Stat-signaling combined with MAPK-signaling stimulates the formation of MGPCs:

CNTF is known to be neuroprotective after excitotoxic damage (Fischer et al. 2004b), and sufficient levels of cell death are required to drive the formation of MGPCs (Fischer and Reh 2001). The neuroprotective effects of CNTF confounded investigation into whether CNTF stimulates the proliferation of MGPCs in damaged retinas. Therefore, we tested whether consecutive daily injections of CNTF were sufficient to induce the formation of MGPCs in the absence of retinal damage (Fischer et al. 2014). Four consecutive daily doses of CNTF failed to stimulate Müller glial proliferation (data not shown). Unlike treatment with CNTF, four consecutive injections of FGF2 are known to be sufficient to stimulate the proliferation of MGPCs in the absence of

damage(Fischer et al. 2014). Since a single injection of FGF2 induced pStat3 in Müller glia (Fig. 3.4), we expected that consecutive daily injections of FGF2 would activate a network of pathways that included gp130 and CNTFR. We found that 3 consecutive doses of FGF2 significantly increased retinal levels of *gp130*, *cntfr*, and *cntf* (Fig 3.5a). Thus, we tested whether co-application of CNTF with FGF2 influenced the proliferation of MGPCs. We found that CNTF combined with FGF2 resulted a significant increase in MGPC-proliferation compared to retinas treated with FGF2 alone (Fig 3.5b-c). This combination failed to influence the proliferation of NIRG cells or microglia/macrophage (Fig 3.5c).

mTor-signaling is downstream and necessary for the effects of glucocorticoid-, Hedgehog-, and Wnt/β-catenin-signaling on the proliferation of MGPCs in the avian retina (Zelinka et al. 2016). Therefore, we tested whether mTor activity is necessary for the mitogenic effects of the combination of FGF2 and CNTF. In retinas treated with FGF2 and CNTF, rapamycin potently inhibited the formation of proliferating MGPCs (Fig 3.5d). MAPK-signaling has also been shown to be necessary for the formation of MGPCs, as well as for the mitogenic effects of Hedgehog-agonists on MGPCs (Fischer et al. 2009a). Thus, we tested whether inhibition of MAPK with U0126, a small molecule inhibitor of MEK, influenced the formation of MGPCs in retinas treated with FGF2 and CNTF. We found that treatment with U0126 significantly inhibited the formation of proliferating MGPCs in retinas treated with FGF2 and CNTF (Fig 3.5e).

In retinas treated with the combination of FGF2 and CNTF there was a significant increase in the levels of CD45 microglia/macrophage, suggesting increased reactivity,

compared to that seen in retinas treated with FGF2 alone (Fig. 3.5f,g). Since reactive microglia/macrophage are known to promote the formation of MGPCs (Fischer et al. 2014), the increased microglial reactivity may have contribute to the increased proliferation of MGPCs seen in retinas treated with FGF2 and CNTF. Therefore, we selectively ablated the microglia/macrophage in the retina with clodronate liposomes (Fischer et al. 2014) and tested whether FGF2 and CNTF influenced the formation of MGPCs. We found that in the absence of reactive microglia, there were significantly fewer proliferating MGPCs in retinas treated with FGF2 and CNTF (Fig. 3.5h-i). Thus, the increased proliferation of MGPCs seen in retinas treated with FGF2 and CNTF may, in part, result from signals provided by reactive microglia/macrophage.

FGF2-induced MGPC-formation requires gp130/Stat3-signaling:

Treatment with FGF2 alone is sufficient to induce the formation of MGPCs in the uninjured avian retina (Fischer et al. 2014). FGF2-induced formation of MGPCs is influenced by glucocorticoid-signaling (Gallina et al. 2014b), Hedgehog-signaling (Todd and Fischer 2015), Wnt/β-catenin-signaling (Gallina et al. 2015) and mTor-signaling (Zelinka et al. 2016). Accordingly, we investigated whether gp130- and Stat-signaling are among the network of pathways that drive the formation of MGPCs in FGF2-treated retinas. We found that inhibition of gp130 with sc144 and inhibition of Stat3 with Stattic significantly reduced numbers of proliferating MGPCs in FGF2-treated retinas (Fig. 3.6a-c).

FGF2-treatment induces pERK, p38 MAPK, pCREB, Egr1, and cFos specifically in Müller glia (Fischer et al. 2009b). We found that levels of pERK, p38 MAPK, pCREB, and Egr1 were not affected by sc144 in FGF2-treated retinas (not shown). However, there was a significant decrease in the levels of cFos in the nuclei of Müller glia treated with FGF2 and sc144 compared to levels seen in the nuclei of Müller glia treated with FGF2 alone (Fig. 3.6d). Inhibition of gp130 in FGF2-treated retinas had no effect on the up-regulation of nuclear β-catenin, Pax6, or Klf4 in Müller glia/MGPCs (data not shown), suggesting that the acquisition of a progenitor-like phenotype by Müller glia was not impaired by sc144. In retinas treated with FGF2 and sc144 there was a decrease in the reactivity of microglia/macrophages compared to that seen in retinas treated with FGF2 (Figs. 3.6f-g). By comparison, the reactivity of microglia/macrophages in FGF2-treated retinas was not affected by Stattic (data not shown). Taken together, these data suggest that FGF2 initiates a signaling network that includes gp130/Stat3- signaling that drives the formation of proliferating MGPCs in the absence of retinal damage.

Inhibition of gp130 biases the fate of MGPC-progeny towards neuronal differentiation at the expense of glial differentiation:

In the avian retina, the majority of MGPCs remain undifferentiated or re-differentiate as Müller glia (Fischer and Reh 2001). This suggests that the mature avian retina provides a gliogenic environment and/or the MGPCs have a cell-intrinsic bias away from neuronal differentiation. gp130/Stat3-signaling has been to promote gliogenesis in the developing nervous system (Guillemot 2007). Furthermore, inhibition

of gp130 in the developing cortex decreases gliogenesis while increasing neurogenesis (Barnabe-Heider et al. 2005). Accordingly, we tested whether the differentiation of progeny of MGPCs are influenced by inhibition of gp130. To test this, we applied NMDA, waited two days to permit the de-differentiation of Müller glia and proliferation of MGPCs, and, thereafter applied doses of gp130-inhibitor. We found a significant decrease in pStat3 in NMDA-damaged retinas treated with sc144 at 3 days after damage (Fig 3.7a). In retinas treated with gp130-inhibitor, we found a significant increase in neuronal differentiation. Compared to control retinas, there was nearly an 80% increase in the percentage of BrdU$^-$positive cells labeled for the neuronal marker HuC/D in the INL of gp130-inhibited retinas (Figs. 3.7b,d). Coincidently, we observed a significant decrease in glial differentiation in sc144-treated retinas. The percentage of BrdU$^-$positive cells labeled for the glial marker glutamine synthetase was decreased by more than 30% in retinas treated with gp130 inhibitor (Fig. 3.7c,e). To provide insight into how inhibition of gp130 might influence neuronal specification from MGPCs, we probed for retinal expression of components of the Notch pathway. We observed decreases in retinal levels of *hes5 and notch1* (Fig. 3.7g). Unexpectedly, we also found a decrease in the pro-neural bHLH factor *ascl1* (Fig. 3.7g). This factor is known to be important for the formation of MGPCs in the zebrafish retina (Ramachandran et al. 2010) and induces the progenitor-like properties of MGPCs in the mouse retina (Pollak et al. 2013; Ueki et al. 2015)

To test whether decreases in *hes5* and *notch1* might underlie increases in neuronal differentiation in damaged retinas treated with sc144 we applied DAPT, a small molecule

Notch-inhibitor. We reasoned that if decreases in *hes5* and *notch1* are downstream of suppressed gp130-signaling, then inhibition of Notch should not further increase neuronal differentiation. Inhibition of Notch is known to increase the neurogenic capacity of MGPCs in the chick retina (Hayes et al. 2007). We found that inhibition of gp130 combined with inhibition of Notch further increased the neuronal differentiation of progeny produced by MGPCs (Fig.3.7f). This finding suggests that signaling through gp130 and Notch may independently influence the neurogenic capacity of MGPCs.

Discussion:

Delineation of the network of cell-signaling pathways that regulate the transition of mature Müller glia to neurogenic MGPCs is imperative to harnessing the regenerative capacity of the retina. Our data suggest that gp130/Jak/Stat-signaling plays an important role in the reprogramming of Müller glia into neurogenic MGPCs in the avian retina. In response to retinal damage, Jak/Stat-signaling is predominantly active in Müller glia and inhibition of gp130, Jak kinases, and Stat transcription factors each reduce the proliferating of MGPCs in damaged retinas. Exogenous CNTF selectively activates Jak/Stat, MAPK, and mTor-signaling in Müller glia, and CNTF augments FGF2-treatment to drive the formation of MGPCs in undamaged retinas. We find that signaling through gp130 and Stat3 is required for FGF2 to stimulate MGPC-formation in undamaged retinas, implicating the recruitment of gp130/Stat3-signaling into the network of pathways active by FGF2-treatment that drives the formation of MGPCs. In addition to

promoting the proliferation of MGPCs, we find that gp130-signaling inhibits the neurogenic capacity of MGPCs.

It is likely that gp130/Stat3-signaling influences Müller glia directly rather than secondarily through reactive microglia/macrophages. In response to damage or exogenous growth factors, pStat3 was detected exclusively in the Müller glia and not in microglia/macrophages. However, damage and exogenous growth factors activated the reactivity of the microglia, consistent with previous reports (Fischer et al. 2014). Reactive microglia/macrophages are known to promote the formation of proliferating MGPCs (Fischer et al. 2014). Although inhibition of gp130 suppressed the reactivity of microglia/macrophage in FGF2-treated retinas, inhibition of gp130 had no detectable effect upon the reactivity of microglia/macrophage in damaged retinas. The FGF2-treatment elicits no detectable damage to retinal neurons (Fischer et al. 2002), whereas NMDA-treatment elicits wide-spread damage and death to inner retinal neurons (Fischer et al. 1998). It is expected that there are many different factors and cell-signaling pathways are activated in damaged retinas that result in the activation of microglial reactivity (Vecino et al. 2016). Thus, we propose that inhibition of gp130-signaling in damaged retinas failed to influence microglial reactivity because gp130-independent pathways stimulated a reactive phenotype. Further, we cannot exclude the possibility that Jak/Stat-signaling,perhaps through Stat1 or Stat2, in the retinal microglia directly influences the reactivity of the microglia or indirectly influences the formation of MGPCs.

In the retina, Jak/Stat-signaling is primarily manifested in the Müller glia, but the effects on cellular phenotype and function appears to vary greatly between vertebrates. In the rat retina, pStat3 is induced by intravitreal injections of a CNTF-analog, light damage, or a retinal needle poke (Peterson et al. 2000). In the mouse retina, CNTF induces pStat3 and pERK, and this effect is abolished in a Müller glia by knockout of gp130 (Rhee et al. 2013). In addition, LIF initiates cell-signaling through gp130 and rapidly activates pStat3 and pERK specifically in Müller glia in the mouse retina (Ueki et al. 2008). Similarly, in the chick retina, we find that pStat3 is rapidly and specifically up-regulated in Müller glia in response to retinal damage, CNTF, FGF2 or insulin. These findings suggest that across vertebrate species, Müller glia are the primary site for Jak/Stat-signaling within the retina, and that this pathway can be quickly activated by damage or growth factors. In the fish retina, knockdown of Stat3 inhibits the formation of proliferating MGPCs in damaged retinas (Nelson et al. 2012). Conversely, activation of Jak/Stat via CNTF is sufficient to induce reprogramming of Müller glia in the undamaged retina, and this effect depends on the gp130 receptor (Kassen et al. 2009; Zhao et al. 2014). In the avian retina, we failed to induce Müller glia reprogramming by activation of Jak/Stat-signaling with CNTF. However, similar to the zebrafish retina, Jak/Stat-signaling in the avian retina is an important player in the network of pathways that drives the proliferation of MGPCs following retinal damage or FGF2-treatment. Unlike in the zebrafish, Jak/Stat-signaling in the avian retina inhibits neuronal differentiation of the progeny produced by MGPCs. The influence of Jak/Stat-signaling on the formation of neurogenic MGPCs in the mammalian retina remains unexplored.

Previous studies have reported that CNTF-treatment and activation of Jak/Stat-signaling induces gliotic phenotypes in Müller glia and promotes neuronal survival in the retinas of fish, birds and rodents (Fischer et al. 2004b; Jiang et al. 2014; Kassen et al. 2009; Rhee et al. 2013; Tassoni et al. 2015). We found that inhibition of gp130 decreased GFAP in Müller glia, whereas cell survival was unaffected when signaling through gp130/Jak/Stat was inhibited following retinal damage. These findings have several implications for CNTF and Jak/Stat-signaling in the avian retina; (1) the survival promoting effects of CNTF and Jak/Stat are potent when applied before, but not after an acute retinal injury, (2) the survival promoting effects of CNTF may be elicited through the Müller glia by cell-signaling pathways in addition to Jak/Stat, and (3) acquisition of gliotic phenotype may be independent of the acquisition of progenitor phenotype. It remains uncertain whether reactive Müller glia are more or less likely to be reprogrammed into progenitor cells. In the zebrafish retina, all Müller glia exhibit reactive properties prior to and during cell cycle re-entry (Thomas et al. 2015). In $p27^{Kip1}$ null mouse retinas, Müller glia up-regulate GFAP and re-enter the cell cycle (Dyer and Cepko 2000; Levine et al. 2000). However, when Lhx2, a stem cell transcription factor, is deleted in Müller glia, these cells exhibit a gliotic phenotype, yet no proliferation occurs (de Melo et al. 2012). In NMDA-damaged chick retina, the Müller glia that express high levels of GFAP tend to remain post-mitotic and do not form proliferating progenitor-like cells(Fischer and Reh 2001). It remains uncertain whether increased expression of GFAP is correlated with or independent of reprogramming of Müller glia into progenitor-like cells.

In the avian retina, consecutive daily treatments of FGF2 induce the formation of proliferating MGPCs by activating a network of signaling pathways. This network is known to include MAPK- (Fischer et al. 2009b), Notch- (Ghai et al. 2010), glucocorticoid- (Gallina et al. 2014b), Wnt/β-catenin- (Gallina et al. 2015), and Hedgehog-signaling (Todd and Fischer 2015). Although MAPK-, Notch-, and Hedgehog-signaling act cooperatively to promote the formation of MGPCs (Todd and Fischer 2015), MAPK-signaling in Müller glia is disrupted by glucocorticoids and the formation of MGPCs is suppressed (Gallina et al. 2014b). These pathways may require activation of mTor for the formation of MGPCs in NMDA-damaged and FGF2-treated retinas (Gallina et al. 2015). By comparison, cross-talk between Jak/Stat- and MAPK-signaling occurs in the fish and rodent retina (Kassen et al. 2009; Peterson et al. 2000; Wahlin et al. 2000). In the zebrafish retina, HB-EGF, insulin, and the combination of FGF and IGF-1 are sufficient to induce proliferating MGPCs and each of these combinations leads to an increase in pStat3 in Müller glia (Wan et al. 2014). Furthermore, inhibition of Jak/Stat-signaling mitigates the ability of HB-EGF, insulin, and the combination of FGF and IGF-1 to stimulate the formation of MGPCs (Wan et al. 2014). Our findings regarding the network of signaling pathways that collectively drive the proliferation of avian MGPCs are strikingly similar to the findings described for the network of pathways that drives the proliferation of zebrafish MGPCs.

Despite the large number of proliferating MGPCs that form in damaged avian retina, the vast majority of these cells remain in an undifferentiated progenitor-like state (Fischer and Reh 2001). The MGPCs that go on to differentiate exhibit a bias towards

gliogenesis, with only a small minority differentiating as neuronal cells (Fischer and Reh 2003). Several different signaling pathways may act to suppress the neurogenic potential of MGPCs. Notch-signaling is known to promote glial fate during retinal development (Furukawa et al. 2000), and Notch suppresses neuronal differentiation from MGPCs in damaged chick retinas (Hayes et al. 2007). Similarly, glucocorticoid-signaling stimulates glial maturation during late stages of retinal development (Moscona and Linser 1983), and inhibition of this pathway increases neurogenesis, at the expense of gliogenesis, from MGPCs in damaged retinas (Gallina et al. 2014b). We report here that inhibition of gp130 increases neurogenesis at the expense of gliogenesis from MGPCs. Our findings are consistent with those of developmental studies wherein gp130/Jak/Stat-signaling plays important roles promoting the differentiation of astrocytes (Barnabe-Heider et al. 2005; Bonni et al. 1997; He et al. 2005; Nakashima et al. 1999). We found that inhibition of gp130 decreased *notch1* and the down-stream effector *hes5.* Decreased Notch-signaling may, in part, underlie how gp130-inhibition increases neurogenesis, given that Notch-signaling is known to maintain cells in an undifferentiated state or to promote a glial identity (Wang and Barres 2000). Interestingly, we find that neurogenesis from MGPCs can be further enhanced by combining inhibition of Notch and gp130, despite diminished levels of *notch1* and *hes5* resulting from gp130-inhibition. This finding suggests that signaling through gp130 may facilitate Notch-signaling, and combined signaling through these pathways suppress neurogenesis from MGPCs. The increase in neural differentiation in retinas treated with gp130-inhibitor cannot be explained by changes in the expression of *ascl1a*; increases in *ascl1a* are expected to accompany

increases in neurogenesis from MGPCs. Given the dynamic nature of Ascl1a-expression (Imayoshi et al. 2013), we cannot exclude the possibility that levels of *ascl1a* were increased early during the gp130-treatment paradigm, or that neurogenic bHLH factors other than Ascl1a influenced neuronal differentiation of the progeny produced by MGPCs.

Conclusions:

We find that Jak/Stat-signaling is an important signaling "hub" during the de-differentiation of Müller glia, proliferation of MGPCs, and specification of progeny produced by MGPCs in the avian retina. Jak/Stat-signaling needs to be active for Müller glia to transition into proliferating MGPCs, but in the later stages of this process Jak/Stat-signaling is an obstacle to neural differentiation. We conclude that within Müller glia there is significant cross-talk between Jak/Stat-, MAPK- and mTor-signaling, and collectively these pathways form a network that drives the proliferation of MGPCs. Therefore, this pathway represents an important therapeutic target to bolster the formation of proliferating neurogenic MGPCs to promote retinal regeneration.

Figure 3.1. Jak/Stat-signaling is rapidly activated in Müller glia after NMDA-treatment. Saline or NMDA was injected into the eyes of P7 chicks. Retinas were obtained from eyes injected with either saline or 1 µmol NMDA and harvested 2hrs, 48hrs or 72hrs later (**a**). Retinal sections were obtain from eyes at 2hrs after NMDA-treatment and labeled with antibodies to pStat3 (green) or TopAp (red) (**b**). Arrows indicate the nuclei of Müller glia labeled for pStat3. The calibration bar represents 50 µm. qRT-PCR was used to measure relative levels of *cntfr and gp130* normalized to *gapdh* (**c,d**). cDNA was generated from that were treated with saline (control) or NMDA (treated), and harvested at 4hrs, 1 day, 2 days or 3 days later.. Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer.

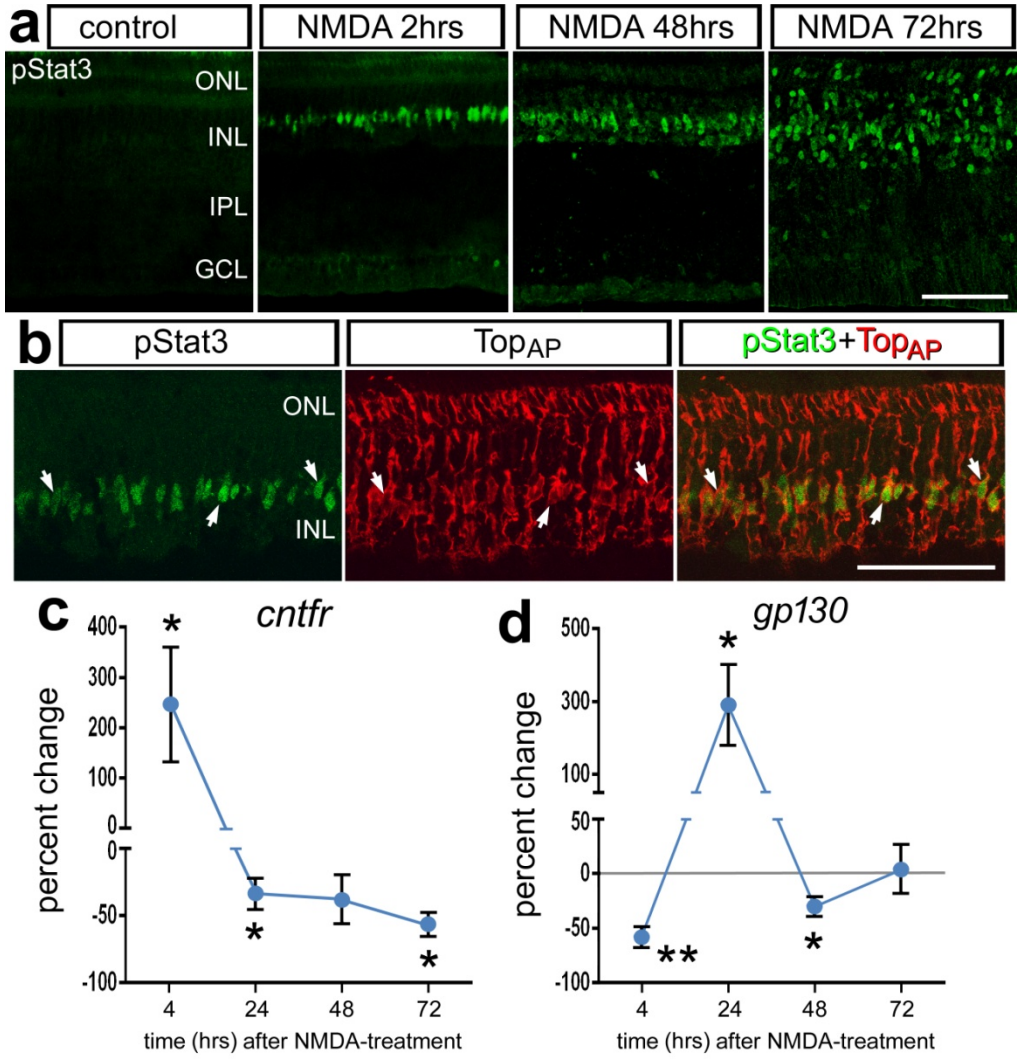

Figure 3.2. The formation of proliferating MGPCs is suppressed by inhibition of Jak/Stat signaling. Eyes were injected with 500 nmol NMDA ± inhibitor at P6, BrdU ± inhibitor at P7 and P8, and retinas harvested at P9. Sections of the retinas were labeled with antibodies to pStat3 (green; **a**), and BrdU (green) and Sox9 (red; **c**), and Nkx2.2 (red) and BrdU (green; **e**) and CD45 (red) and BrdU (green; **f**). Panel **a** includes regions of pixels or ROIs (regions of interest) above threshold outlined in magenta. The histogram in **b** illustrates the percent change in density sum for pStat3 (SD; n=4) in the nuclei of Müller glia treated with NMDA versus NMDA + sc144. Arrows indicate $BrdU^+/Sox9^+$ MGPCs, and small double-arrows indicate $BrdU^+/Sox9^-$ microglia. The scale bar in panel **c** represents 50 µm and in panels **e** &**f** 10 µm. Histograms illustrate the mean (±SD) numbers of proliferating MGPCs (**d**), NIRG cells (**e**), and microglia/macrophages (**f**). Mean numbers of proliferating cells in retinas (±SD; n≥6) treated with NMDA + vehicle (control) and NMDA + gp130 inhibitor (sc144) (**c**), Stat-inhibitor (stattic), or Jak-inhibitor (JSI124). **g**: qRT-PCR was used to measure genes that are changed in response to the gp130-inhibitor sc144 in NMDA-damaged retinas. These genes included *hes5*, *notch1*, *dll4*, *ascl1a,* and *gli2*. Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer, ROI – regions of interest.

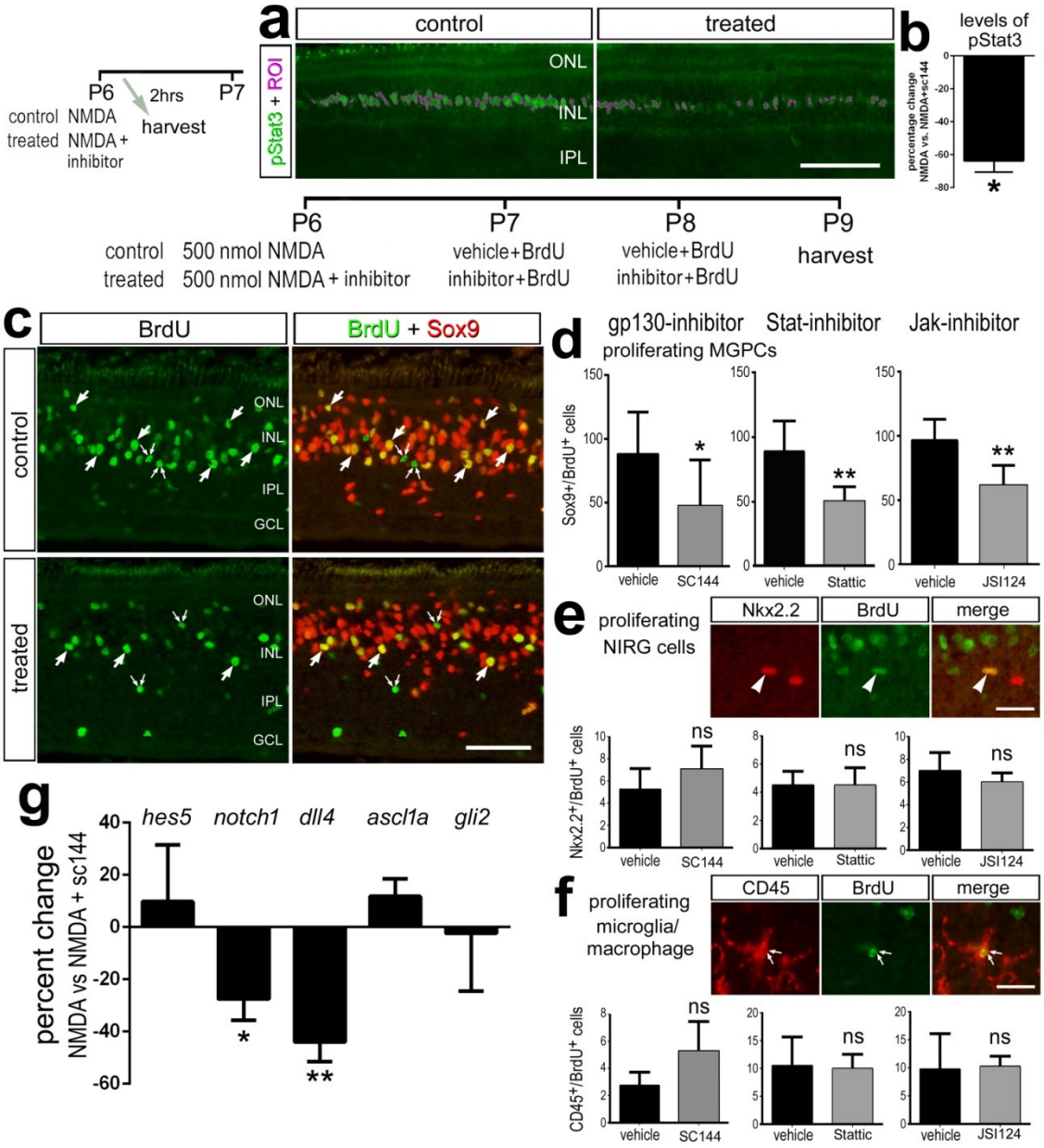
a
control
treated
P6
2hrs
P7
harvest
control NMDA
treated NMDA + inhibitor
pStat3 + ROI
ONL
INL
IPL
b
levels of pStat3
percentage change NMDA vs. NMDA+sc144
P6
P7
P8
P9
harvest
control 500 nmol NMDA
treated 500 nmol NMDA + inhibitor
vehicle+BrdU
inhibitor+BrdU
vehicle+BrdU
inhibitor+BrdU
c
BrdU
BrdU + Sox9
control
treated
ONL
INL
IPL
GCL
d
gp130-inhibitor
Stat-inhibitor
Jak-inhibitor
proliferating MGPCs
Sox9+/BrdU+ cells
vehicle
SC144
Stattic
JSI124
e
proliferating NIRG cells
Nkx2.2
BrdU
merge
Nkx2.2+/BrdU+ cells
ns
f
proliferating microglia/ macrophage
CD45
CD45+/BrdU+ cells
g
hes5
notch1
dll4
ascl1a
gli2
percent change NMDA vs NMDA + sc144

Figure 3.3. Inhibition of gp130 in NMDA-damaged and CNTF-treated retinas suppresses the reactivity of Müller glia, whereas microglia appear to be unaffected. Eyes were injected with 500 nmol NMDA ± sc144 at P6, ± sc144 at P7 and P8, and retinas harvested at P9 (**a**-**c**). Alternatively, eyes were injected with CNTF ± sc144 at P6, P7 and P8 and retinas were harvested at P9 (**d**,**e**). Sections of the retinas were labeled with antibodies to GFAP (**a, e**), and CD45 (**c**). The histogram in **b** and **c** illustrates the mean density sum for CD45 and GFAP immunofluorescence. The histogram in **d** illustrates the mean density sum for GFAP. The scale bar (50 μm) in panel **a** applies to **a** and **e**, and the bar in **c** applies to **c** alone.

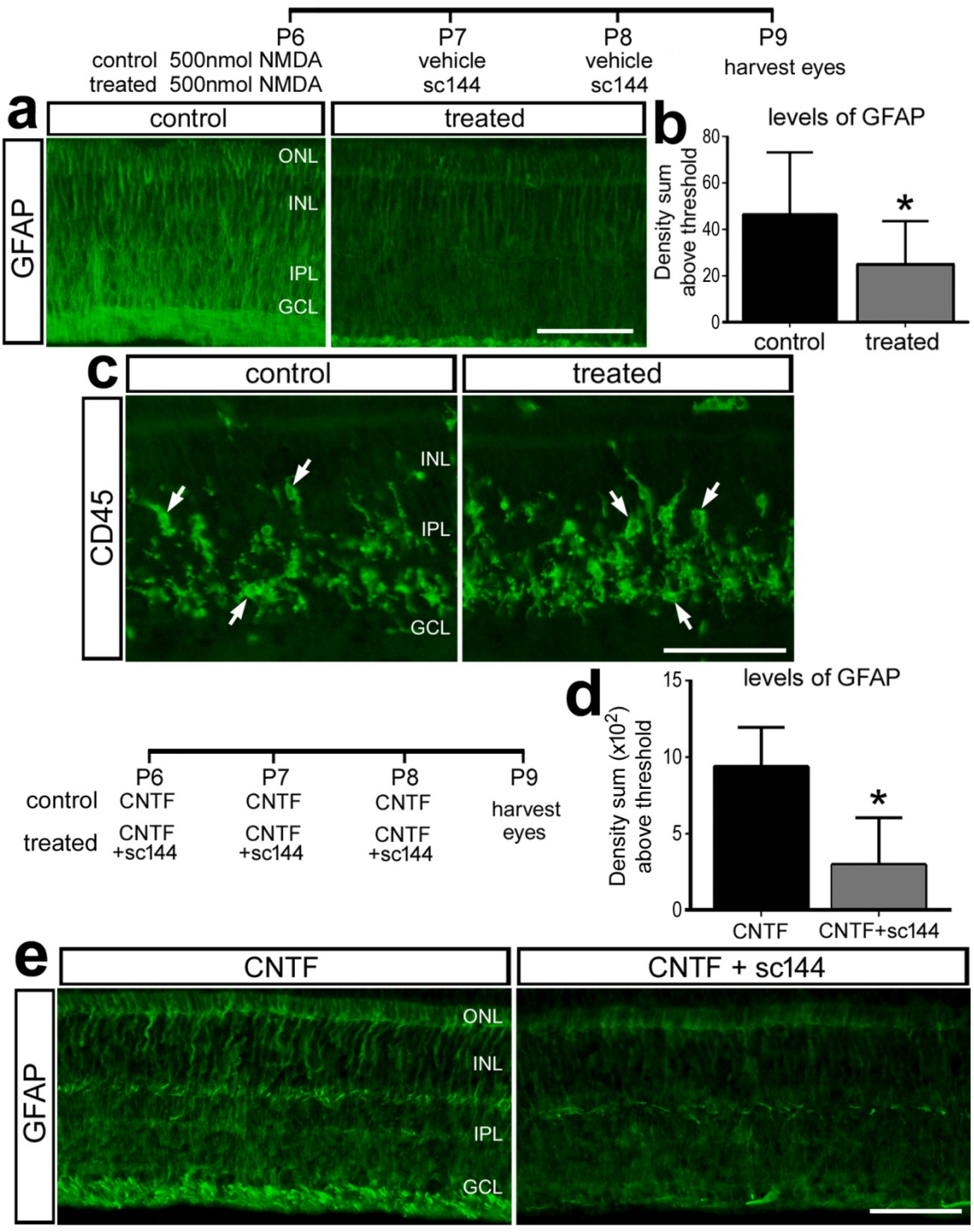
P6
P7
P8
P9
control 500nmol NMDA
treated 500nmol NMDA
vehicle sc144
vehicle sc144
harvest eyes
a
control
treated
GFAP
ONL
INL
IPL
GCL
b
levels of GFAP
Density sum above threshold
80
60
40
20
0
*
control
treated
c
control
treated
CD45
INL
IPL
GCL
P6
P7
P8
P9
control CNTF
CNTF
CNTF
harvest eyes
treated CNTF +sc144
CNTF +sc144
CNTF +sc144
d
levels of GFAP
Density sum (x10²) above threshold
15
10
5
0
*
CNTF
CNTF+sc144
e
CNTF
CNTF + sc144
GFAP
ONL
INL
IPL
GCL

Figure 3.4. Müller glia-specific activation of Jak/Stat-, MAPK- and mTor-signaling in retinas treated with FGF2, CNTF or insulin. Eyes were injected at P6 with saline (**b,c,d,e**), CNTF (**a-c**), insulin (**d**), FGF2 alone or FGF2+UO126 (**e**) at P7 and retinas were harvested 4hrs later. Retinal sections were labeled for pStat3 (green; **a,d,e**), TopAP (red; **a**), Sox2 (red; **b**), pS6 (green; **b**), and pERK (green; **c**). The histogram in **f** illustrates the mean density sum for pStat3 immunofluorescence. Arrows indicate the nuclei of Müller glia, and small double-arrows indicate microglia and/or NIRG cells. The scale bar (50 μm) in panel **a** applies to **a** alone, the bar in **c** applies to **c** alone, the bar in **d** applies to **b** and **d**, and the bar in **e** applies to **e** alone.

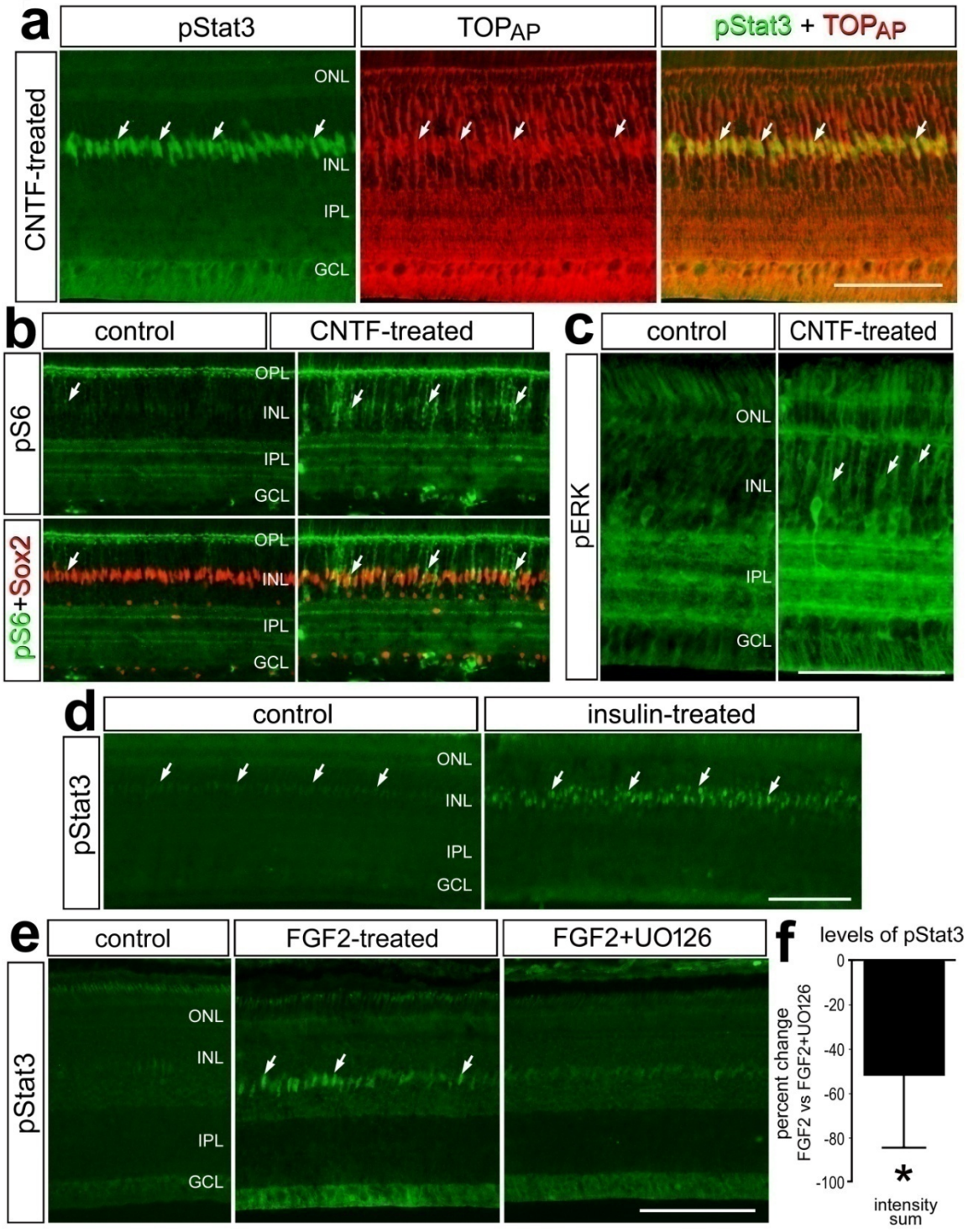
a
pStat3
TOPAP
pStat3 + TOPAP
CNTF-treated
ONL
INL
IPL
GCL
b
control
CNTF-treated
pS6
pS6+Sox2
OPL
INL
IPL
GCL
c
control
CNTF-treated
pERK
ONL
INL
IPL
GCL
d
control
insulin-treated
pStat3
ONL
INL
IPL
GCL
e
control
FGF2-treated
FGF2+UO126
pStat3
ONL
INL
IPL
GCL
f
levels of pStat3
percent change FGF2 vs FGF2+UO126
0
-20
-40
-60
-80
-100
*
intensity sum

Figure 3.5. The formation of proliferating MGPCs in FGF2-treated retinas is potentiated by co-application of CNTF in the absence of retinal damage, but is suppressed by inhibition of mTor and MAPK. qRT-PCR were used to measure levels of *gp130, cntfR and cntf* after three consecutive daily doses of FGF2 starting at P6 ending at P8 (**a**). The histogram illustrates the mean percent change in levels for *gp130, cntfR, cntf* standardized to *gapdh*. Eyes were treated with different injection regimens involving FGF2, CNTF, rapamycin and/or UO126, and BrdU to label proliferating cells (**b**-**g**). Alternatively, eyes were treated with IL6 ± clodronate liposomes, to selectively ablate microglia, 3 days prior to treatment with FGF2 and CNTF (**h** and **i**). Retinal sections were labeled for BrdU (green; **b**,**h**), Sox9 (red; **b**,**h**), or CD45 (green; **f**,**h**). The histograms in **c**,**d**, **e** and **i** represent the mean number of proliferating MGPCs in control and treated retinas. The histogram in **g** illustrates the mean density sum for CD45 in control and treated retinas. The scale bars in panels **b**, **f** and **h** represents 50 μm.

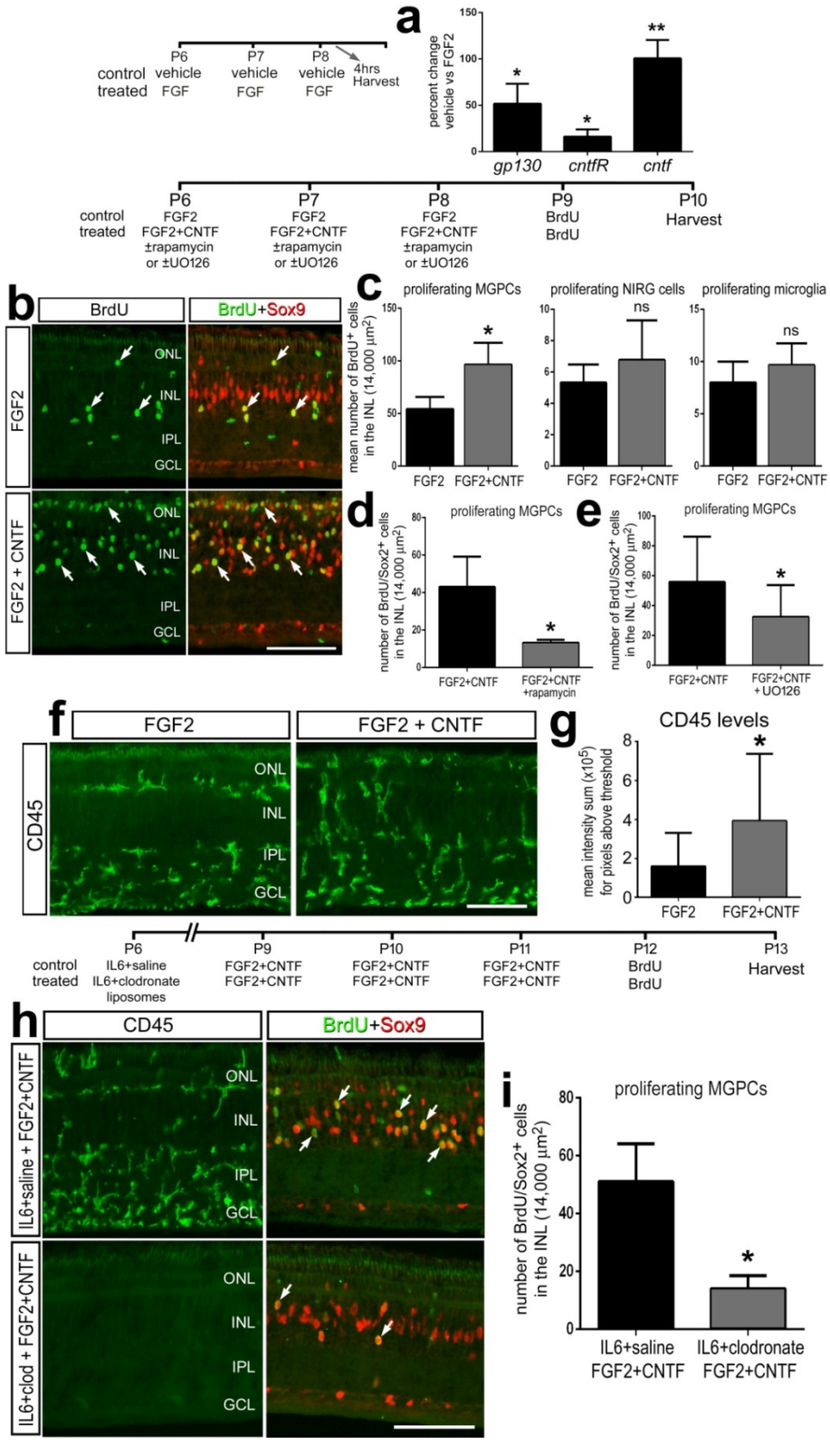
a
control treated
P6 vehicle FGF
P7 vehicle FGF
P8 vehicle FGF
4hrs Harvest
percent change vehicle vs FGF2
gp130
cntfR
cntf
control treated
P6 FGF2 FGF2+CNTF ±rapamycin or ±UO126
P7 FGF2 FGF2+CNTF ±rapamycin or ±UO126
P8 FGF2 FGF2+CNTF ±rapamycin or ±UO126
P9 BrdU BrdU
P10 Harvest
b
BrdU
BrdU+Sox9
FGF2
FGF2 + CNTF
ONL
INL
IPL
GCL
c
proliferating MGPCs
proliferating NIRG cells
proliferating microglia
mean number of BrdU+ cells in the INL (14,000 µm2)
ns
FGF2
FGF2+CNTF
d
proliferating MGPCs
number of BrdU/Sox2+ cells in the INL (14,000 µm2)
FGF2+CNTF
FGF2+CNTF +rapamycin
e
proliferating MGPCs
FGF2+CNTF
FGF2+CNTF + UO126
f
FGF2
FGF2 + CNTF
CD45
g
CD45 levels
mean intensity sum (x10^5) for pixels above threshold
control treated
P6 IL6+saline IL6+clodronate liposomes
P9 FGF2+CNTF FGF2+CNTF
P10 FGF2+CNTF FGF2+CNTF
P11 FGF2+CNTF FGF2+CNTF
P12 BrdU BrdU
P13 Harvest
h
CD45
BrdU+Sox9
IL6+saline + FGF2+CNTF
IL6+clod + FGF2+CNTF
i
proliferating MGPCs
number of BrdU/Sox2+ cells in the INL (14,000 µm2)
IL6+saline FGF2+CNTF
IL6+clodronate FGF2+CNTF

Figure 3.6. The formation of proliferating MGPCs in FGF2-treated retinas is suppressed by inhibition of gp130 or Stat3. Eyes were injected with FGF2 ± sc144 or Stattic at P6, P7, P8, P9 and P10, and retinas harvested at P11. Retinal sections were labeled for Sox2 (red; **a,d**), BrdU (green; **a**), cFos (green; **d**), and CD45 (green; **f**). The histograms in **b** and **c**, represent the mean numbers of proliferating MGPCs in retinas treated with FGF2 + vehicle (control) and FGF2 + gp130 inhibitor (sc144) or Stat-inhibitor (stattic). The histogram in **e** and **g** illustrates the mean density sum for cFos and CD45. The scale bar (50 μm) in panel **f** applies to **a,d** and **f**.

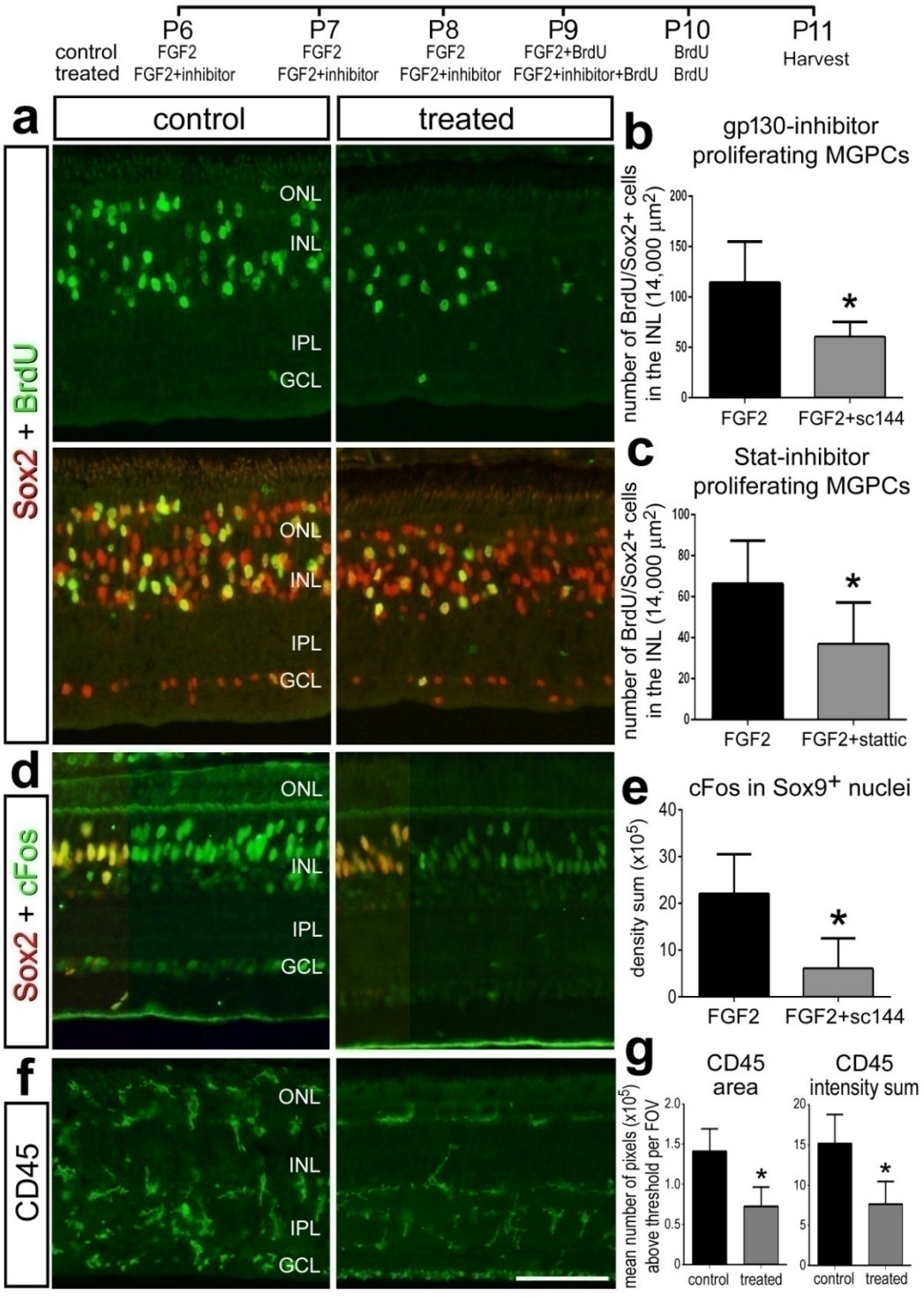
P6
P7
P8
P9
P10
P11
control
treated
FGF2
FGF2+inhibitor
FGF2
FGF2+inhibitor
FGF2
FGF2+inhibitor
FGF2+BrdU
FGF2+inhibitor+BrdU
BrdU
BrdU
Harvest
a
control
treated
Sox2 + BrdU
ONL
INL
IPL
GCL
b
gp130-inhibitor
proliferating MGPCs
number of BrdU/Sox2+ cells in the INL (14,000 µm2)
FGF2
FGF2+sc144
c
Stat-inhibitor
proliferating MGPCs
number of BrdU/Sox2+ cells in the INL (14,000 µm2)
FGF2
FGF2+stattic
d
Sox2 + cFos
e
cFos in Sox9+ nuclei
density sum (x10^5)
FGF2
FGF2+sc144
f
CD45
g
CD45
area
CD45
intensity sum
mean number of pixels (x10^5) above threshold per FOV
control
treated
control
treated

Figure 3.7. Neuronal differentiation is enhanced, while glial differentiation is suppressed, by inhibition of gp130 in NMDA-damaged retinas. Eyes were injected with NMDA at P7 and at P9,10, and P11 control eyes received either DMSO+BrdU (vehicle) (**a,c,d**) or SC144+BrdU (control; **e**) and treated eyes received Sc144+BrdU (**a,c,d**) or Sc144+DAPT+BrdU (**f**). Retinas were harvested at P12 and labeled for HuC/D (red; **a**), BrdU (green; **a**), and glutamine synthetase (red; **b**). **b**; representative image of an ectopic HuC/D-BrdU-positive cell in the distal INL (left) and an orthotopic HuC/D-BrdU-positive cell among the amacrine cells in the proximal INL (right). **c**; the panels to the right are enlarged for the area boxed-out in yellow to demonstrate BrdU+/GS+ Müller glia and BrdU+/GS- cells. The histogram in **d, e** and **f** illustrates the mean percent of Brdu+/HuD+ or BrdU+/GS+ in control vs. treated retinas .**g**: qRT-PCR was used to measure genes that are changed in response to the gp130-inhibitor sc144 in NMDA-damaged retinas. These genes included *hes5*, *notch1*, and *ascl1a*. Sections of the retinas were labeled with antibodies to pStat3 (**a**). The histogram in **a** illustrates the mean density sum for pStat3 immunofluorescence in the INL and ONL. The scale bar (50 μm) in panel **a** applies to **b** and **c.**

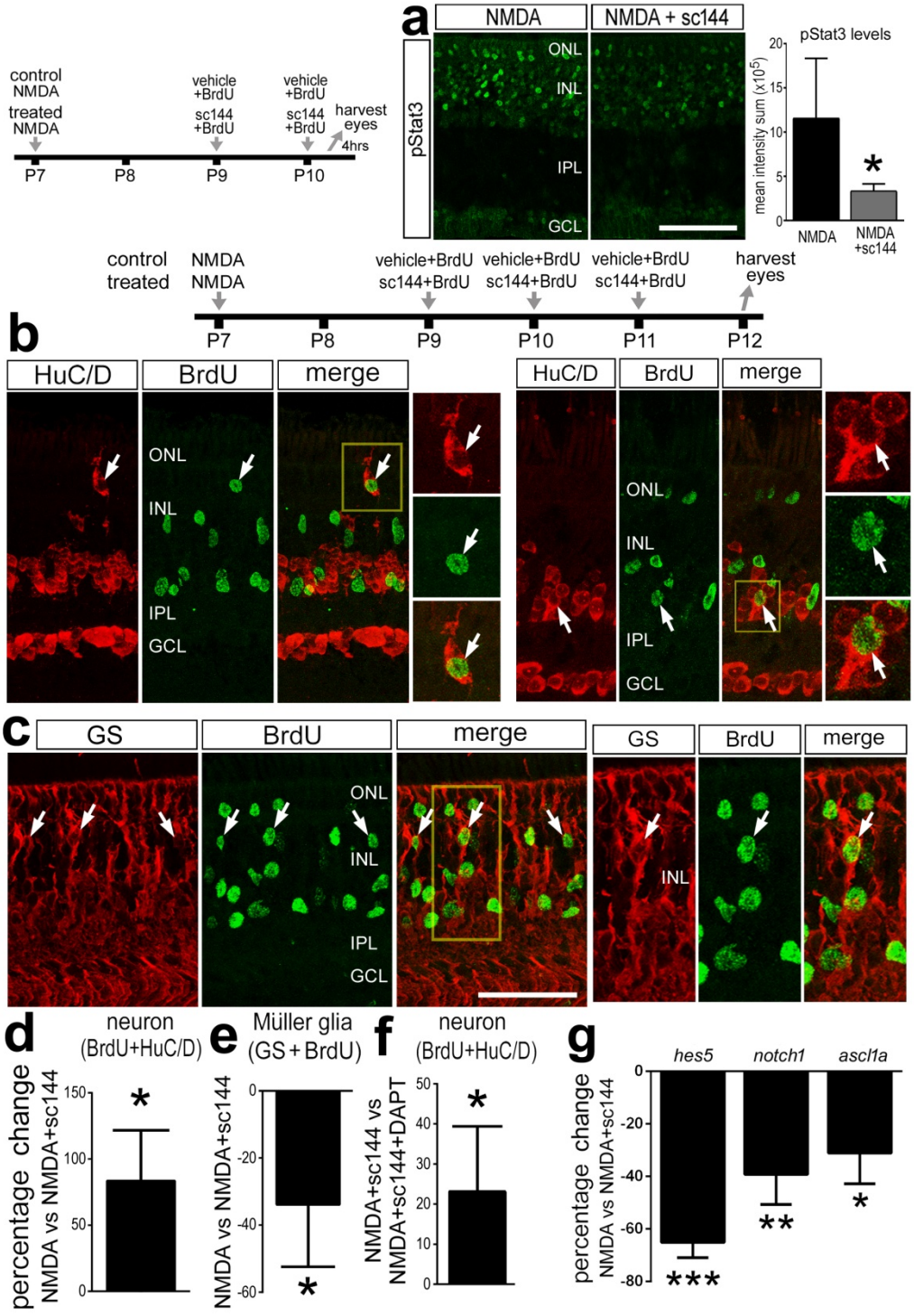
a
NMDA
NMDA + sc144
pStat3
ONL
INL
IPL
GCL
pStat3 levels
mean intensity sum (x10^5)
NMDA
NMDA +sc144
control NMDA treated NMDA
vehicle +BrdU
sc144 +BrdU
harvest eyes
4hrs
P7
P8
P9
P10
b
HuC/D
BrdU
merge
P11
P12
c
GS
d
neuron (BrdU+HuC/D)
percentage change NMDA vs NMDA+sc144
e
Müller glia (GS + BrdU)
NMDA vs NMDA+sc144
f
neuron (BrdU+HuC/D)
NMDA+sc144 vs NMDA+sc144+DAPT
g
hes5
notch1
ascl1a

Table 3.1: Forward and reverse primer sequences (5' – 3') and predicted product sizes (in brackets).

Gene name	Forward	Reverse	Product size (bp)
notch1	GGC TGG TTA TCA TGG AGT TA	CAT CCA CAT TGA TCT CAC AG	(154)
hes5	GGA GAA GGA GTT CCA GAG AC	AAT TGC AGA GCT TCT TTG AG	(143)
dll4	GGT CTG CAG CGA GAA CTA CT	TGC AGT ATC CAT TCT GTT CG	(182)
cntfr	CTG TGA GAA GGA CAT CTT CC	CTT TAC TAT GGC GAA CTC GT	(151)
gli2	CGT GGA AGG CCG GAA AAA TG	TCT GGA GGG GGA TGG CTT TA	(190)
cntf	TAG AAG GCT GAC TTG GAA GA	CTC CAG ATG CTT TAT TTG CT	(157)
ascl1a	AGG GAA CCA CGT TTA TGC AG	TTA TAC AGG GCC TGG TGA GC	(187)
gapdh	CAT CCA AGG AGT GAG CCA AG	TGG AGG AAG AAA TTG GAG GA	(161)
gp130	AGC AGT GTT GTA GCA TCA GTT	GCC AAA GTC AAG GCA ACT CT	(196)

Table 3.2. Antibodies, working dilutions, host and source.

Antigen	Working dilution	Host	Clone or catalog number	Source
BrdU	1:200	rat	OBT00030S	AbDSerotec
BrdU	1:100	mouse	G3G4	Developmental Studies Hybridoma Bank (DSHB)
CD45	1:200	mouse	HIS-C7	Cedi Diagnostic
cFos	1:400	rabbit	K-25	Santa Cruz Immunochemicals
Egr1	1:1000	goat	AF2818	R&D Systems
GFAP	1:2000	rabbit	N1506	Dako
Glutamine Synthetase	1:2000	mouse	AB125724	Abcam
HuC/D	1:300	mouse	21271	Invitrogen
Nkx2.2	1:80	mouse	74.5A5	DSHB
p38 MAPK	1:400	rabbit	12F8	Cell Signaling Technologies
Pax6	1:50	mouse	PAX6	DSHB
PCNA	1:1000	mouse	M0879	Dako
pCREB	1:500	rabbit	87G3	Cell Signaling Technologies
pERK1/2	1:200	rabbit	137F5	Cell Signaling Technologies
pS6	1:750	rabbit	2211	Cell Signaling Technologies
pStat3 (Tyr705)	1:50	rabbit	9131	Cell Signaling Technologies
Sox2	1:1000	goat	Y-17	Santa Cruz Immunochemicals
Sox9	1:2000	mouse	AB5535	Chemicon
Top_{AP}	1:100	mouse	2M6	Dr. P. Linser University of Florida
transitin	1:80	mouse	EAP3	DSHB
vimentin	1:400	rabbit	H5	DSHB

Chapter 4

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

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.

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 in these experiments was in accordance with the guidelines established by the National Institutes of Health and the Ohio State University. Newly hatched wild type leghorn chickens (*Gallus gallusdomesticus*) were obtained from Meyer Hatchery (Polk, Ohio). Postnatal chicks were kept on a cycle of 12 hours light, 12 hours dark (lights on at 8:00 AM). Chicks were housed in a stainless steel brooder at 25^{o}C and received water and Purina^{tm} chick starter *ad libitum*.

Intraocular injections:

Chickens were anesthetized via inhalation of 2.5% isoflurane in oxygen and intraocular injections performed as described previously (Fischer et al., 1999). For all experiments, the right eyes of chicks were injected with the "test" compound and the contra-lateral left eyes were injected with vehicle as a 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), liarozoledihydrochloride (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). Working dilutions and sources of antibodies used in this study are listed in table 4.1.

None of the observed labeling was due to non-specific labeling of secondary antibodies or autofluorescence because sections labeled with secondary antibodies alone were devoid of 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:

Immunolabeled sections were fixed in 4% formaldehyde in PBS for 5 minutes at

room temperature, washed for 5 minutes with PBS, permeabilized with 0.5% Triton X-100 in PBS for 1 minute 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. Sections were washed with PBS for 5 minutes and further processed for immunofluorescence as required.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL):

To identify dying cells that contained fragmented DNA the TUNEL method was used. We used an *In Situ* Cell Death Kit (TMR red; Roche Applied Science), as per the manufacturer's instructions.

Photography, measurements, cell counts and statistics:

Photomicrographs were obtained using a Leica DM5000B microscope equipped with epifluorescence and Leica DC500 digital camera. Confocal images were obtained using a Leica SP8 imaging system at the Hunt-Curtis Imaging Facility at The Ohio State University. Images were optimized for color, brightness and contrast, multiple channels overlaid and figures constructed by using Adobe Photoshop. Cell counts were performed on representative images. To avoid the possibility of region-specific differences within the retina, cell counts were consistently made from the same region of retina for each data set.

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). Identical illumination, microscope, and camera settings were used to obtain images for quantification. Retinal areas were sampled from 5.4 MP digital images. These areas were randomly sampled over the inner nuclear layer (INL) where the nuclei of the bipolar and amacrine neurons were observed. Measurement for content in the nuclei of Müller glia/MGPCs were made by selecting the total area of pixel values ≥70 for Sox2 or Sox9 immunofluorescence (in the red channel), and copying Klf4 or Pax6 (in the green channel). This copied data was pasted into a separate file for quantification or onto 70% grayscale background for figures. Measurements were made for regions containing pixels with intensity values of 68 or greater (0 = black and 255 = saturated). The total area was calculated for regions with pixel intensities > 68. The average pixel intensity was calculated for all pixels within threshold regions. The density sum was calculated as the total of pixel values for all pixels within threshold regions. These calculations were determined for retinal regions sampled from ≥5 different retinas for each experimental condition. The mean area, intensity and density sum was calculated for the pixels within threshold regions from ≥5 retinas for each experimental condition. GraphPad Prism 6 was used for statistical analyses.

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 CMZ. The identity of EdU-labeled cells was determined based on

previous findings that 100% of the proliferating cells in the chick retina are comprised of Sox2/9^{+} Müller glia in the INL/ONL, Sox2/9/Nkx2.2^{+} Non-astrocytic Inner Retinal Glial (NIRG) cells in the IPL, GCL and NFL (the NIRG cells do not migrate distally into the retina), and CD45^{+} (Sox2/9^{-}) microglia (Fischer et al. 2010; Zelinka et al. 2012). Sox2^{+} nuclei in the INL were identified as Müller glia based on their large size and fusiform shape which are distinctly different from the Sox2^{+} nuclei of cholinergic amacrine cells which are small and round (Fischer et al. 2010).

Where significance of difference was determined between two treatment groups accounting for inter-individual variability (means of treated-control values) we performed a two-tailed, paired t-test. Where significance of difference was determined between two treatment groups we performed a two-tailed, unpaired t-test.

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. 4.1). Many of these cells were destroyed or down-regulated CRABP by 1 day

after NMDA-treatment (Fig. 4.1). 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. 4.1). By 3 days after NMDA-treatment, CRABP was detected in the nuclei of $Sox2^{+}$ MGPCs (Fig. 4.1). 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. 4.2a-b). By comparison, TTNPB did not affect the proliferation of Non-astrocytic Inner Retinal Glial (NIRG) cells and microglia (Figs. 4.2c,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. 4.2e,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. 4.2g).

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.4.3a,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. 4.3c,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 (levels of damage) or levels of

CD45 in microglia (data not shown) which is diagnostic of 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, 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. 4.4a-c). The potentiating effects of RA and TTNPB were specific to Müller glia as microglia and NIRG cell proliferation was unaffected (Figs. 4.4c,d,f,g). TTNPB increased levels of cFos-expression in the nuclei of FGF2-treated Müller glia/MGPCs (Figs. 4.4h,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.4.4j-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. 4.5a,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. 4.5c,d). In addition, we found that BMS493 inhibited the proliferation of NIRG cells, whereas the proliferation of microglia was not affected (Figs. 4.5c,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. 4.5e,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. 4.6a,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. 4.6c,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. 4.6b). 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).

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 such as IGF1 and FGF2 (Fischer et al. 2002). Accordingly, we tested whether RA influenced the proliferation of CMZ progenitors. We found that 3 consecutive daily intraocular injections of RA alone had no effect upon the proliferation of CMZ progenitors (not shown). By comparison, the combination of RA with IGF1 significantly increased the proliferation of retinal progenitors in the CMZ compared to IGF1 alone (Figs. 4.7a,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. The percentage of cells that differentiated as HuC/D+ neurons was increased nearly 150% (Figs. 4.7c,e,f), and the percentage of cells that differentiated as Otx2+ neurons was increased by nearly 60% (Figs. 4.7d,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.

Figure 4.1. 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.

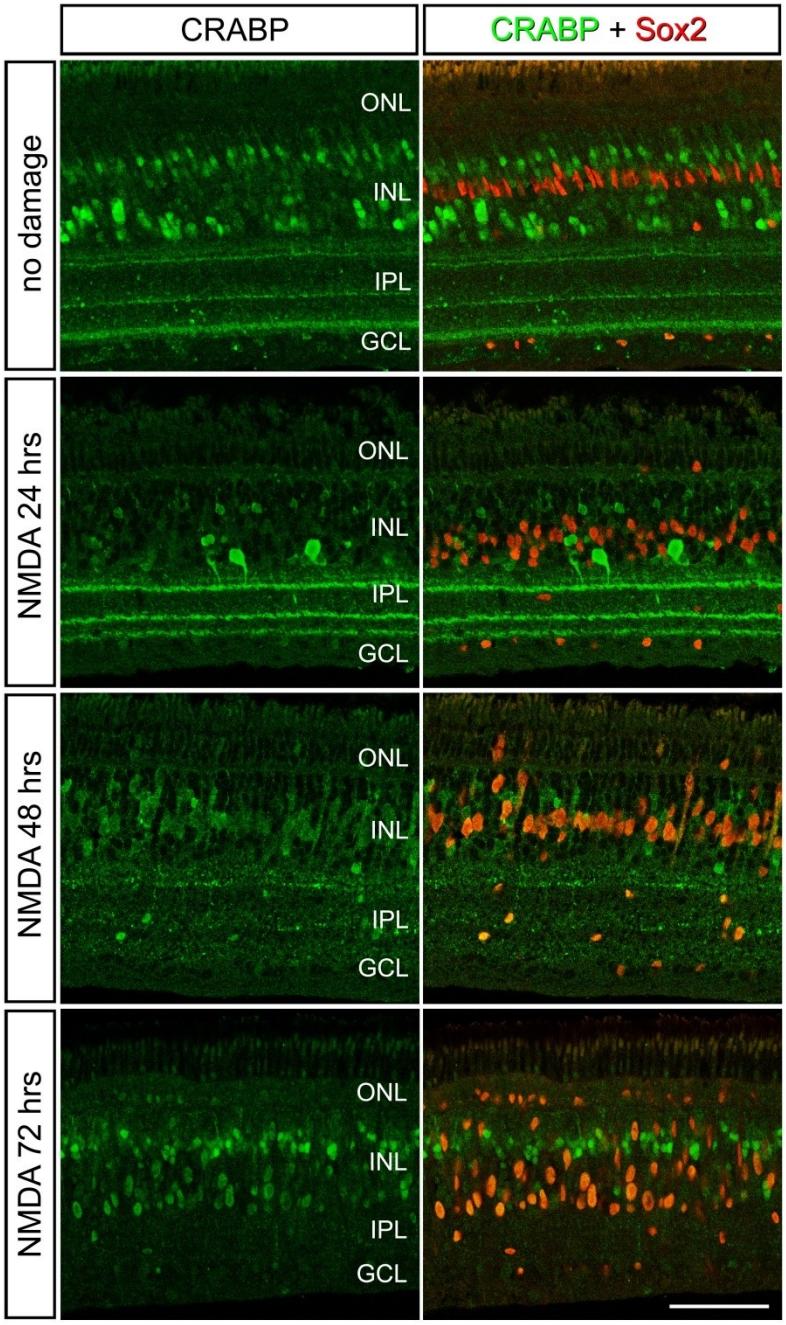

Figure 4.2. Activation of RA-signaling stimulates the proliferation of MGPCs in damaged retinas. Eyes were injected with a relatively low dose (60 nmol) of NMDA (control) or NMDA + RAR agonist (TTNBP; treated) at P6, vehicle + EdU (control) or TTNBP + EdU (treated) at P7 and P8, and tissue harvested 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 illustrate the mean, upper extreme, lower extreme, upper quartile and lower quartile. Significance of difference (***p<0.0001) was determined by using a t-test (**b**,**c**,**d**,**g**) 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.

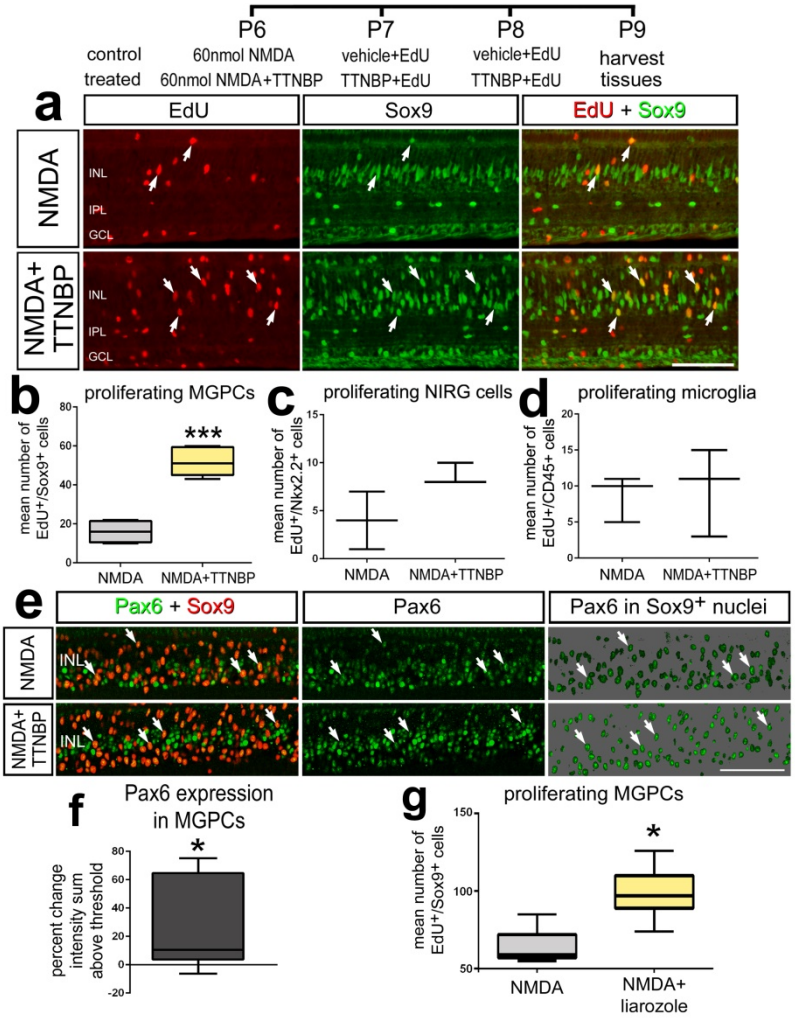

Figure 4.3. Inhibition of RA-signaling suppresses the proliferation of MGPCs in damaged retinas. Eyes were injected with a relatively high dose (500 nmol) of NMDA alone (control) or NMDA+RAR antagonist (BMS493) at P7, vehicle+EdU or RAR antagonist+ EdU at P8 and P9, and tissues harvested at P10. Sections 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. 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.

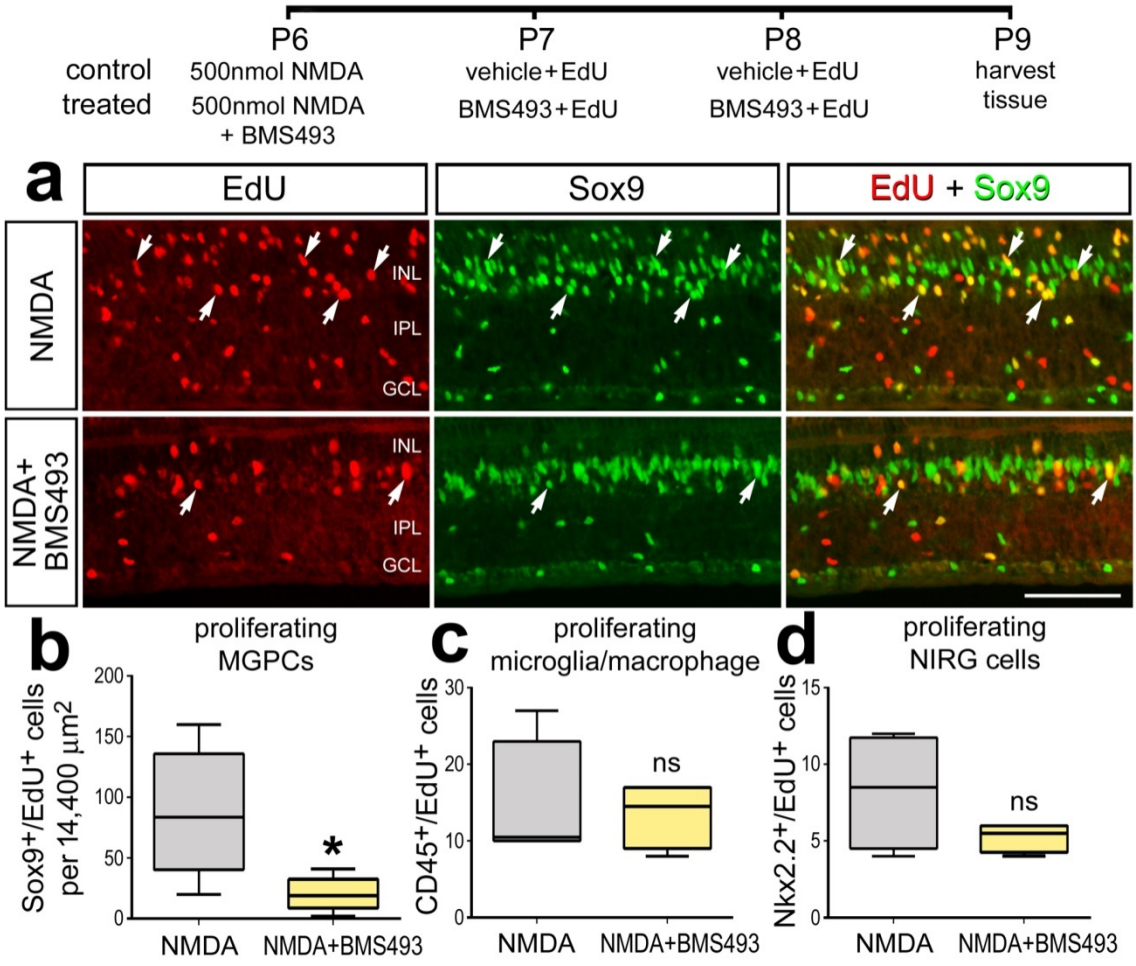

Figure 4.4. In the absence of retinal damage, activation of RA-signaling stimulates the formation of proliferating MGPCs in FGF2-treated retinas. (**a**-**d**) Eyes were injected with FGF2 alone (control) or FGF2+RA (treated) at P6 and P7, FGF2+EdU or FGF2+EdU+RA at P8, EdU alone at P9, P10 and P11, 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. 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.

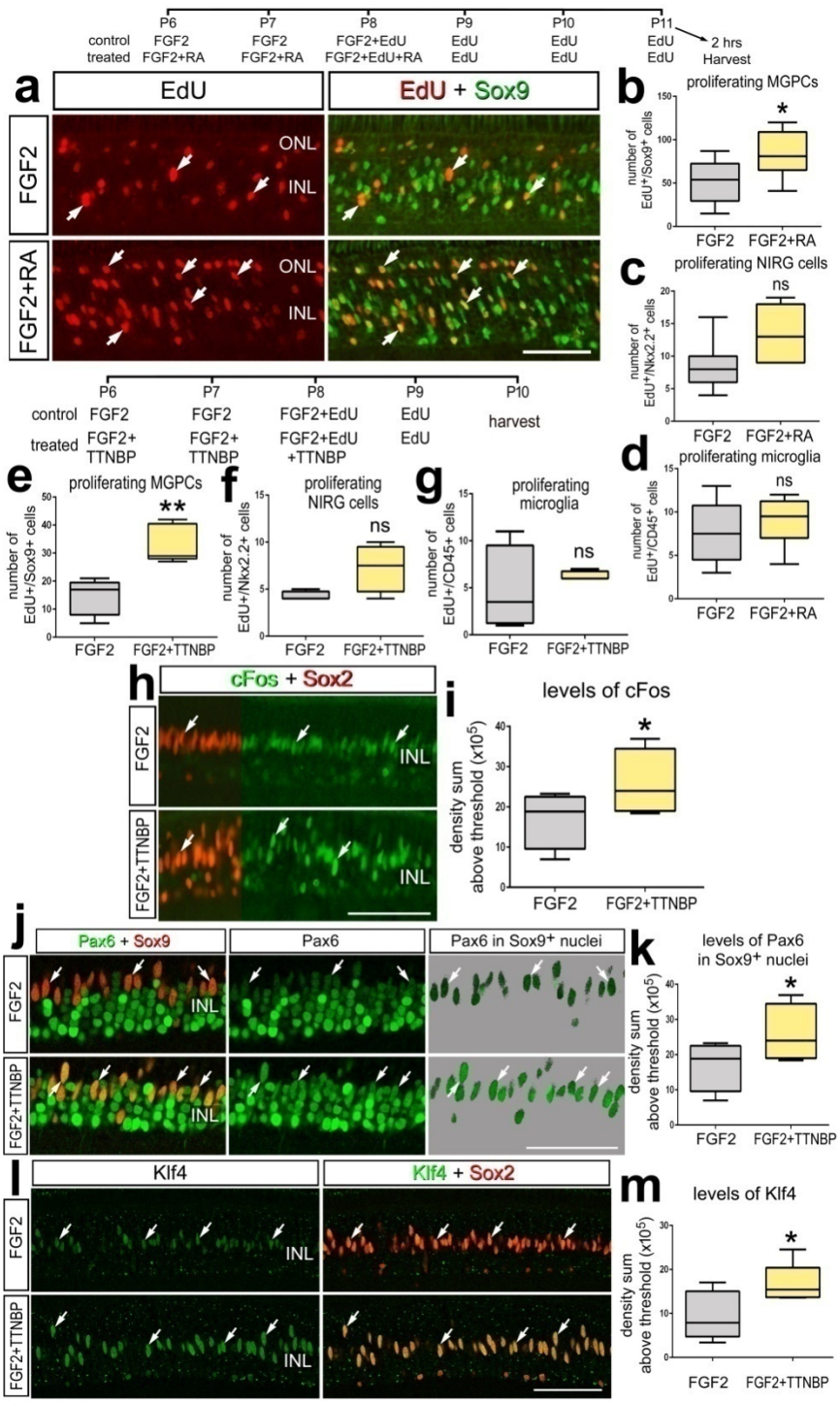
P6
P7
P8
P9
P10
P11
control
treated
FGF2
FGF2+RA
FGF2+EdU
FGF2+EdU+RA
EdU
EdU
EdU
EdU
EdU
EdU
2 hrs
Harvest
a
EdU
EdU + Sox9
FGF2
FGF2+RA
ONL
INL
b
proliferating MGPCs
number of EdU+/Sox9+ cells
FGF2
FGF2+RA
c
proliferating NIRG cells
number of EdU+/Nkx2.2+ cells
ns
d
proliferating microglia
number of EdU+/CD45+ cells
ns
P6
P7
P8
P9
P10
control
treated
FGF2
FGF2+ TTNBP
FGF2
FGF2+ TTNBP
FGF2+EdU
FGF2+EdU +TTNBP
EdU
EdU
harvest
e
proliferating MGPCs
**
f
proliferating NIRG cells
ns
g
proliferating microglia
ns
FGF2
FGF2+TTNBP
h
cFos + Sox2
FGF2
FGF2+TTNBP
INL
i
levels of cFos
density sum above threshold (x10^5)
*
j
Pax6 + Sox9
Pax6
Pax6 in Sox9+ nuclei
k
levels of Pax6 in Sox9+ nuclei
l
Klf4
Klf4 + Sox2
m
levels of Klf4

Figure 4.5. 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. 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.

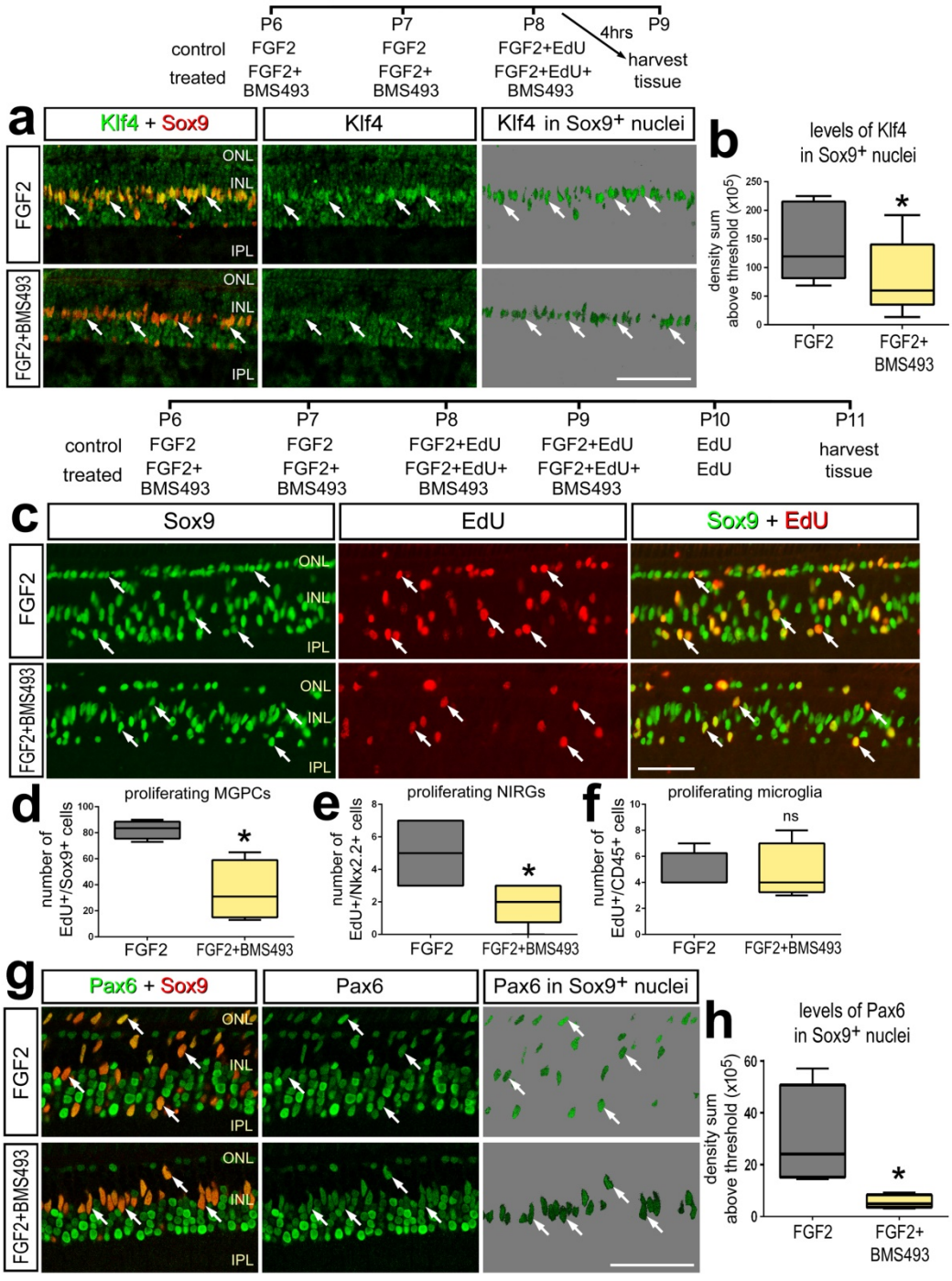
P6
P7
P8
4hrs
P9
control
FGF2
FGF2
FGF2+EdU
harvest tissue
treated
FGF2+ BMS493
FGF2+ BMS493
FGF2+EdU+ BMS493
a
Klf4 + Sox9
Klf4
Klf4 in Sox9+ nuclei
FGF2
FGF2+BMS493
ONL
INL
IPL
b
levels of Klf4 in Sox9+ nuclei
density sum above threshold (x10^5)
FGF2
FGF2+ BMS493
P10
P11
EdU
c
Sox9
EdU
Sox9 + EdU
d
proliferating MGPCs
number of EdU+/Sox9+ cells
e
proliferating NIRGs
number of EdU+/Nkx2.2+ cells
f
proliferating microglia
number of EdU+/CD45+ cells
ns
g
Pax6 + Sox9
Pax6
Pax6 in Sox9+ nuclei
h
levels of Pax6 in Sox9+ nuclei

Figure 4.6. 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. 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.

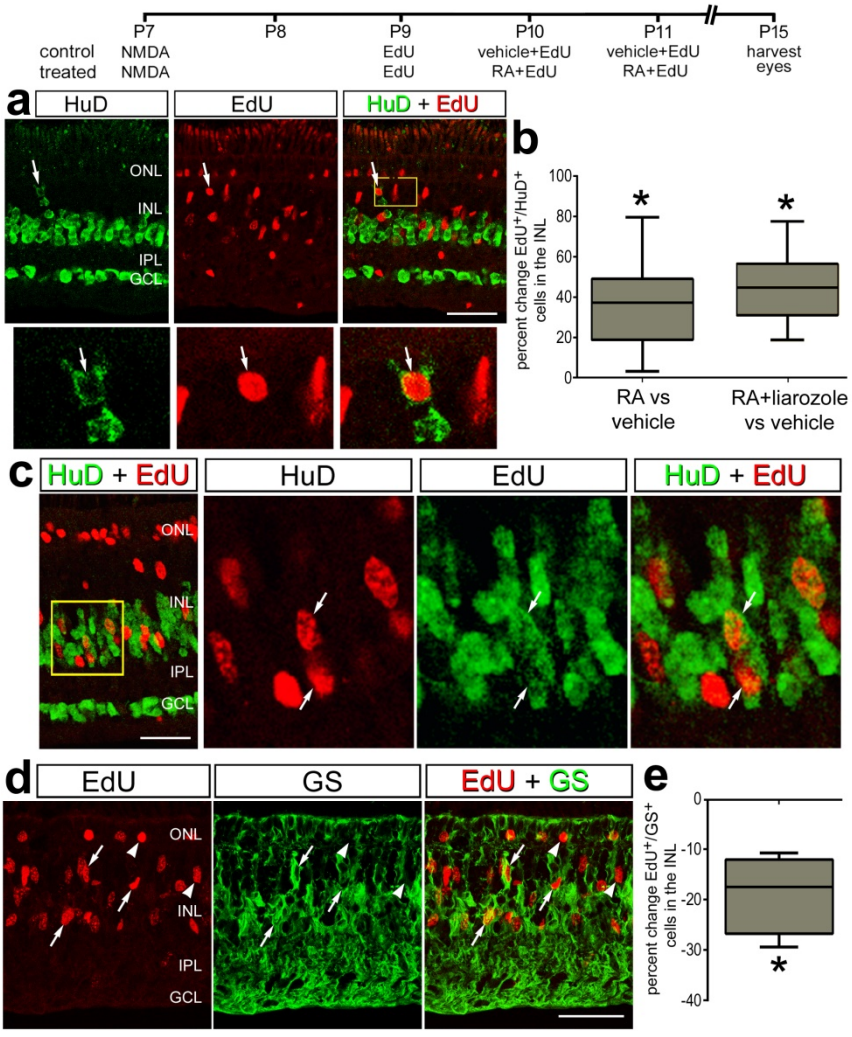

Figure 4.7. Activation of RA-signaling stimulates the neuronal differentiation of progeny of CMZ progenitor cells. Eyes were injected with IGF1+EdU (control) or IGF1+EdU+RA (treated) at P1, P2 and P3, EdU alone at P4, and tissues harvested at P5. Sections of the peripheral retina and CMZ were labeled 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. 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.

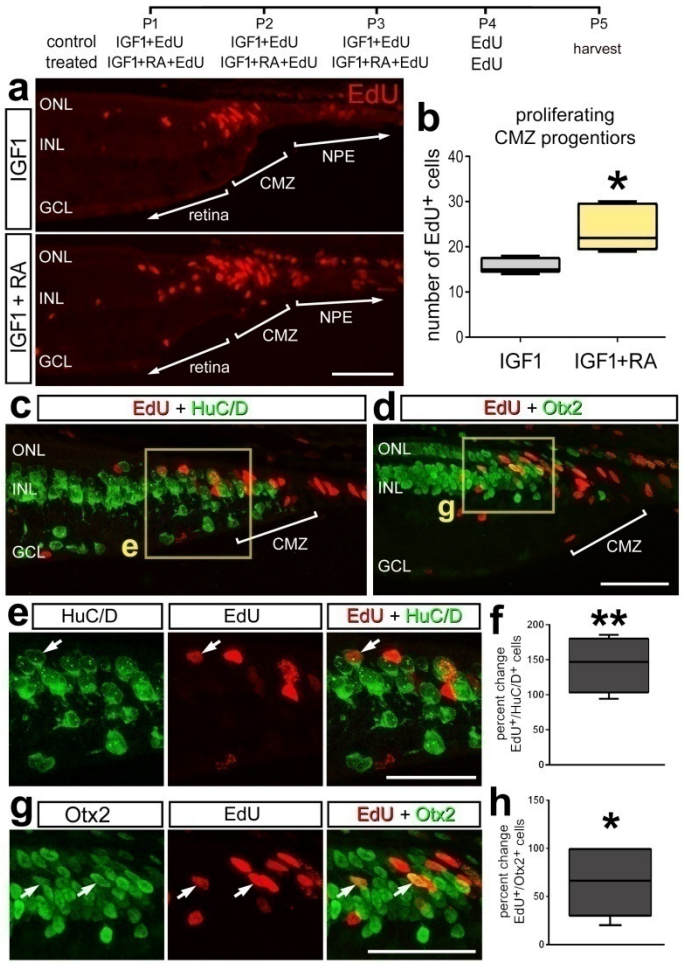

Table 4.1. Antibodies, sources and working dilutions. Patterns of labeling and stimulus-dependent changes in levels of immunolabeling using these antibodies are consistent with previous reports (Fischer and Omar, 2005; Fischer et al., 2009a, 2009b; Fischer et al., 2014; Todd and Fischer, 2015).

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

Chapter 5

Summary and Future Directions

Synopsis of Findings

This dissertation focuses on the cell-signaling mechanisms that regulate the ability of Müller glia to reprogram into neurogenic progenitor cells. This work addresses aspects related to Müller glia homeostasis, cell signaling interactions, and cell fate choice. This work provides novel data that Hedgehog-, Jak/Stat- and Retinoic Acid-signaling pathways are crucial regulators of MGPC formation. These data represent significant advances in increasing the regenerative capacity of Müller glia in the avian retina.

In the second chapter, we investigated the role that Hedgehog (HH)-signaling has on the formation of MGPCs in the chick retina. We find that the HH-pathway components are upregulated in NMDA-damaged retinas and that the Shh-ligand is potentially released by ganglion cells and received by Müller glia. We find that HH-agonists increase Müller glia proliferation in both damaged and FGF2-treated retinas. Consistent with this finding, inhibition of HH-signaling at the level of the Shh-ligand, Ptc receptor, or Gli transcription factors attenuates Müller glia proliferation. Our data also shows that Hedgehog-signaling is recruited by MAPK signaling and these pathways are upstream of the Notch-pathway. We observe that HH-signaling promotes Müller glia reprogramming evidenced by the up-regulation of the stem cell transcription factors Klf4

and Pax6. Collectively, these novel findings implicate the HH-pathway as a key regulator of MGPC formation.

In the third chapter, we examined the influence of Jak/Stat-signaling on retinal regeneration in the avian retina. We show that Jak/Stat-signaling is rapidly activated in Müller glia in response to NMDA-damage. We also find that inhibition of the gp130-receptor, JAK kinase, or Stat3 transcription factor each suppresses MGPC proliferation in damaged and FGF2-stimulated retinas. These data suggest that Jak/Stat-activity promotes MGPC formation. Consistent with this notion, we find that the Jak/Stat ligand CNTF activates Jak/Stat-, MAPK-, and mTOR-signaling in Müller glia. Furthermore, CNTF combined with FGF2 leads to greater MGPC proliferation than FGF2 alone. Importantly, we show that inhibition of gp130 biases MGPC-progeny towards neurogenesis at the expense of gliogenesis. Taken together, this chapter suggests that the Jak/Stat-pathway plays a crucial role in retinal regeneration.

In the fourth chapter, we investigated how Retinoic Acid (RA)-signaling impacts the proliferative and neurogenic capacity of MGPCs in the chick retina. We found that the RA-pathway component CRABP was upregulated specifically in Müller glia two days after NMDA damage. We show that RA-agonists increase MGPC formation in damaged and FGF2-treated retinas. Consistent with the notion that RA-signaling is mitogenic, inhibition of the RA-receptor decreases proliferation in damaged and FGF2-treated retinas. We provide data that RA-signaling is one of the many pathways recruited by FGF2 and that RA-signaling is necessary for FGF2/MAPK-stimulated MGPC formation. We report that RA increases the percentage of MGPC-progeny that

differentiate into neurons. We also find that RA increases neurogenesis from CMZ progenitors found at the retinal margin. These data are the first to implicate RA-signaling as an important component of MGPC mediated retinal regeneration. Taken together, the data in this dissertation provides a rationale for multiple avenues of future investigations. The remainder of this section will suggest future research strategies to harness the regenerative capacity of MGPCs inspired by the work in the preceding chapters.

Prospective studies for Hedgehog signaling in retinal regeneration

In Chapter 2, we provide data that HH-signaling promotes the reprogramming of Müller glia into MGPCs in the avian retina. We also provide data that HH-signaling stimulates proliferation of CMZ progenitors. The data from chapter 2 suggest interesting follow up experiments for both the chick and mouse model. This section will describe prospective studies involving HH-signaling and retinal regeneration.

Novel combinations of pharmaceuticals to elicit MGPC reprogramming

It will be important to determine whether HH-agonists in novel combinations with other signaling pathway manipulations can drive MGPC formation in the absence of damage or FGF2. We observed that Müller glia de-laminate and migrate to the ONL in uninjured retinas treated with the HH-agonist, SAG, combined with IGF1. This combination was not sufficient to up-regulate the progenitor markers Pax6 or Klf4. This suggests that SAG and IGF1 may partially initiate MGPC reprogramming but not

sufficiently for cell cycle re-entry. However, in the CMZ, the combination of SAG and IGF1 does stimulate proliferation of retinal progenitors. These data were intriguing considering that FGF2 application has been shown to be required in all contexts where MGPC formation occurs in the absence of damage (Fischer et al. 2002; Fischer et al. 2014). It would be worthwhile to test whether in the absence of exogenous FGF2, novel combinations of growth factors and small molecule inhibitors can stimulate MGPC formation in the uninjured retina. Pharmacological manipulations using HH-agonists, IGF1, CNTF, RA,TGFB-inhibitors, GCR-inhibitors and Wnt-activators have all proven to boost MGPC formation in the avian retina (Fischer et al. 2002; Gallina et al. 2015; Gallina et al. 2014b; Todd and Fischer 2015; Todd et al. 2017; Todd et al. 2016). An attractive approach would be to examine whether novel combinations of these growth factors and small molecule inhibitors could stimulate Müller glia proliferation without FGF2 in the uninjured retina. Data from these experiments would help answer questions regarding whether FGF2 is an essential component of MGPC formation in the chick retina and whether alternative strategies exist to elicit Müller glia reprogramming in the absence of damage.

Consistent with this approach, recent investigations have determined an effective "chemical cocktail" that can reprogram fibroblasts into induced neural stem cells without genetic manipulation (Zhang et al. 2016). This "chemical cocktail" consisted of nine components and included molecules aimed at activating HH-signaling, RA-signaling, FGF2/MAPK-signaling, SMAD1/2/5/8-activity, and Wnt-signaling combined with DNA and histone methyltransferase inhibitors (Zhang et al. 2016). A pharmacological approach

to stem cell reprogramming helps avoid tumorigenic side effects that occur with viral-based reprogramming and helps reduce costs and technical obstacles associated with stem cell engineering (Bengoa-Vergniory and Kypta 2016). A similar chemical approach will be useful in the future to reprogram Müller glia into a therapeutically useful source of retinal neurons.

HH-mediated MGPC formation in the mammalian retina

The data provided in Chapter 2 in the avian retina provides rationale to examine whether HH-signaling may boost MGPC-mediated retinal regeneration in the mammal. To date, only one report has suggested Shh application can stimulate Müller glia proliferation in the damaged rat retina (Wan et al. 2007). However, in this study retinal damage was elicited at P7 and Shh was applied for three subsequent days. Because Müller glia are not fully differentiated at this developmental stage, this raises concern that late stage progenitors could be responsible for the proliferation seen in response to Shh (Close et al. 2005; Nelson et al. 2011). Therefore, the effect HH-signaling has on mammalian MGPCs has yet to be elucidated.

The AAV capsid variant ShH10 can specifically transfect Müller glia from intravitreal injections (Klimczak et al. 2009). This viral capsid can be engineered to investigate whether HH-activation in Müller glia is sufficient to stimulate proliferation in the healthy and damaged mouse retina. Packaging the ShH10-virus with the SMO-M2 plasmid (adgene plasmid #64628) would result in active HH-signaling specifically in Müller glia. The SMO-M2 is a constitutively active version of the HH-receptor

Smoothened and previous reports have confirmed that viral expression of SMO-M2 induces ligand-independent activation of the HH-pathway (Yu et al. 2006). Examining the efficiency of Müller glia reprogramming in retinas transfected with SMO-M2 via the ShH10 virus would provide data on the impact of HH-activation on mammalian MGPC formation.

The ShH10 virus can also be engineered to examine the necessity of HH-signaling in MGPC formation in mouse retina. The ShH10 virus can be packaged with the CRE recombinase enzyme. This virus can then be injected into mice harboring floxed sites surrounding Gli2 (Jackson mouse # 007926). The HH-pathway manifests its effects through three different Gli transcription factors Gli1, Gli2, and Gli3 (Ruiz i Altaba et al. 2002). Floxed alleles exist for each Gli transcription factor, but data in the mouse and chick retina suggests Gli2 may be the most attractive target for preliminary studies (Todd and Fischer 2015; Wall et al. 2009). It would be interesting to examine whether deletion of Gli2 impacts the ability of Müller glia to reprogram in response to NMDA+ growth factor application. Data from these experiments will help determine whether HH-activation is a critical component of Müller glia reprogramming in mammalian retinas.

HH-signaling and the LRP2 receptor in the mouse retina

Consistent with previous reports, we observed that HH-activation increased proliferation of CMZ progenitors in the chick retina (Moshiri et al. 2005). In further support of this notion, mutant mice that contain a single functional allele of the Ptc-receptor have a gain of function in HH-signaling and contain a zone of progenitors

reminiscent of the CMZ (Moshiri and Reh 2004). A recent report has implicated LRP2, an auxiliary Shh-receptor, in regulating CMZ quiescence in the mouse (Christ et al. 2015). LRP2 is expressed in the retinal margin of mice where it regulates endocytic clearance of the Shh-ligand (Christ et al. 2015). Genetic loss of LRP2 allows Shh to stimulate CMZ-like progenitors in the retinal margin (Christ et al. 2015). It would be interesting to further investigate whether LRP2 is enriched in the retinal margin of other mammalian species including humans, as LRP2 may play a causative role in the mitotic quiescence of the retinal margin in higher vertebrates. Furthermore, LRP2 may be responsible for limiting the ability of HH-signaling to stimulate MGPC formation in the uninjured mouse retina. Deletion of LRP2 in Müller glia may yield interesting results.

Future studies for Jak/Stat signaling in retinal regeneration

In Chapter 3, we show that Jak/Stat signaling influences the proliferative and neurogenic potential of Müller glia in the chick retina. This work implicates Jak/Stat signaling as a target to boost the regenerative potential of mammalian Müller glia. This section will describe potential research avenues involving Jak/Stat signaling retinal regeneration.

Does a loss or gain in Jak/Stat signaling influence the formation of MGPCs in the mouse retina?

We provide data that Jak/Stat signaling promotes Müller glia reprogramming into proliferating MGPCs in the chick retina. This work provides rationale to examine

whether Jak/Stat plays a similar role in the mammalian retina. To test whether a gain-of-function in the Jak/Stat-pathway increases Müller glia reprogramming, mice from Jackson Laboratory that possess loxP sites flanking exon 2 of *Socs3* (Jax# 010944) could be utilized. Socs3 acts to inhibit the activity of the JAK kinase and prevent phosphorylation of Stat3. Therefore, deletion of Socs3 results in a gain of function in Jak/Stat-signaling (Carow and Rottenberg 2014). Injecting Socs3 floxed mice with the ShH10-Cre virus will result in a Müller glia-specific loss of Socs3 and an increase in Jak/Stat activation. For the loss of function experiments, the ShH10-Cre virus could be applied to mice harboring the floxed Stat3 allele (Jax#016923). Investigating how each of these genetic manipulations impacts the reprogramming capacity of Müller glia will shed light on Jak/Stats role in mammalian retinal regeneration.

Does gp130 inhibition increase neural differentiation of MGPC-progeny?

While advances have been made in stimulating the proliferation of mammalian Müller glia, very little has been discovered to increase the neurogenic capacity of these cells. Similar to the chick, the percentage of proliferating Müller glia that differentiate into neurons in the murine retina is very low. In response to NMDA+EGF less than 4% of BrdU+ MGPCs express neuronal makers *in vivo* (Karl et al. 2008). It would be worthwhile to test whether Jak/Stat signaling can modify the regenerative capacity of murine MGPCs. As shown in Chapter 3, the temporal dynamics of gp130 inhibition is critical. Inhibition of gp130 acutely after damage decreases Müller glia proliferation while inhibition after cell cycle re-entry increases neurogenesis. Due to the time sensitive

nature of gp130 activity, a permanent Cre-mediated excision is not appropriate. Therefore, a pharmacological approach to examine the impact of gp130 on neurogenesis is needed. The gp130 small molecule inhibitor should be applied in the narrow window of time that follows MGPC proliferation but proceeds differentiation of MGPC-progeny. The peak of proliferation of MPGCs occurs two days after NMDA+EGF treatment (Karl et al. 2008). Thus, beginning at two days after NMDA+EGF, the small molecule gp130 inhibitor should be applied to bias cell fate. Including EdU in the injection paradigms will label proliferating cells and allow quantification of neuronal vs. glial differentiation. By examining the percent of EdU+ cells that co-label with neuronal markers in gp130-inhibitor treated retinas compared to vehicle treated controls will determine whether gp130 influences the neurogenic capacity of MGPCs in the murine retina.

Potential directions for Retinoic acid signaling in retinal regeneration

In Chapter 4, we show that RA-signaling bolsters the proliferative and neurogenic capacity of MGPCs in the chick retina. The data presented in Chapter 4 indicates the RA-pathway as a novel contributor to the signaling network underlying MPGC formation. Whether the RA-pathway has similar effects on MGPC formation in the fish and mouse retina has yet to be determined. This section suggests follow up work that will help elucidate how RA-signaling influences retinal regeneration.

What are the targets of RA-signaling in the avian retina during MGPC formation?

We provide data that RA-signaling is recruited into the network of pathways that regulate FGF2/MAPK-mediated MGPC formation. However, the precise targets of RA-activation in Müller glia are unknown. Discovering the mechanisms in which RA-signaling controls MGPC formation will provide the field of retinal regeneration with novel targets to explore. RNA-seq analysis of sorted Müller glia from RA-treated retinas would help accomplish this aim. Intraocular injections of DiY accumulate in Müller glia nuclei (Jeon and Masland 1993). Using FACS to isolate DiY+ Müller glia, RNA-seq analysis can be performed specifically on Müller glia. Examining the full transcriptome of Müller glia from the experimental groups (1) RA-alone vs. vehicle controls, (2) FGF2 vs. FGF2+RA, and (3) NMDA vs. NMDA+RA treated retinas would reveal potential targets that the RA-pathway exerts its proliferative and neurogenic effects through.

RA-signaling and photoreceptor regeneration in the avian retina

In the chick retina MGPCs have been shown to be capable of regenerating bipolar cells, amacrine cells and ganglion-like cells (Fischer and Reh 2001; Fischer and Reh 2002). However, regeneration of photoreceptors has yet to be observed from chick MGPCs. RA-signaling is known to promote photoreceptor fate in fish, chick, rodent, monkey, and human (Hyatt et al. 1996; Kelley et al. 1994; Osakada et al. 2008; Stenkamp et al. 1993). Intriguingly, we show in Chapter 4 that RA-agonists stimulate CMZ progenitors to increase production of Otx2+ neurons. Otx2 is a critical transcription factor necessary for photoreceptor development (Brzezinski and Reh 2015). Additionally, we observed an increased amount of EdU+ MGPCs residing in the photoreceptor layer in

retinas treated with RA (unpublished observations). However, these cells failed to express the photoreceptor markers Otx2, Lim3, or recoverin. This may be due to the fact that NMDA-damage leaves photoreceptors intact (Fischer et al. 1998). It is possible that MGPCs do not regenerate photoreceptors when the ONL is intact due to spatial constraints or lack of replacement signals from dying photoreceptors (Nelson et al. 2013). Therefore, it would be interesting to develop photoreceptor injury models using constant light exposure or focal laser ablation to test whether RA-agonists can lead to photoreceptor regeneration in these contexts. Developing models of photoreceptor injury in the avian retina will be useful for screening compounds that may increase photoreceptor regeneration in higher vertebrates.

Can RA increase the neural differentiation of MGPC-progeny in the mouse retina?

As discussed previously, the neurogenic capacity of mammalian MGPCs is insufficient for functional regeneration. Developing strategies to increase the neural differentiation from these cells is one of the most important objectives in developing therapies for Müller glia-mediated cell replacement. Therefore, it is imperative to test whether RA-application can increase the regenerative capacity of the mammalian retina. A previous report found that RA-treated retinas increased bipolar cell production from MGPCs, however this effect failed to be replicated (Karl et al. 2008; Ooto et al. 2004). It has yet to be examined whether consistent treatment of RA-agonists in damaged retinas increase the proliferative and neurogenic capacity of mammalian MGPCs. It would also be interesting to combine RA with other compounds such as GSK3B inhibitors, EGF,

HB-EGF, or in retinas with Ascl1 overexpression to see if RA can boost neurogenesis in these contexts of increased MGPC formation (Jorstad et al. 2017; Karl et al. 2008; Osakada et al. 2007; Todd et al. 2015).

The current state of retinal regeneration and the path forward

Significant advances have been made since Müller glia were originally reported to be a source for retinal regeneration over 15 years ago (Fischer and Reh 2001). We now know many of the signaling pathway components that regulate the de-differentiation and proliferation of Müller glia in response to retinal injury (Gallina et al. 2014a). We have also uncovered a complex interacting network that centers on the MAPK pathway that orchestrates MGPC reprogramming in the absence of damage. Recent work has also implicated microglia in MGPC formation, although the nature of the signals that microglia provide remain unknown (Fischer et al. 2014). It wasn't until 2006 that Müller glia were discovered to be the cells responsible for the remarkable regeneration that occurs in the fish retina (Fausett and Goldman 2006). In the succeeding decade a complex network of cell signaling pathways and transcription factors were discovered to control MGPC formation in the fish (Lenkowski and Raymond 2014). As described in Chapter 1 of this dissertation, the network of pathways underlying MGPC formation in the zebrafish is very similar to that in chicks. This inspires confidence that the networks that drive MGPC formation are conserved across vertebrate species and that lessons learned from lower vertebrates will be applicable to mammalian models. Studies from the fish and chick model have inspired investigations into stimulating retinal regeneration in

the mouse retina. Recent work has described genetic strategies that lead to the Müller glia transdifferentiation into functional neurons (Jorstad et al. 2017). However, even in the best cases the extent of neurogenesis is too limited to provide functional restoration of vision in the chick and mouse. The next era of investigations will need to tackle the obstacle of how to increase the neurogenic capacity of MGPCs as well as how to facilitate functional integration of newly generated neurons.

Concluding remarks

Over 12 million Americans currently suffer from uncurable vision-threatening diseases. Using Müller glia as an endogenous source of retinal neurons is a promising thereapeutic target to restore lost vision. The work presented in this dissertation builds on the existing knowledge regarding the mechansims underlying the reprogramming of Müller glia into proliferating neurogenic progenitor cells. This work provides a foundation for future studies examining Müller glia-mediated retinal regeneration.

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