Fatty Acid Binding Proteins Facilitate Reprogramming of Müller Glia into Progenitor Cells

Warren Campbell, Sydney Blum, Maddie Hathoot,

Thanh Hoang[2], Seth Blackshaw[2], Andy J. Fischer[1]*

Abstract

Degenerative retinal diseases result in neuronal cell death, which causes in permanent vision loss in humans. The response to the loss of retinal neuron across species is diverse, with some species capable of complete and functional retinal regeneration. One source of these regenerated neurons is Müller glia (MG), the primary glial cell of the retina. In zebrafish, MG respond to damage by dedifferentiating into progenitor cells (MGPCs) which proliferate before they undergo neurogenesis. While mice completely lack a regenerative response by MG, chick MG form progenitors with limited neurogenesis, proving to be an effective phenotype for understanding molecular pathways that influence reprogramming and cell fate. Preliminary data has indicated that fatty acid binding protein 8 (FABP8) is highly upregulated early in the regenerative response to damage in the chick retina. FABPs are a class of lipid binding proteins that traffic polyunsaturated fatty acids such as docosahexaenoic acid and arachidonic acid which have been collectively implicated in signal transduction, neurotransmission, proliferation, differentiation, and neuronal migration. **We hypothesize that FABP8 is significantly influencing the early reprogramming phase of MG, and can be utilized to drive the formation of MGPCs in mammalian retinas**. To test this hypothesis, we will first characterize the spatiotemporal expression pattern of FABP8 in damaged and growth factor treated chick retinas. Subsequently, we will test the effect of new and established pharmacological inhibitors of FABP proteins on the

formation of retinal progenitors in chick. Single cell sequencing identified FABP5, FABP7, and FABP8 as significantly increasing in Müller glia after NMDA damage. FABP8 mRNA expression correlated to protein localization in Müller glia after damage. Pan inhibitor of FABPs BMS309403 was found to significantly reduce the formation of progenitor cells. Further research into the potential role of endocannabinoids and FABPs is being evaluated in the mechanism of action for affecting Müller glia's reprogramming response.

A. Introduction

dRetinal disease is the most common cause of blindness in children, and is the most common cause of irreversible adult blindness in the Western world.[1,2] Vision is perceived to be the most important sense for daily life, and there are over 25 million adults in the United States living with visual impairment leading to economic burden of over $180 billion.[3,4] The decrease in visual acuity in retinal disease is due to the loss of retinal neurons. Currently there are no therapeutics available to restore lost retinal neurons, only prophylactic treatment to delay neuronal death in diseases such as diabetic retinopathy and glaucoma. Vision research has focused on therapeutic strategies to restore neuronal populations through endogenous mechanisms of regeneration, such as Müller glia (MG) reprogramming.

MG are the primary glial cell of the retina that serves pleiotropic functions including neurotransmitter recycling, osmotic balance, structural support, potassium siphoning, and bicarbonate transport.[5] Importantly, MG retain stem cell properties and are capable of replacing lost neurons. However, this regenerative potential is variable among species (Figure 1). Zebrafish can regenerate all retinal neurons, while mice fail to form any Müller glia derived progenitor cells (MGPCs). Chick retinas exhibit an intermediate phenotype with significant MGPC formation but limited neurogenesis into a few neuronal subtypes.

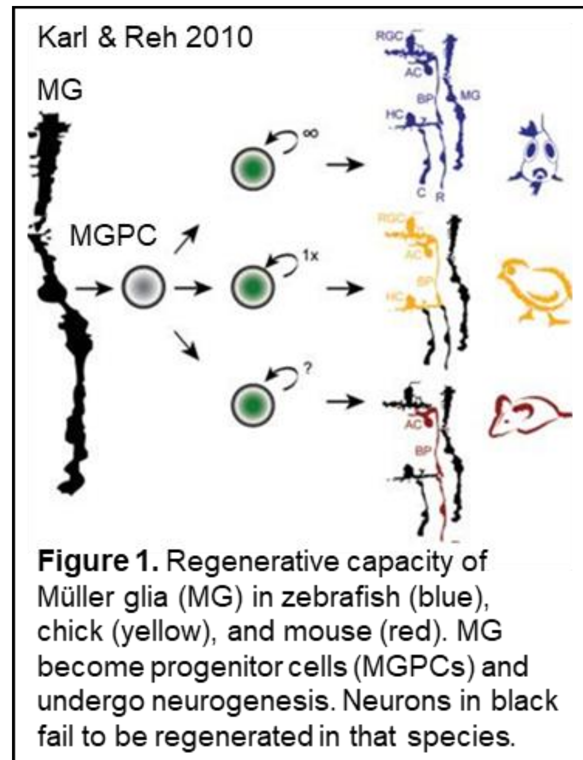

Figure 1. Regenerative capacity of Müller glia (MG) in zebrafish (blue), chick (yellow), and mouse (red). MG become progenitor cells (MGPCs) and undergo neurogenesis. Neurons in black fail to be regenerated in that species.

The limited regenerative potential in chicks serves as an exemplary in-vivo model to determine inhibitory and potentiating factors of regeneration.

For MG derived neuronal replacement to occur, MG must proceed through four phases: de-differentiation, proliferation, neurogenesis, and integration. In zebrafish, the cellular pathways that drive the initial de-differentiation reprogramming mechanism include Mapk-ERK, Gsk3β-β catenin, and Jak-stat. Similarly, factors such as let-7, TGFβ, and insulinoma-associated 1a(Insm1a) drives progenitors out of the cell cycle for subsequent neurogenesis.[6] Many of these important cell signaling cascades can be recapitulated in chick models of retinal damage to induce greater MGPC formation and modulate neurogenesis.[7–11]

These results imply that regenerative mechanisms are conserved, and with proper modulation of murine MG, can be induced to regenerate retinal neurons. And indeed, recent research using a ASCL1a overexpression model can form a few MGPCs when driven in conjunction with retinal damage.[12] Nonetheless, this approach only produces a few MGPCs, requires damage, and only form bipolar cells. To further enhance the regenerative potential of murine MG, our lab and collaborators have conducted extensive research into the transcriptomic and epigenetic landscape through single cell RNA sequencing (scRNA-seq), assay for transposase accessible chromatin (ATAC)-sequencing, and chromatin immunoprecipitation (CHIP) sequencing.[13] Utilizing these techniques on the developing and damaged retina, our research focus is on identifying important biological targets to influence MGPC formation and neurogenesis with greater efficacy.

One target identified by this research has been the fatty acid binding proteins (FABPs). The FABPs are a class of intracellular lipid-binding proteins that possess a highly conserved tertiary structure.[14] Additionally, each of these FABPs proteins are conserved among species from drosophila melanogaster and Caenorhabditis elegans, to mouse and human.[15] FABPs contain a

hydrophobic binding pocket capable of binding to polyunsaturated fatty acids (PUFAs) and other hydrophobic ligands. In the mammalian system, FABP3, 5, and 7 have been characterized by their expression in the brain, retina, and radial glia differentiation and their role in fate determination.[16–18] The importance of FABPs in development is hypothesized to be a result of its ability to bind to PUFAs including arachidonic acid and docosahexaenoic acid which have been established to modulate signal transduction, neurotransmission, proliferation, differentiation, and neuronal migration.[19–22] Additionally, evidence of the proliferative functions of FABP proteins has been identified in aggressive cancers, including CNS malignancies.[23–25] However, there has not been a strong scientific consensus, with additional research implicating the role of CNS FABP expression to include modulation of cannabinoid signaling, Peroxisome proliferator-activated receptor (PPAR), NF-kB, and CREB signaling.[21,22,26,27]

Interestingly, FABP8 has very low levels of expression in the central nervous system (CNS) in mammals, but does have a high level of expression in the peripheral nervous system, specifically in the myelin sheaths. Currently, the specific function of this isoform is not well characterized. In zebrafish, knockdown of FABP8 affected the development of motor neurons causing a failure of axonal outgrowth from the notochord.[28] Our lab has found that in the retinas of chicks, FABP8 is significantly overexpressed in MG in the context of retinal damage or growth factor stimulation. While there are other FABP proteins expressed in the chick retina, this homolog is specific to early phase reprogramming of MG. Hence, our preliminary data has led us to the hypothesis that **FABP8 plays a critical role in early reprogramming and de-differentiation of Müller glia that has the potential to significantly enhance MGPC formation in other vertebrate species.**

FABP8 is a novel target in the field of retinal regeneration and requires thorough characterization using pharmacologic and genetic manipulation in chick models (Aim1). Implementing MG

FABP8 ectopic expression in an Ascl1 overexpression mouse line has not been previously attempted and can elucidate their impact on murine retinal regeneration models (Aim 2). When FABP8 expression is modulated, retrospective analysis of the cell signaling cascades and transcription factors to further understand subcellular FABP function in a neurological context (Aim 3). The findings of this research will be significant in the field of retinal regeneration by **validating FABP8 as a target to influence regeneration**, **the mechanism by which they influence glial cellular biology**, **and importance of FABP proteins in neurodevelopment and stem cell biology**.

Methods and Materials

Animals:

The animals approved for use in these experiments was in accordance with the guidelines established by the National Institutes of Health and IACUC at The Ohio State University. Newly hatched P0 wildtype leghorn chicks (Gallus gallus domesticus) were obtained from Meyer Hatchery (Polk, Ohio). Post-hatch chicks were maintained in a regular diurnal cycle of 12 hours light, 12 hours dark (8:00 AM-8:00 PM). Chicks were housed in stainless-steel brooders with enrichment at 25o C and received water and Puranam chick starter food and supplement ad libitum.

Fertilized eggs were obtained from the Michigan State University, Department of Animal Science. Eggs were incubated at a constant 37.5°C, with a 1hr period room temperature cool down every 24hrs. Additionally, the eggs were rocked every 45 minutes, and held at a constant relative humidity of 45%. Embryos were harvested at various time points after incubation and staged according to guidelines established by Hamburger and Hamilton (1951).

Intraocular injections:

Chicks were anesthetized with 2.5% isoflurane mixed with oxygen from a non-rebreathing vaporizer. The technical procedures for intraocular injections were performed as previously described [29]. With all injection paradigms, both pharmacological and vehicle treatments were administered to the right and left eye respectively. Compounds were injected in 20 µl sterile saline with 0.05 mg/ml bovine serum albumin added as a carrier. Compounds included: NMDA (38.5nmol or 154 µg/dose; Sigma-Aldrich), FGF2 (250 ng/dose; R&D systems), purified mouse midkine (1 µg/dose; MyBioSource), recombinant chicken midkine (1 µg/dose, MyBioSource), insulin (1µg/dose; Sigma), Sodium Orthovanadate (NA3VO4, Sigma-Aldrich), and IPA-3 (Tocris Bioscience). 5-Ethynyl-2´-deoxyuridine (EdU) was intravitreally injected to label the nuclei of proliferating cells. Injection paradigms are included in each figure.

Single Cell RNA sequencing of embryonic and mature chick retinas

Retinas were obtained from embryonic and postnatal chicks. Embryonic retinas were harvested E5, E8, E12, and E15. Isolated retinas tissue were dissociated in a 0.25% papain solution in Hank's balanced salt solution (HBSS), pH = 7.4, for 30 minutes, and suspensions were frequently triturated. The dissociated cells were passed through a sterile 70µm filter to remove large particulate debris. Dissociated cells were assessed for viability (Countess II; Invitrogen) and cell-density diluted to 700 cell/µl. Each single cell cDNA library was prepared for a target of 10,000 cells per sample (n = 2 per sample). The cell suspension and Chromium Single Cell 3' V2 reagents (10X Genomics) were loaded onto chips to capture individual cells with individual gel beads in emulsion (GEMs) using 10X Chromium Controller. cDNA and library amplification for an optimal signal was 12 and 10 cycles respectively. Sequencing was conducted on Illumina HiSeq2500 (Genomics Resource Core Facility, John's Hopkins University) or HiSeq4000 (Novogene) with 26 bp for Read 1 and 98 bp for Read 2. Fasta sequence files were de-multiplexed, aligned, and

annotated using the chick ENSMBL database (Chick-5.0, Ensembl release 94) and Cell Ranger software. Gene expression was counted using unique molecular identifier bar codes, and gene-cell matrices were constructed. Using Seurat toolkits, t-distributed stochastic neighbor embedding (tSNE) plots or Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) plots were generated from aggregates of multiple scRNA-seq libraries [30,31]. Compiled in each tSNE/UMAP plot of individual retinal cells are two sequencing library replicates for each embryonic stage, saline treated, and 24 hrs, 48 hrs, and 72 hrs after NMDA damage. Identification of different types of retinal cells that were clustered together in tSNE/UMAP plots was accomplished by probing for well-established cell-distinguishing genes. Seurat was used to construct violin/scatter plots. Significance of difference in violin/scatter plots was determined using a Wilcoxon Rank Sum test with Bonferroni correction. Monocle was used to construct unbiased pseudo-time trajectories and scatter plotters for Müller glia and MGPCs across pseudotime [32–34]. Genes that were used to identify different types of retinal cells included the following: (1) Müller glia: GLUL, VIM, SCL1A3, RLBP1, (2) MGPCs: PCNA, CDK1, TOP2A, ASCL1, (3) microglia: C1QA, C1QB, CCL4, CSF1R, TMEM22, (4) ganglion cells: THY1, POU4F2, RBPMS2, NEFL, NEFM, (5) amacrine cells: GAD67, CALB2, TFAP2A, (6) horizontal cells: PROX1, CALB2, NTRK1, (7) bipolar cells: VSX1, OTX2, GRIK1, GABRA1, and (7) cone photoreceptors: CALB1, GNAT2, OPN1LW, and (8) rod photoreceptors: RHO, NR2E3, ARR3. The Müller glia have an over-abundant representation in the scRNA-seq databases. This likely resulted from fortuitous capture-bias and/or tolerance of the Müller glia to the dissociation process. scRNA-seq libraries are available at: https://proteinpaint.stjude.org/F/2019.retina.scRNA.html

Fixation, sectioning and immunocytochemistry:

Retinal tissue samples were formaldehyde fixed, sectioned, and labeled via immunohistochemistry as described previously [35,36]. Antibody dilutions and commercial sources for images used in this study are described in table 2. Observed labeling was not due to off-target labeling of secondary antibodies or tissue autofluorescence because sections incubated exclusively with secondary antibodies were devoid of fluorescence. Secondary antibodies utilized include 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 and 0.2% Triton X-100.

Labeling for EdU:

For the detection of nuclei that incorporated EdU, immunolabeled sections were fixed in 4% formaldehyde in 0.1M PBS pH 7.4 for 5 minutes at room temperature. Samples were 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 a buffer consisting of 100 mM Tris, 8 mM CuSO4, and 100 mM ascorbic acid in dH2O. The Alexa Fluor 568 Azide (Thermo Fisher Scientific) was added to the buffer at a 1:100 dilution.

Photography, measurements, cell counts and statistics:

Microscopy images of retinal sections were captured with the Leica DM5000B microscope with epifluorescence and the Leica DC500 digital camera. High resolution confocal images were obtained with a Leica SP8 available in The Department of Neuroscience Imaging Facility at The Ohio State University. Representative images are modified to have enhanced color, brightness, and contrast for improved clarity using Adobe Photoshop. In EdU proliferation assays, a fixed region of retina was counted and average numbers of Sox2 and EdU co-labeled cells. The retinal

region selected for investigation was standardized between treatment and control groups to reduce variability and improve reproducibility.

For quantification of immunofluorescence, densitometry measurements were collected and compared between control and treatment conditions. Cell-specific quantifications were taken from images with identical staining and exposure times to collect pixel in changes where cell specific markers were colocalized. ImagePro 6.2, ImageJ, and Microsoft Excel were used for data and calculations respectively.

For statistical evaluation of differences in treatments, a two-tailed paired T-test was applied for intra-individual variability where each biological sample also served as its own control. For two treatment groups comparing inter-individual variability, a standard two-tailed unpaired T-test was applied. For multivariate analysis, an ANOVA with the associated Tukey Test was used to evaluate any significant differences between multiple groups.

Results

Single cell RNA sequencing identifies FABPs upregulation in Müller glia during reprogramming.

FABP8 protein expression has been validated in the chick retina. To support MG expression during reprogramming and proliferation, we generated a single cell RNA sequencing library analyzing the transcriptomic change in MG in response to NMDA damage (Figure 1). Individual cells are plotted and clustered in t-stochastic neighbor embedding (tSNE) plots showing transcriptomic similarities between cells and highlighting the cell type, library ID/replicate, and heat map expression of FABP8 (Figure 1A-B). Of note, MG change their gene expression in response to NMDA damage, as shown by the dark green (control MG), forest green (24hr NMDA damage), and light green (48-72hr NMDA) clusters (Figure 1A). As part of this reprogramming phase,

FABP8 can be seen upregulated in MG as high as 64-fold in some cells (Figure 1 C,D). Transcriptionally distinct progenitors start forming 48-72hrs after damage (as seen by the neon green cluster), and can be highlighted by cell cycle markers Ascl1, Nestin, Rax, PCNA, and Cdk1. These replication competent cells also maintain high levels of FABP8 expression, further implicating FABP8 in the dedifferentiation and proliferation processes in the retina.

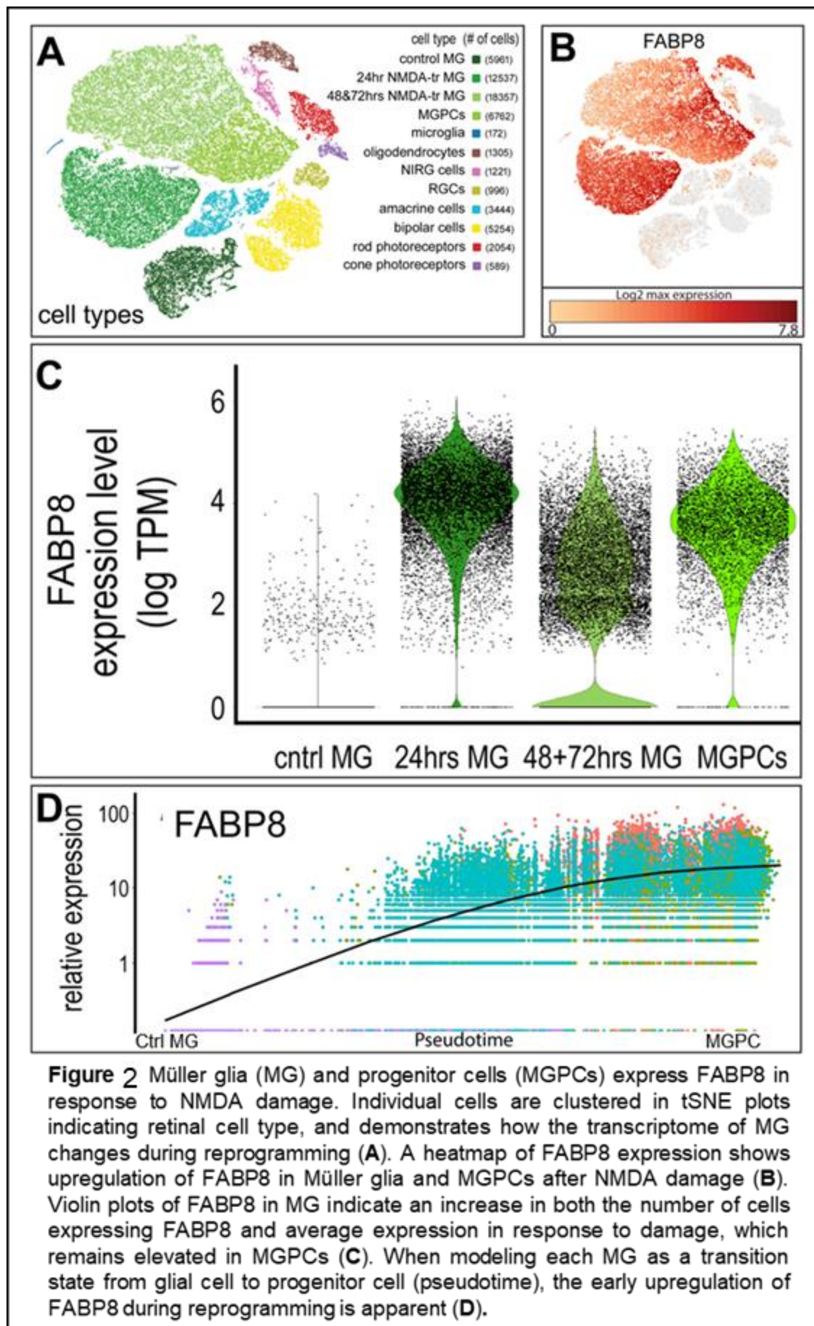

Figure 2 Müller glia (MG) and progenitor cells (MGPCs) express FABP8 in response to NMDA damage. Individual cells are clustered in tSNE plots indicating retinal cell type, and demonstrates how the transcriptome of MG changes during reprogramming (**A**). A heatmap of FABP8 expression shows upregulation of FABP8 in Müller glia and MGPCs after NMDA damage (**B**). Violin plots of FABP8 in MG indicate an increase in both the number of cells expressing FABP8 and average expression in response to damage, which remains elevated in MGPCs (**C**). When modeling each MG as a transition state from glial cell to progenitor cell (pseudotime), the early upregulation of FABP8 during reprogramming is apparent (**D**).

***FABP8 is expressed during MG reprogramming in the chick retina*.**

FABP8 is a novel target in understanding the mechanisms of MG reprogramming, and has not been previously identified in the central nervous system in birds. Thus, little is known about its potential function in the retina or the formation of MGPCs. To study regeneration in the chick, we use a well characterized model of induced MG regeneration through the intravitreal injection of N-methyl D-aspartate (NMDA).[37] NMDA induces excitotoxic cell death of primarily amacrine cells, which in turn induces MG to express retinal progenitor factors and form MGPCs. Of those MG that dedifferentiate, a subset of these neurons undergoes cell division primarily 24 to 48 hours after damage. Another small subset of these post-mitotic MGPCs will form amacrine and ganglion cells.[38] Similarly, our studies have found that four consecutive doses of FGF2 is sufficient to induce progenitor formation by stimulating muller glia through a mitogen activated protein kinase pathway with upregulation of pERK, phospho-cyclicAMP response (pCREB), cFos, and early growth response 1 (EGR1).[36,39] These two paradigms provide different methods to evaluate MG regeneration in the chick retina and how novel targets respond to either facilitate or inhibit MGPC formation and neurogenesis.

Using this well characterized response of MG to regenerate in chick, we initially characterize the expression of FABP8 in response to NMDA damage (Figure 3). In the undamaged retina, FABP8 is localized to oligodendrocytes. After damage, the retina dramatically upregulated FABP8 spanning the outer nuclear layer (ONL) to the nerve fiber layer (NFL), developing a staining pattern of MG. MG proliferation is optimal at around 48 hours. This proliferative window is when the expression of FABP8 is observed to be at maximal expression, and was confirmed to be localized in MG using Sox2, glutamine synthetase, and FABP8 colocalization. The expression of this protein in MG returns to physiologic levels 72 hours following damage, and MGPCs begin to differentiate into new neurons.

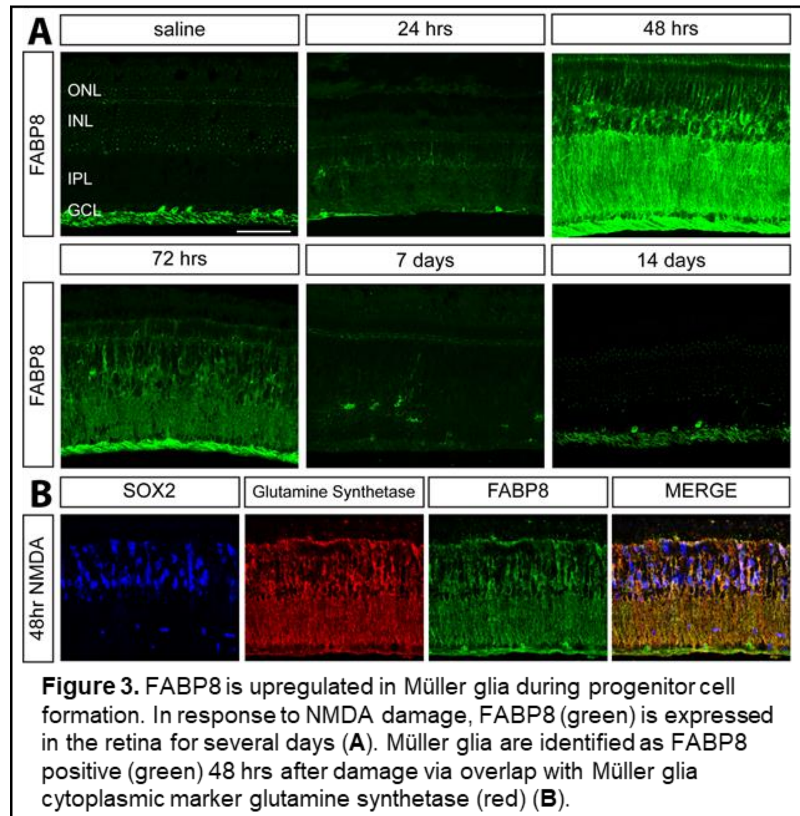

Figure 3. FABP8 is upregulated in Müller glia during progenitor cell formation. In response to NMDA damage, FABP8 (green) is expressed in the retina for several days (**A**). Müller glia are identified as FABP8 positive (green) 48 hrs after damage via overlap with Müller glia cytoplasmic marker glutamine synthetase (red) (**B**).

Inhibition of FABPs reduces the formation of Müller glia derived progenitor cells.

FABP proteins share common tertiary functions, and have unique binding efficiencies for fatty acids.[40] To modulate FABP intracellular function, pharmacological inhibitors that interact with the binding pocket were developed. One of the first inhibitors identified was for FABP4 called BMS309403. Utilizing a fluorescent 8-anilino-8-naphthalene sulfonate (ANS) binding displacement assay of palmitic acid, they observed K_i values of <2nM for FABP4, and K_i values

of 250nM and 350nM for FABP3 & 5 respectively.[41] This has been used in-vivo to reduce atherosclerosis phenotypes presumably through alterations in fatty acid trafficking in macrophages and adipocytes that results in an inhibition of NFkB.[42–45]

The apparent physiological benefit of FABP inhibition has inspired additional work to identify new inhibitors. Dr. Ojima at Stony Brook university has identified a new inhibitor, SBFI-26 with selectivity toward FABP5 with comparable K_i values to BMS309403.[46] Similar to FABP4 inhibition, there was a change in fatty acid trafficking that resulted in analgesic effects, presumably through the polyunsaturated fatty acid anandamide.[26,47] Between BMS309403 and SBFI-26, there is an opportunity to pharmacologically modify the function of FABP proteins to screen for physiologic changes.

These small molecule drugs will be tested in our chick intraocular injection NMDA paradigms that have been previously described.[29] After several days of sustained inhibition with NMDA damage, there was a significant decrease in MGPCs (Figure. 4). The Edu marking proliferating cells was significantly reduced with the administration of BMS309403. This overlap with Sox2 indicated that these cells are MGPC derived, and was found to be reproducible in large cohorts of chicks (n >8).

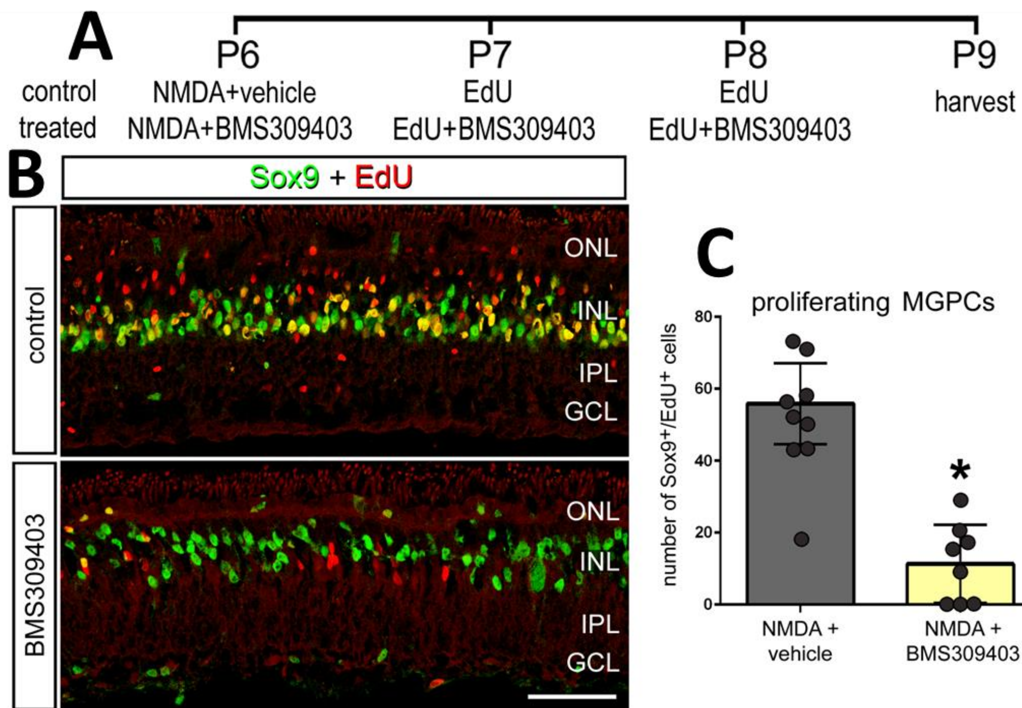

Figure 4. FABP inhibition reduces the formation of MGPCs after NMDA damage. Chicks were injected with excitotoxin NMDA intravitreally with the presence of FABP inhibitor BMS309403 (A). Retinal sections were stained for Sox2 and Edu for the identification of Muller glia derived progenitor cell nuclei seen in yellow (B). The reduction of progenitor cells was quantified (n>8) (C).

Discussion

The research described here seeks to understand the understudied FABP proteins, and investigate their function as a novel target in early reprogramming of MG. FABP proteins are often detected on large genomic screens in highly proliferative states, such as cancer and development, but relatively little is known about their mechanisms of influencing cell biology. FABP8 is a novel target for understanding MG reprogramming and requires innovative approaches to better understand their impact on retinal regeneration.

FABP proteins have been identified in many different contexts, as different isoforms of the FABP family have tissue specific expression. Even so, there has been research associating various tissue FABPs with progenitor cells and regeneration. FABP1, or liver fatty acid binding protein, is upregulated in all states of hepatocyte cell division, and in liver regeneration with 70% resection surgeries.[48–51] FABP4, found in endothelial cells, caused intimal hyperplasia with AAV overexpression vectors leading to vessel stenosis and proteinurea in the heart and kidney respectively.[52,53] In the developing mouse brain, FABP3, 5, and 7 are invested as a potential target to influence dedifferentiation and proliferation.[16–18] FABP8 has been a longtime molecular identifier of peripheral myelin in mammalian systems, and has been researched on its role in structural integrity and axonal regeneration. However, the role of FABP8 in the CNS and retina of nonmammalian systems with robust regeneration phenotypes have not been investigated.

The FABP protein class exhibits high tertiary structure conservation, and may serve redundant overlapping functions with the other isoforms. For example, FABP5$^{-/-}$ knockout is a developmentally normal mouse with no gross neurological abnormalities.[54] This result would limit our capacity to interpret different biological models of FABP expression in the retina. Under the assumption that different isoforms compensate for important cellular functions, we will expand

our characterization to other FABP proteins identified in CNS glia and neurons, such as FABP3, 5, and 7. These proteins have been previously observed in the retina, albeit limitedly, and offer an interesting comparison between species where no prior studies have been conducted.[55,56]

Our lab will create a plasmid construct for MG specific inducible expression of Cas9 and a FABP8 specific gRNA to generate a MG FABP8 knockout in one retina. We will create a construct with the following design: Tol2-RLBP1-Cas9-P2A-rtTA-Tol2:tetO-gRNA. Tol2 is a transposase originally identified in teleost fish that is capable of introducing transposons into the genome of vertebrates.[57] When integrated at an early stage of development with co-transfection with a CAG-transposase, all progeny of early retinal progenitors will contain the gene of interest (mosaic retinal expression). Retinaldehyde binding protein (RLBP1) promoter is specific to MG in the retina, and will drive the expression of Cas9 and the mutant reverse tetracycline transactivator (rtTA). The rtTA is inactive until tetracycline administration which binds to the tetracycline operator (tetO) and drives transcription of the gRNA. Collectively, this will allow for MG specific knockouts of FABP8. Conversely, for simple inducible upregulation of FABP8, we can use a simplified construct: RLBP1-rtTA-Tol2:tetO-FABP8. To determine what cells have been transfected, cells can be stained for Cas9 or rtTA expression and analyze the effect on MG with the quantification. Furthermore, experimentation on these factors in models of mouse retinal damage may give further information about the translational potential of these factors in influencing regeneration in a mammalian model.

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