Microglia-Specific Expression and Transcriptional Regulation of *Apolipoprotein C1 (apoc1)*

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Authorization to Submit Thesis

This thesis of Emma K. J. Esposito submitted for the degree of Master of Science with a Major in Neuroscience and titled "Microglia-Specific Expression and Transcriptional Regulation of *Apolipoprotein C1 (apoc1)*," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Our recent transcriptome analysis[1] found that *apoc1*, which encodes an apolipoprotein with poorly understood central nervous system functions, is highly expressed by zebrafish microglia. We became interested in microglial expression of this gene because *APOC1* has been shown to have a genetic association with human neurodegenerative diseases. Even with disease association, very little is known about the function of Apoc1 in the central nervous system. In fact, microglia-specific expression of this gene, or whether this expression is developmentally regulated, has yet to be definitively demonstrated.

First, we examined the orthologous relationship between human *APOC1* and zebrafish *apoc1* and concluded that these genes are orthologous. We characterized expression patterns of *apoc1* in the developing zebrafish central nervous system using an anti-sense RNA probe. *Apoc1* transcript was localized to microglia in 3- and 5-day old zebrafish retina and brain. There was a dramatic increase in *apoc1* transcripts on a per microglial cell basis during development as well. We also found that *apoc1* mRNA localized exclusively to microglia in adult zebrafish retinas.

To provide insight into the regulation of *apoc1* expression in microglia, we performed *in silico* analysis of the 5' UTR and a 5kb sequence upstream of the predicted zebrafish *apoc1* start site. The transcription factor (TF) binding site analysis indicated that the 5kb region upstream of *apoc1* contains several predicted TF binding sites. We identified TF binding sites included those for RAR:RXR and PPARα:RXR. We therefore hypothesized that the ligands for RAR:RXR and/or PPAR:RXR nuclear receptors may modulate expression of *apoc1*. Using agonists of these nuclear hormone receptors, we found that *apoc1* expression can be upregulated in an RXR dependent manner, but that this RXR mediated induction does not involve the RAR binding partner.

Future experiments will aim to reveal the function of *apoc1* expression by microglia in the CNS and its regulation. Understanding this gene may lead to further discoveries in its role in the CNS and may lead to treatments for neurodegenerative diseases.

Acknowledgements

First and foremost, I would like to thank the members of my committee. Dr. Diana Mitchell has been the most caring and understanding mentor that I could have asked for. She has provided knowledge and guidance that allowed me to complete my program and gave me skills that I will continue to use throughout my career. I thank Dr. Deborah Stenkamp for her extensive knowledge and experience that she brought to this committee. I thank Dr. Allan Caplan for his expertise in the molecular aspects of this project, and knowledge he brought to the committee. I would also like to thank the other members of the Mitchell and Stenkamp labs for their support and assistance throughout this entire project. I also thank the faculty and staff of the Department of Biological sciences for providing a friendly and supportive learning environment. This research was supported in part by the Idaho INBRE program, NIH NIGMS P20 GM103408.

Dedication

I would like to thank my family for all their support throughout my time at the University of Idaho. Debbie and Ralph Esposito have always worked to provide me with a quality education and a home that I could thrive in. I could not be where I am today without their love and support. I also thank my siblings, Paige and Leah, for their constant encouragement to pursue a research career and for all their support.

Table of Contents

List of Tables

List of Figures

Statement of Contribution

The research work presented in this thesis was performed by Emma K.J. Esposito, with the exception of one contribution from Zachary I. Blume (referenced data not shown on page #21).

Chapter 1: Introduction

Macrophages are phagocytotic immune cells that have a variety of functions during tissue development, homeostasis, and repair. A specialized population of macrophages resides in the vertebrate central nervous system (CNS), and are called microglia. In mice, microglia have been shown to regulate vascular endothelial tissue development in the retina, as well as remodel vascular tissue in the eye by mediating apoptosis[2]. During zebrafish development, microglia have a similar role of removing apoptotic cells in the head and eyes[3,4] and in the brain[5]. Macrophages also secrete growth factors, cytokines, and other proteins that can remodel the extracellular matrix, which possibly indicates involvement in organogenesis[6]. In addition, microglia have been recently discovered to have roles in synaptic pruning in mice in the CNS and retina[7]. In healthy developing brains of mice, microglia made direct and transient connections with neuronal synapses based on the amount of neural activity[8]. Presynaptic and postsynaptic cellular components were found inside of microglial lysosomes, providing further evidence that immune cells are actively working in synaptic pruning[8].

Tissue damage propagates an immune response by macrophages that leads to phagocytosis of cellular debris and influences healing of damaged areas. Macrophages undergo phenotypic changes when tissue damage occurs, regulate other fibrotic or pro-inflammatory macrophages, and produce mediators that regulate progenitor cells during tissue regeneration[6]. However, while this macrophage-mediated regeneration process in non-CNS tissue has been shown in both mouse[9] and zebrafish[10], tissue regeneration does not occur in damaged CNS tissue of mammals. Due to the presence of the blood brain/retina barrier, under normal conditions macrophage precursors (which circulate as monocytes) cannot enter CNS tissue, and instead the CNS has the resident microglia population. These microglial cells originate from the hematopoietic stem cell precursors in the yolk sac, that travel to populate the brain and differentiate from macrophages into primitive microglia, making them genetically similar and ontogenetically related to macrophages[11]. Microglia are involved in phagocytosis and maintaining homeostasis of CNS tissue[12,13].

Microglia are abundantly present in the vertebrate retina and actively communicate with other retinal cells, such as Müller glia[11]. In the mammalian retina, microglia respond to damage and can initiate inflammatory responses[11]. This response is necessary for clearing cellular debris but can lead to damage if it becomes chronic, and since the retina is a part of the CNS retinal tissue regeneration does not occur for mammals. When damage occurs in CNS tissue of mammals, glial cell accumulation (including microglia and in addition, often macrophages from outside of the retina) and scar

formation occurs, preventing regeneration of tissue[11,14,15]. However, other vertebrate species possess the ability to regenerate CNS tissue. One of these vertebrates, *Danio rerio* (aka zebrafish), has this unique ability, and for this reason, zebrafish have gained popularity as a model organism for research in retinal regeneration and degenerative diseases[16–18]. Further, the ease of imaging in the eye and brain in early developmental stages makes them a popular organism to use to gain insight into conserved developmental processes in vertebrates[4,12].

There have also been many discoveries of genetic orthologs and conserved function between zebrafish and human innate immune system cellular mechanisms[19–23]. Due to the similarities of vertebrate innate immunity, zebrafish are an excellent model organism for understanding the immune systems role in homeostasis, damage, and regeneration of CNS tissue[22]. It is becoming clear that zebrafish are an appropriate model for microglial biology[1,5,12,24–27]. Zebrafish have even been used to visualize single cellular interactions of microglia, as well as entire networks of glia in the larval brain[12] and phagocytic events in real-time[4,28]. In recent research, the presence and morphological activation of macrophages and microglia in the retina of zebrafish have been correlated with retinal regeneration after tissue damage[29]. Mitchell et al. (2018) showed that immune cells accumulate in response to induced lesions of the adult zebrafish retina. Interestingly, these responding immune cells appear to include both resident microglia and macrophages that infiltrate from outside the retina[29]. In addition, microglia retain their morphological appearance for several weeks during retinal regeneration[29], suggesting they have a function during retinal regeneration, though such functions remain to be determined.

As discussed above, zebrafish are becoming a popular model organism to uncover microglial biology. In order to suggest putative functions of microglia during CNS regeneration, and to reveal the genetic expression patterns and signatures of microglia in zebrafish, a transcriptome analysis was performed on microglial cells present during retinal regeneration[1]. From this experiment and analysis, hundreds of genes were found to be highly expressed by zebrafish retinal microglia. The function of many of these genes are not known, both in general and/or in the context of microglial biology. Interestingly, several of the genes identified with enriched expression in zebrafish microglia were predicted orthologs of genes considered to be expressed by “Disease Associated Microglia” in mouse models of neurodegenerative diseases[30,31]. One of such genes, *apoc1*, which encodes an apolipoprotein, was found to be highly expressed by zebrafish microglia during retinal regeneration[1]. Another published RNA-seq study indicates that this gene is also highly enriched in zebrafish microglia in homeostasis[25].

The function of microglia expressed *apoc1* has not been identified in any species, but interestingly, *APOC1* appears to also be highly expressed by microglia in humans[32]. Gosselin et al. (2017) also showed that mouse microglia express relatively low levels of *Apoc1* compared to human microglia. Therefore, we consider that zebrafish is a preferred model organism to study this gene in the context of microglial function in the CNS. In further support of this approach, comparisons of human *APOC1* and zebrafish *apoc1* indicate that these genes have an orthologous relationship (See Chapter 2).

Although, the function of *APOC1* in the CNS is not understood, *APOC1* has been linked to neurodegenerative diseases in humans. *APOC1* has been proposed as a risk factor for Alzheimer's Disease (AD)[33]. It was found that different *APOE* alleles, a gene well known for its association with AD, and located immediately upstream of the *APOC1* in humans (with similar chromosomal organization in zebrafish), may affect expression of neighboring genes, including *APOC1*[33]. In addition, the APOC1 protein was also found at elevated levels in glioblastomal cysts in human patients[34]. Evangelou et al. (2019) also found that the mean mRNA levels of *APOC1* were the highest in glioblastoma cysts and hypothesized that *APOC1* plays some role in the cyst formation.

While *APOC1* has been linked to neurodegenerative diseases, its normal function in both human and zebrafish CNS tissue is not known. In the context of the peripheral body (outside of the CNS), proteins in the apolipoprotein family are known have roles in the assembly of lipoproteins, which are soluble proteins that combine with fats or lipids to facilitate transport and cellular uptake, such as VLDL, LDL, and HDL[35]. They also facilitate metabolic processing of endogenous and exogenous lipids in cells, act as ligands for some receptors, and modulate activity of other proteins[35]. In the periphery, *Apoc1* is mainly expressed in the liver, where it is involved in lipid transport and metabolism[35]. In macrophages, it is highly upregulated during monocyte differentiation *in vitro*[36], which makes sense given that peripheral macrophages are involved in lipoprotein metabolism. In a study that silenced *APOC1* in monocyte derived macrophages using targeted siRNAs, macrophage uptake of LDL was reduced[37].

Due to the fact that human *APOC1* and zebrafish *apoc1* are highly orthologous and have a poorly understood central nervous system function yet this gene is correlated to neurodegenerative diseases, zebrafish provide an excellent system to characterize and study the regulation and function of this gene. Towards this end, microglial-specific expression patterns of *apoc1 in situ* in developing zebrafish embryos and adult zebrafish retinas were demonstrated and characterized (Chapter 2). We also analyzed the putative upstream *apoc1* promoter region and found that there were predicted RAR:RXR and PPAR:RXR transcription factor binding sites. We therefore hypothesized that ligands

for these nuclear hormone receptors regulate *apoc1* expression in microglia. To test this hypothesis, we determined the effects of RAR and RXR agonists on *apoc1* expression in vivo (Chapter 3). In order to make tools to facilitate the study of *apoc1 in vivo*, we also attempted to generate a zebrafish transgenic reporter line that had the putative *apoc1* promoter driving expression of GFP (Chapter 4).

Chapter 2: Expression Characterization of Zebrafish *apoc1*

Introduction

In this chapter, we provide evidence that human *APOC1* and zebrafish *apoc1* are evolutionarily related and therefore we predict have a conserved function in both species. We demonstrate that *apoc1* expression is exclusive to microglia in the developing zebrafish CNS and in adult zebrafish retina, using an in-house generated RNA probe specific to *apoc1*. We determined that *apoc1* is expressed in microglia at as early on as 3 days post fertilization (dpf) in developing zebrafish, and that *apoc1* transcript levels increase dramatically in microglia from 3 to 5 dpf.

Methods and Materials

Animal care

All procedures using zebrafish were performed in compliance with IACUC (Institutional Animal Care and Use Committee) approved protocols at the University of Idaho. Adult zebrafish (*Danio rerio*) were maintained on a 14:10 hour light:dark cycle in 28.5°C recirculating, monitored system water, and were housed and propagated following Westerfield (2007). Zebrafish lines used in this work include a wild-type strain, referred to as SciH, originally obtained from Scientific Hatcheries (now Aquatica Tropicals), and *mpeg1*:mCherry [38] (originally obtained from Zebrafish International Resource Center, ZIRC). Embryos were collected into glass beakers in the morning, with light onset considered to be zero hours post fertilization (hpf), and water was refreshed daily until experimental endpoints. Zebrafish cannot be sexed before reproductive maturity and so could not be determined for experiments involving embryonic zebrafish; adult zebrafish of both sexes were used for collection of adult retinal tissue.

RNA preparation, cDNA synthesis, PCR, and apoc1 cDNA cloning

Following dark adaption, whole eyes were enucleated from one year male and female SciH adult zebrafish. Retinas were dissected from eyes as described [29] and the retinal pigmented epithelium (RPE) was removed. Retinas were submerged in lysis buffer (Machery-Nagel RNA extraction kit) and homogenized using a pellet pestle. RNA was extracted using an RNA extraction kit (Machery-Nagel), following the manufacturer's protocol. A Nanodrop® ND-1000 Spectrophotometer was used

to check RNA yield and quality. Synthesis of cDNA was performed using SuperScript® IV Reverse Transcriptase kit (Invitrogen), with random hexamer and oligodT primers.

Three separate primer pairs (Table 2.1) were used to amplify selected *apoc1* cDNA sequences. Primers were designed based on the Ensembl database, using the zebrafish genome build 11 (GrcZ11). PCR reactions were performed using Q5 polymerase Master Mix (NEB). A volume of 1 μL of cDNA was used as template, and the manufacturer's recommended cycling conditions were used. PCR products were transferred to gel electrophoresis (2% agarose, with TAE (Tris base, acetic acid, EDTA) Buffer) and imaged using a Bio-Rad Gel Doc-1000 and Quantity One imaging software. Subsequently, using a blue light box (Clare Chemical Research Dark Reader® Transilluminator), bands were excised from the agarose gel then extracted using a NEB Monarch Gel Extraction kit. To increase product yield, the extracted PCR products were used as templates in a second PCR re-amplification reaction using the same primer pairs. The re-amplified PCR products were then again run on an agarose gel and extracted.

Purified PCR products were ligated into the pMiniT vector using the NEB® PCR cloning kit, following the manufacturer's instructions. A ratio of 3:1 (insert:vector) was used. The ligation product was transformed into NEB 10-beta E. coli competent cells following the manufacturer's protocol, and transformants were plated and grown on LB-Amp plates. Single colonies were then selected to inoculate liquid cultures. Plasmids were extracted using QIAprep® Spin Miniprep kit (Qiagen). Plasmids were screened for successful ligation using restriction enzyme digestion. Plasmids containing inserts of correct size were verified by Sanger Sequencing using the Cloning Analysis Primers provided by the NEB cloning kit. Sanger Sequencing was performed at Washington State University for Reproductive Biology Core (WSU CRB). Sequences mapped to the expected exons 1-4 of *apoc1*, which included the 5' and 3' UTR, and excluding the intronic regions (Supplemental Figure 1). Of the three primer pairs, the cDNA product from primer pair 2 (99% ID to *Danio rerio apoc1* mRNA consensus sequence) was selected for use as a template to generate RNA probes.

Table 2.1 Primer sequences for zebrafish *apoc1* cDNA cloning

	Forward Primer (5'→3')	**Reverse Primer (5'→3')**
Primer Pair 1	CGAGAGATGAACGCGAGGAA	AAATGTGCCAGTCGGCTCAA
Primer Pair 2	AAGCGAGTGATTGCAGGAGG	AATGTGCCAGTCGGCTCAAC
Primer Pair 3	AGGGACAAGCCATCTGTGGG	GCCAGTCGGCTCAACAGTTT

Generation of RNA probes for in situ hybridization

Purified plasmid containing *apoc1* cDNA insert (primer pair 2 product) was linearized with PacI or BamHI, then precipitated with 1.5 volume of cold 100% Ethanol, and stored at -80C for 2 hours or overnight. Linearized plasmids were then spun twice at 14,000xg for 10 min, with one rinse in 1 mL 70% Ethanol in between spins. The supernatant was decanted, and the pellet was air dried, then resuspended in 50 µL of RNAse-free water. 1 µg of the linearized template was used for in vitro RNA transcription, using either T7 or SP6 polymerase (to generate both sense and anti-sense probes), and DIG-labeled RNA nucleotides using the DIG RNA Labeling Kit (SP6/T7) (Millipore-sigma). At the end of the reaction, tubes were spun twice for 15 minutes at 14,000xg with one rinse with 70% Ethanol in between. Supernatant was then decanted, and the RNA pellet was air dried, then resuspended in 50 µL RNAse-free water. Probe concentration was measured using a Nanodrop® ND-1000 Spectrophotometer, then aliquoted and stored at -20°C until use.

In situ hybridization of fixed tissue

Whole retinas were dissected from 6 month old *mpeg1*:mCherry transgenic [38] fish using the same protocol described above. The retinas were fixed in a 4% PFA in 1X PBS RNAse-free solution overnight at 4°C, washed in 100% methanol, and stored at -20°C in 100% methanol. The *in situ* hybridizations were carried out as previously described [39]. In brief, the tissue was rehydrated in a decreasing concentration serios of methanol, treated with (10 µg/mL) proteinase K for 30 minutes, and hybridized overnight at 56°C with 1 mg/ml probe in probe hybridization solution. An anti-DIG-POD antibody (Millipore-sigma), followed by tyramide signal amplification with a Fluorescein fluorophore (Perkin Elmer® TSA™ kit), was used to amplify the probe hybridization signal for fluorescent detection.

At 3 and 5 dpf, *mpeg1*:mCherry zebrafish embryos were anesthetized and fixed in a 4% PFA in 1X PBS RNAse-free solution overnight at 4°C, washed in 100% methanol, and stored at -20°C in 100% methanol. The *in situ* hybridizations were carried out as described before for the whole retina protocol with minor changes. Dehydration in 100% methanol and a Xylene wash were included before the rehydration in order to clear the pigment accumulated at this stage in development. The proteinase K treatments were also shortened to 10 minutes for the 3 dpf embryos and 20 minutes for the 5 dpf embryos.

Immunolabeling of whole fixed tissue

Due to degradation of the transgenic fluorescent signal during the *in situ* procedure, immunolabeling was performed to detect the mCherry protein. After the *in situs* were performed the tissue was washed with PBST (phosphate buffered saline, 0.1% Tween) then placed into antibody dilution buffer over

night at 4°C with agitation. The tissue was washed again in PBST then placed into primary antibody solution containing rabbit anti-mCherry at 1:100 dilution (Genetex) and DAPI (Thermofisher) overnight at 4°C with agitation. The tissue was washed in PBST and placed into secondary antibody solution containing donkey anti-rabbit Cy3 antibody (Jackson Immunoresearch) overnight at 4°C with agitation. The tissue was washed again in PBST and received a final wash in PBS (phosphate buffered saline). Whole retinas were flattened and mounted on glass slides in Vectashield Vibrance (Vector Laboratories) and embryos were mounted in glycerol and imaged using 1.0 coverslip glass bottom dishes (MatTek Corporation).

Microscopy and imaging

Images were acquired using a Nikon Andor spinning disk confocal microscope equipped with a Zyla sCMOS camera and computer running Nikon Elements software. Imaging was performed using a 20X air objective. For whole retinas and embryos, z stacks were obtained at 3-5 μm intervals. Z stack images were visualized using Nikon Elements software.

Statistical Analysis

Graphing and statistics were performed using GraphPad Prism software. For comparisons of two experimental groups, analysis of variances was performed using a F test and normality was analyzed using the Shapiro-Wilk test. If the data was normally distributed and the variances were equal, an Unpaired t- test was performed to analyze difference between groups. For the cell counts (Figure 3E) a Fisher's exact test was used to analyze the difference. When comparing more than two experimental groups One-way ANOVA (Kruskal-Wallis) was performed, then if indicated, a post-hoc Dunnett's multiple comparisons test to analyze statistically significant differences. P values below the cut-off ($p<0.1$) are reported in the text, figures, and figure legends.

Results

Orthology of human APOC1 and zebrafish apoc1

We first compared the chromosomal region containing the human (*APOC1*), mouse (*Apoc1*), and zebrafish (*apoc1*) genes. All three species show similar organization including chromosomal clustering of apolipoprotein genes with the *apoeb* (zebrafish; *APOE*: human, *Apoe*: mouse) gene upstream of *apoc1* in all three species (Figure 2.1A-C). Other similarities include other apolipoprotein genes (*apoc2* and *apoc4*: zebrafish)(*APOC2 and APOC4*: human)(*Apoc2 and Apoc4*: mouse), are found downstream of *apoc1* in all three species. Although, *Tomm40* (mouse) and *TOMM40* (human) are upstream of *apoc1* in mice and humans, respectively zebrafish *tomm40* lies roughly 2Mb downstream, and in the opposite orientation, compared to *apoc1* in zebrafish (Figure 2.1A-C). In

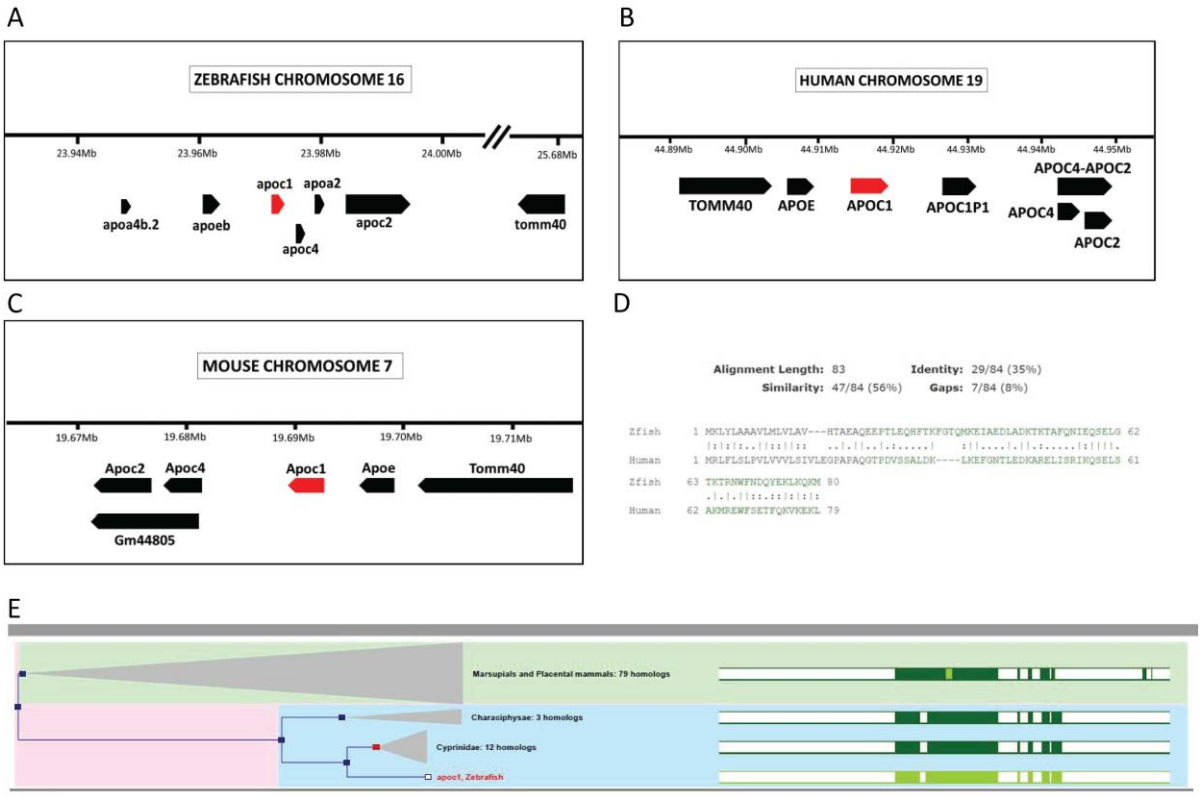

Figure 2.1. Orthology of human *APOC1* and zebrafish *apoc1*. Chromosomal regions pertaining to the location of human *APOC1* **(A)** zebrafish *apoc1* **(B)** and mouse *Apoc1* **(C)** genes. In all three species, the apolipoprotein genes show chromosomal clustering. The immediate upstream and downstream genes of *apoc1* in zebrafish are similar in humans and mouse, indicating evolutionary retention of these genes amongst species. Amino acid alignment (Uniprot) of human and zebrafish APOC1 are shown in **(D)**. The lines indicate fully conserved residue, a colon (:) indicates conservation between groups with strongly similar properties, a period (.) indicates conservation between groups with weakly similar properties, and the green letters indicate sequences that are found in conserved domains database (CDD) **(D)**. **(E)** Gene tree of *apoc1* (image taken from Ensembl) tracing lineage of the gene through different species (Dark green bars represent consensus sequences, and light green bars represent protein alignments).

humans, there is also a pseudogene (*APOC1P*) downstream of *APOC1* that is not annotated in mouse or zebrafish genomes. Another difference in apolipoprotein gene clustering is that *apoa4b.2* is found upstream of *apoc1* in zebrafish, but in humans (*APOA4*) it is found on chromosome 11 and in mice (*Apoa4*) it is found on chromosome 9. An amino acid alignment (UniProt) was also performed between the two species showing similarity of 56%, and conserved identity of 35% (Figure 2.1D). We also used Ensembl to create a gene tree for *apoc1*, to further investigate the relationship of the zebrafish *apoc1* gene to other species. Based on this gene tree, there is an apparent a common ancestral *apoc1* gene that gave rise to both the mammalian and zebrafish genes (Figure 2.1E).

To further examine orthologous relationship of human *APOC1* and zebrafish *apoc1*, we used the DRSC Integrative Ortholog Prediction Tool (DIOPT). The DIOPT is an ortholog and paralog search tool that compares ortholog predictions from multiple algorithms, such as Compara, eggnog and OrthoDB[40]. The DIOPT score was 10 for *apoc1* when comparing human and zebrafish genes,

Tool	Simple Score	Weighted Score	Original Tool Information			
			BLAST Result	Score	Score Type	Cluster ID
Compara	1	0.930				C1151282
eggNOG	1	0.900				E1_2E8B8
Hieranoid	1	1.000				
Homologene	0	0.000	*Not matched by this tool.*			
Inparanoid	1	1.050	53	1.000	Inparanoid score	I12312
OMA	0	0.000	*Not matched by this tool.*			
OrthoDB	0	0.000	*Not matched by this tool.*			
OrthoFinder	1	1.000				FOG0006587
OrthoInspector	1	1.000				T1603566
orthoMCL	0	0.000	*Not matched by this tool.*			
Panther	1	1.100			LDO	PTHR16565
Phylome	0	0.000	*Not matched by this tool.*			
RoundUp	1	1.030		avgDist	Average_Evolutionary_Distance	R8175
TreeFam	1	0.960				
ZFIN	1	1.500				
	10	**10.470**				

Input Order	Search Term	Zebrafish GeneID	ZFINID	Zebrafish Symbol	Species 2	Human GeneID	Human Species Gene ID	Human Symbol	DIOPT Score	Weighted Score	Rank	Best Score	Best Score Reverse	Prediction Derived From	Alignment & Scores	Feedback	Gene2Function Details
1	apoc1	570638	ZDB-GENE-030131-1074	apoc1	Human	341	607	APOC1	10	10.47	high	Yes	Yes	Compara, eggNOG, Hieranoid, Inparanoid, OrthoFinder, OrthoInspector, Panther, RoundUp, TreeFam, ZFIN	View	Add	APOC1 details

Figure 2.2. Output data from the DRSC Integrative Ortholog Prediction Tool. The different ortholog prediction algorithms that DIOPT compares are on the left. The simple score from each algorithm predicts whether *apoc1* is orthologous (1) or not (0) to human *APOC1*. The weighted score is based on each of the algorithms prediction score that DIOPT assigns it[40]. Overall, the DIOPT tool gave zebrafish *apoc1* and human *APOC1* high likelihood of being orthologues.

meaning that 10 of the algorithms that DIOPT uses, recognized the human and zebrafish genes as orthologs (Figure 2.2). DIOPT analysis also showed that the orthology was ranked "high" meaning that the pairs had the best scores for either forward or reverse searches and had an overall score of above 2 (Figure 2.2). Collectively, it can be concluded that human *APOC1* and zebrafish *apoc1* are orthologs. This orthologous relationship supports that the zebrafish is an appropriate model organism to study this gene.

Apoc1 expression by microglia

In our previous report describing the transcriptome of zebrafish microglia isolated from regenerating retinas[1], *apoc1* was the most significant differentially expressed gene enriched in microglia. We therefore used the tools at www.zfregeneration.org[41] to re-examine *apoc1* expression in another published study[25], which described the transcriptome of zebrafish brain microglia as well as our own RNA-seq experiment (Figure 2.3A-B). In the zebrafish brain, microglia abundantly express *apoc1* (normalized transcript count over 7000) when compared to other brain cells (Figure 2.3A). Following acute damage of the brain, *apoc1* is still highly expressed (normalized counts over 3000), but at lower

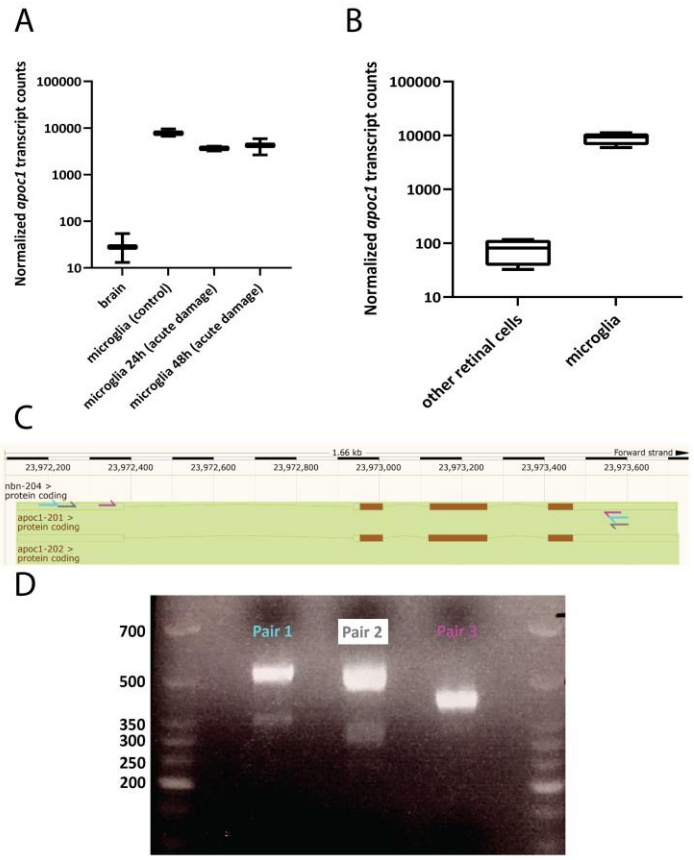

Figure 2.3. Expression of *apoc1* in the zebrafish CNS measured by RNAseq and RT-PCR. (A-B) The expression of *apoc1* in microglia in the adult zebrafish CNS based on RNAseq. **(A)** Normalized transcript level of *apoc1* for microglia isolated from steady-state brain (control) and from brains during acute tissue damage (24h, 48h post damage) [25]. **(B)** The transcript levels of *apoc1* in microglia and other cell types from regenerating retinas [1]. The fkbm generated for these graphs was obtained from zfregeneration.org. **(C-D)** RT-PCR was used to amplify *apoc1* cDNA from adult wild type zebrafish retinas in order to confirm expression in steady state retinas. **(C)** Three primer pairs depicted as three different colors in the figure (pink, aqua, and gray) were used to perform the RT-PCR that had various alignments to the predicted mRNA of *apoc1*. The three products were visualized using gel electrophoresis **(D).**

levels than when compared to steady state microglia (Figure 2.3A). During zebrafish retinal regeneration, microglia also express *apoc1* at highly abundant levels (normalized transcript counts over 10000) than other retinal cells (Figure 2.3B).

In order to confirm expression of *apoc1*, specifically in the zebrafish retina, we first extracted mRNA from adult zebrafish retinas and performed reverse transcription to make cDNA. To amplify cDNA corresponding to *apoc1* mRNA transcripts, we designed three primer pairs for PCR (Figure 2.3C). These primer pairs hybridize in the 5'UTR/first exon and 3'UTR/last exon of a*poc1*, and are expected to detect both previously described transcript variants of zebrafish *apoc1*[42]. Gel electrophoresis revealed RT-PCR products at the expected size from each primer pair (Figure 2.3D). These amplified cDNAs were cloned and sequenced revealing identity comparisons to be 99% for all three primer

Figure 2.4. Sequence analysis for *apoc1* primer products. Sanger sequencing results for cloned RT-PCR products obtained from the three primer pairs **(A-C).** Sequences were blasted against the NCBI zebrafish genome database, and all three products had alignment identities of 99% to *apoc1* consensus sequence **(right)**. The sequences were also blasted against the Ensembl zebrafish genome database, revealing alignment only to the exons of *apoc1* **(left)**.

pairs (Figure 2.4A-C). We chose the product from primer pair 2 to serve as a template for generation of sense and anti-sense RNA probes to detect *apoc1* transcripts *in situ*.

We first examined expression of *apoc1 in situ* in embryonic zebrafish at 3 and 5 dpf, to determine if microglia express *apoc1* during early brain and retinal development. We chose these time points because microglia colonization of the retina and brain occurs by 3 dpf[4,26,43,44]. In order to confirm that *apoc1* was expressed by microglial cells, *in situs* were performed using *mpeg1*:mCherry fish, which is a transgenic line in which macrophages and microglia are labeled by mCherry[4,29,45]. Validity of the probe was confirmed by the fluorescence only being present in the presence of the anti-sense probe and not the sense probe (Figure 2.5A-B). Validity of the probe was further supported by visible

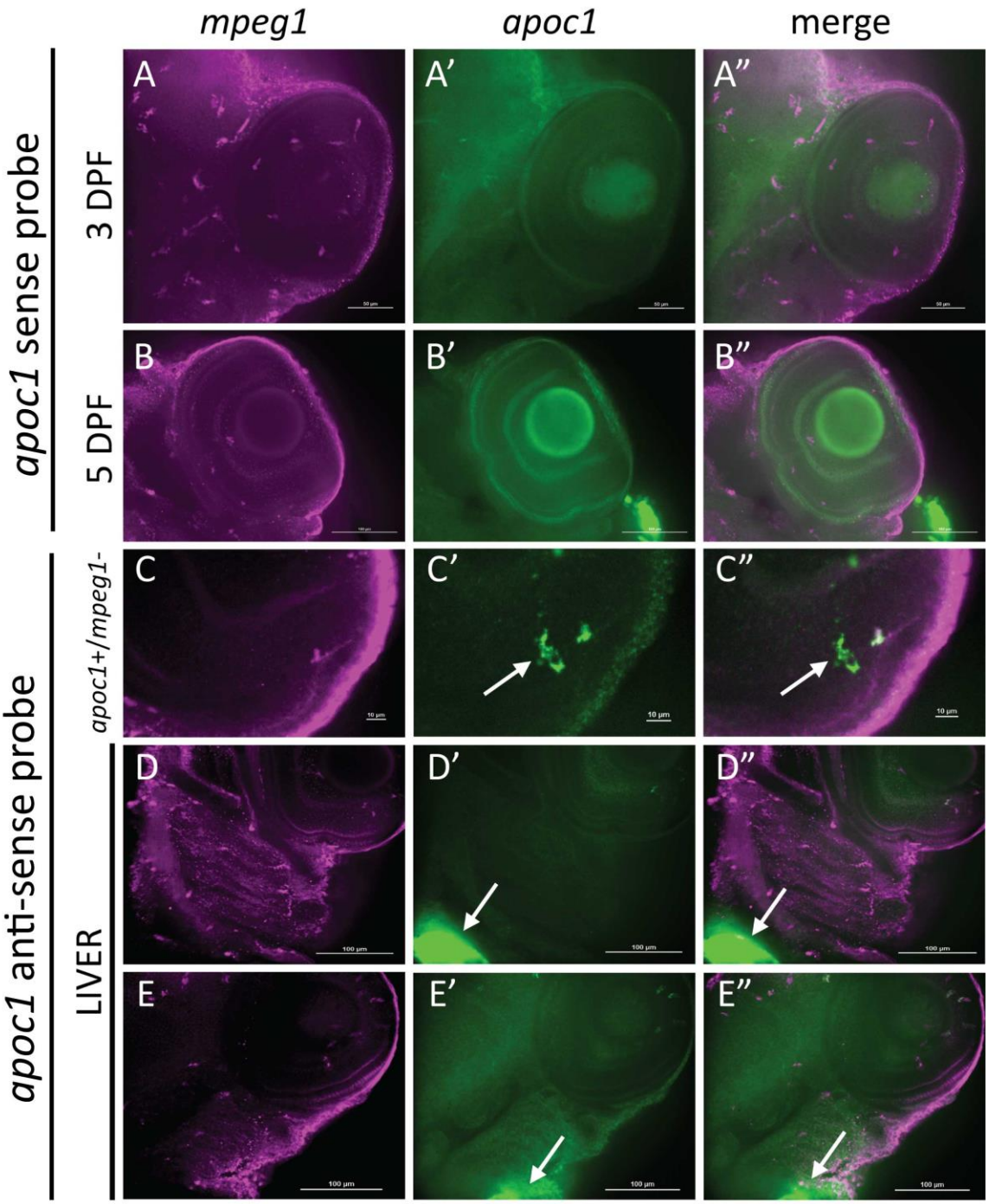

Figure 2.5. Validation of *apoc1 in situ* probes. The validity of the *apoc1* probe was verified by the absence of fluorescence from the sense probe in 3dpf (A) and 5dpf (B) *mpeg1*:mCherry (magenta) larvae. (C) Using the anti-sense probe, an image of a cell (possibly a microglia cell, based on morphology), only expressing *apoc1* (green, arrow) and lacking expression of *mpeg1*:mCherry (magenta). (D) Due to presumed high expression of *apoc1* in the developing zebrafish liver, we confirmed strong fluorescent of the *apoc1* anti-sense probe (green fluorescence) in the region of the developing liver (arrow). Images shown are flattened z projections and each scale bar represents 100µm (B, D, E), 50µm (A), and 10µm (C).

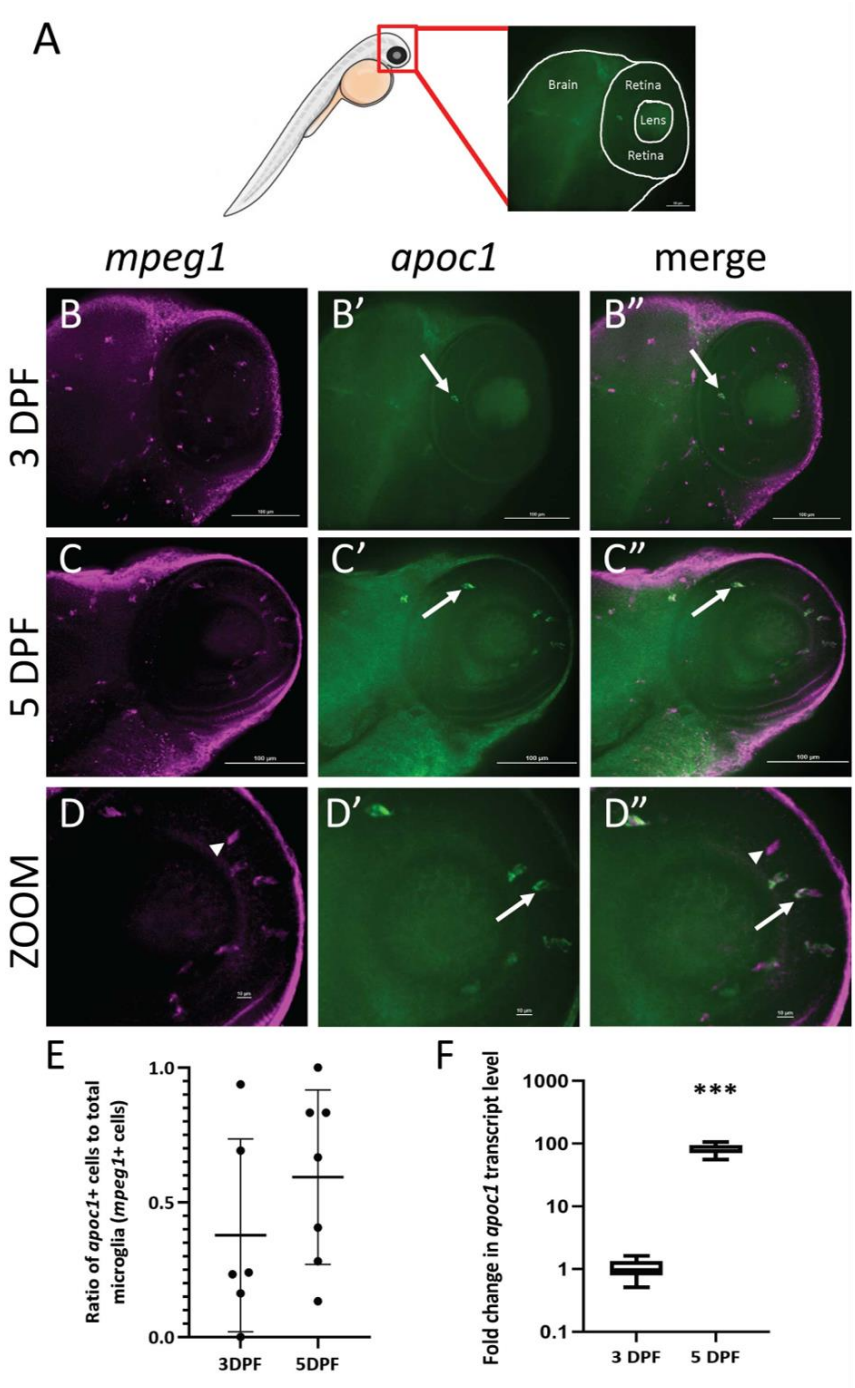

Figure 2.6. Expression of *apoc1* by zebrafish microglia during early CNS development. *In situ* hybridization was performed using *mpeg1*:mCherry transgenic zebrafish larvae [45], in which microglia are labeled by mCherry (magenta) fluorescence [29]. **(A)** Shows the orientation of the embryos when images were taken and outline of anatomical structures (Larval drawing provided by Mind the Graph). Green fluorescence corresponds to *apoc1* transcript. **(B-B'')** Images of embryos at 3 dpf (n=6). **(C-C'')** Images of embryos at 5dpf (n=7). *Apoc1* transcripts co-localized with microglia (white arrows). **(D-D'')** Zoomed image of microglia in the retina of a 5dpf embryo that express both *apoc1* and *mpeg1* (arrow) and microglia that only express *mpeg1* (arrowhead). **(E)** Quantification of the ratio of *apoc1+mpeg1+* cells out of total *mpeg1+* cells in the retinas of embryos/larvae at 3 dpf and 5dpf. Each circle represents an individual embryo/larva and the bars represent standard deviation. **(F)** Quantitative RT-PCR analysis of *apoc1* transcript levels in heads from 3 and 5dpf embryos ($p<0.001$, Unpaired t-test). Images shown **(A-D)** are flattened z projections and each scale bar represents 100µm **(B and C)** and 10µm **(D)**.

fluorescence in the region of the developing liver in the presence of the anti-sense probe, where *apoc1* is highly expressed[35,42] (Figure 2.5D-E).

Co-expression of *mpeg1*:mCherry and *apoc1* confirmed that microglial cells in the developing brain and retina at both 3 and 5 dpf express *apoc1*, and essentially all the *apoc1* transcripts were localized to microglia (Figure 2.6). There were only very rare instances of *apoc1* signal that was not localized to microglia (Figure 2.6 and Supplemental Figure 2.5C); however, morphological pattern of signal suggest these are also microglial cells that may not yet express the *mpeg1*:mCherry reporter. *Mpeg1:*mCherry+ microglia not expressing *apoc1* were a more common occurrence in both 3 and 5 dpf embryos (Figure 2.6B, 2.6C).

We quantified the number of *apoc1*+ microglia out of total microglia in the retina at 3 and 5 dpf, to determine if more microglia begin to express *apoc1* over this developmental period. Although there was possibly a subtle increase in these numbers, ratios varied widely in individual embryos, and the differences from 3 to 5 dpf were not statistically significant (Fig 2.6E). Further, RT-qPCR revealed a nearly 100 fold increase (p-value < 0.0001) in *apoc1* transcript levels in the heads of zebrafish between 3 and 5 dpf (Figure 2.6F). This indicates that from 3 to 5 dpf, while more microglia may begin to express *apoc1*, *apoc1* transcript levels are strongly increased on a per microglial cell basis. We next examined adult zebrafish retinas for microglial expression of *apoc1*. Expression of *apoc1* was confirmed in adult *mpeg1*:mCherry retinas using *in situ* hybridizations (Figure 2.7). In the adult retina, most microglia express *apoc1*, as nearly all *mpeg1*+ cells co-expressed *apoc1* (Figure 2.7B), though the expression of *apoc1* in each individual cell appears to be heterogenous (Figure 2.7C). Each microglial cell appeared to express varying amounts of transcript (based on intensity of visible fluorescent signal), and probe signal was variable in different areas of the cell, such as the soma, microglial processes and appendages (Figure 2.7B" and 2.7C"). In fact, the fluorescent signal from

apoc1 transcript appeared to more uniformly label the microglial cell, including the cell body and processes, than *mpeg1*:mCherry signal (Figure 2.7C").

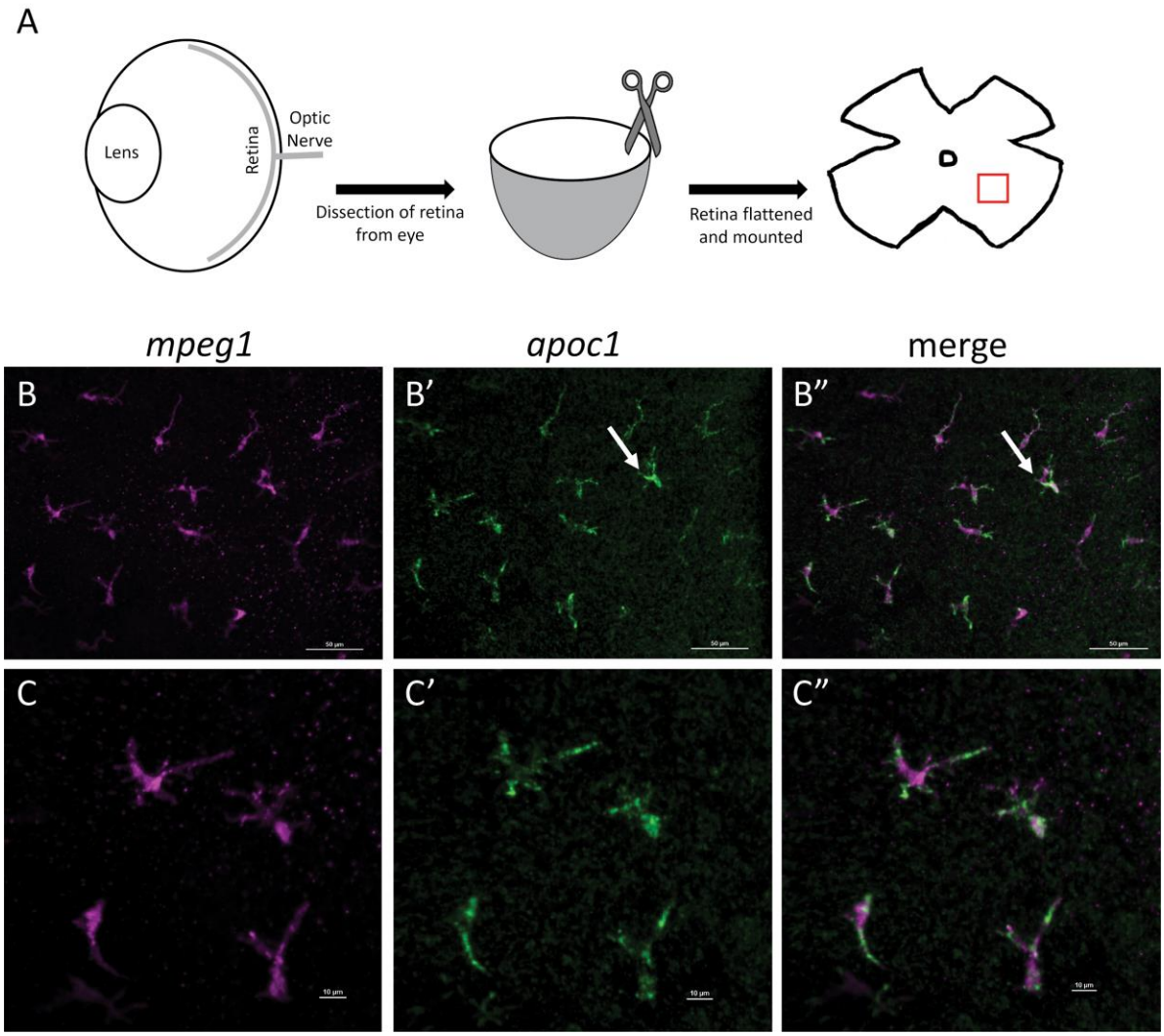

Figure 2.7. Expression of *apoc1* in adult zebrafish retinal microglia. *In situ* hybridization was performed using retinas from *mpeg1*:mCherry transgenic adult zebrafish[45], in which microglia are labeled by mCherry (red) fluorescence [29]. **(A)** Diagram showing the basic procedure for dissection and imaging of whole retinas. The red square on the flattened retina represents area of imaging. **(B-B'')** In adult retinas (n=4) *apoc1* transcripts are localized to microglia (as indicated by the arrows) (Scale bar represents 50μm). **(C-C'')** Enlarged images to visualize the localization of *apoc1* expression in microglia cells. In individual microglia the expression of *apoc1* appears to be heterogeneously expressed throughout the entire cell, while *mpeg1* does not always label the entire cell (processes, etc.) (Scale bar represents 10μm). Images shown **(B-C)** are flattened z projections.

Conclusions and Discussion

In this chapter, we show evidence of the orthologous relationship between human *APOC1* and zebrafish *apoc1*. Both species have similar chromosomal arrangement with neighboring genes and the protein sequences are well conserved. The gene tree also indicates a common ancestral gene that gave rise to human *APOC1* and zebrafish *apoc1*. Next we examined the expression of *apoc1* in the zebrafish CNS. Using *in situ* hybridization, we found that *apoc1* was exclusively expressed by microglia in the retina and brain of developing embryos and adult zebrafish. Onset of *apoc1* expression by microglia begins during early CNS development, when microglia colonize the brain and retina. Microglia-specific expression of *apoc1* also appears to be maintained into adulthood.

Due to the orthologous nature of human *APOC1* and zebrafish *apoc1* as discussed in this chapter, the zebrafish is the appropriate model organism to study the function of *apoc1* in microglia. This is further supported by our evidence of *apoc1* expression being exclusive to microglia in CNS tissue. Our results also coincide with other studies that show *apoc1* expression is exclusive to microglia via transcriptome analysis[1,25]. It has also been noted that mice may not be top choice for studying microglia in all contexts[32]. Gosselin et al. (2017)[32] performed a transcriptome analysis on human and mice microglia and related the results to neurodegenerative associated disease genes. One notable result from this experiment was that mouse microglia do not express *Apoc1* to the same extent that human microglia express *APOC1*[32]. The result from the Gosselin et al. (2017)[32] study, the orthologous relationship between human *APOC1* and zebrafish *apoc1*, and our results showing that zebrafish microglia, like human microglia, express high levels of *apoc1* transcripts all suggest that the zebrafish is a highly suitable model organism to study *apoc1* in the context of microglial biology.

Some studies have been done to investigate the function of *apoc1* in CNS tissue. One study knocked out *Apoc1* in mice, and found that the mutated mice had impaired memory functions[46]. These results are interesting because in a previous study done by the same group, they showed impaired memory function in mice that were forced to express human *APOC1*[47]. Since mice seem to express *Apoc1* to a lesser extent than humans in microglia, it may have a different function in mice or be expressed by a different cell type, such as astrocytes. The impaired memory functions in the knockout mice could be caused by the lack of *Apoc1* resulting in disrupted synaptic function, as *Apoc1* may be necessary for lipid recycling and uptake at the synapses. They also found there was no change in brain morphology or sterol concentrations in the knockout mice[46]. *Apoe* mRNA levels were also reduced but Apoe protein levels were not affected in *Apoc1* knockout mice[46]. This could be due to *Apoc1* possibly being a microglia specific gene, and *Apoe* being expressed by multiple cell types, including astrocytes and microglia. mRNA of some proinflammatory markers were also increased significantly in the mutant

mice[46]. The perplexing results of this knockout study in mice give us further reason to believe that the zebrafish is a highly suitable model organism to study *apoc1* in CNS tissue, and *apoc1* knockout studies should be performed in zebrafish to understand the function of *apoc1* in microglia.

Chapter 3: Regulation of Expression of *apoc1* in Zebrafish Microglia

Introduction

In this chapter, we identified predicted binding sites for nuclear hormone receptors PPAR:RXR and RAR:RXR in the putative zebrafish *apoc1* promoter region. We then provide evidence that RXR ligands, but not retinoic acid, increase *apoc1* expression specifically in microglia. Microglia express *apoeb*[48], however they are not the only cell to exclusively express it in the retina, as Müller glia also express *apoeb*[16]. Due to this fact, we also examined expression of *apoeb* to determine if *apoc1* and *apoeb* might be coordinately regulated in an RXR dependent manner.

Methods and Materials

Animal Care

As described in Chapter 2. In the experiments in this chapter, only the SciH (wildtype) strain was used.

Transcription factor analysis

We used the PROMO virtual laboratory[49,50] tool to analyze the 5 kb region upstream of the *apoc1* transcription start site. We used the PROMO website (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), selected "human factors" to search for human consensus binding sites, and entered the 5 kb sequence corresponding to the 5kb upstream of the transcription start site of *apoc1*. The dissimilarity rate applied was 15%. We selected human factors for this analysis due to zebrafish transcription factors not being well annotated and results were minimally informative.

Bexarotene and 9-cis retinoic acid treatments

Stock solutions of bexarotene (Millipore-sigma) and 9-cis retinoic acid (9-cis RA; Millipore-sigma) were prepared in dimethylsulfoxide (DMSO; Millipore-sigma), aliquoted, and stored in the dark at -20°C. The 9-cis RA was also stored under nitrogen. Embryos were collected, reared, and treated as described previously[51] with treatments performed at 28.5°C, beginning at around 52 hpf, and lasting 24 hours total. Once the 24-hour treatment was complete, embryos were anesthetized, and the heads were surgically separated from the body using dissecting scissors to ensure transcript measurements were from the eyes and brain, and not the developing liver (which has high expression of apolipoproteins)[35,52].

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR or qPCR)

Total RNA extraction, and cDNA synthesis from zebrafish embryo heads was performed using the same methods as described above. The template cDNA was diluted in a 1:20 ratio (cDNA:water). Amplification was performed on an Applied Biosystems Step OnePlus Real-Time PCR System using SYBR-Green PCR Master Mix (Thermo Fisher) and transcript specific primer pairs (Table 3.1). Relative quantification of gene expression between control and treated samples was determined using the constitutively expressed gene *actb2* and the 2^ddCt method. The gene *lws1* was used as a positive control for the effectiveness of retinoid treatments, as it has been shown previously that retinoic acid increases the expression of *lws1*[51]. The *mpeg1* gene (a macrophage specific gene also expressed in microglia) was used to ensure that treatments were not affecting all genes expressed by microglia.

HCR in situ hybridization of fixed tissue

After the 24 hour bexarotene treatment (300 nM), embryos were anesthetized Tricane (MS222) and fixed in a 4% PFA in 1X PBS RNAse-free solution for one hour at room temperature, washed in 100% methanol, and stored at -20°C in 100% methanol. The *in situ* hybridizations were carried using the Hybridization Chain Reaction (HCR) kit (Molecular Instruments). The HCR protocol carried out is similar to the protocol previously described (Chapter 2) for the whole retina with minor changes. Rehydration procedures took place in methanol/PBST (phosphate buffered saline, 0.1% Tween) solutions, embryos were treated with (10 μg/mL) proteinase K for 10 minutes, and hybridization was incubated overnight at 37° C with 2 pmol probe in 500 μL probe hybridization solution. Two probe sets were used in multi-plex, one for detecting *apoc1* transcripts and one for detecting *mpeg1* transcripts. After hybridization, embryos were washed with wash buffer (provided by the HCR kit), and SSCT (saline sodium citrate buffer, 0.1% Tween), before the signal was amplified, using HCR hairpins, overnight at room temperature. The hairpins resulted in labeling of detected transcripts with AlexaFluor 547 (for *apoc1* transcripts) and AlexaFluor 488 (for *mpeg1* transcripts). Embryos were then washed in SSCT and imaged in glycerol as described previously (Chapter 2).

Statistical Analysis

Statistical analysis used were the same as the methods described in Chapter 2.

Table 3.1 Primer sequences for qPCR

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	ZFIN ref-seq	Citation
apoc1	AAGACCAAAACCGCCTTCCA	AAATGTGCCAGTCGGCTCAA	ZDB-GENE-030131-1074	1
apoeb	CTCTTGTGGTATTCTTTGCTCTGGCAGTTT	TTGCACCATGCCGTCAGTTTGTGTGTTGAG	ZDB-GENE-980526-368	53
mpeg1	CATGTCGTGGCTGGAACAGA	GGGGGTGTAAGGTAAATGGGG	ZDB-GENE-030131-734	1
lws1	CCCACACTGCATCTCGACAA	AAGGTATTCCCCATCACTCCAA	ZDB-GENE-990604-41	51
actb2	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	ZDB-GENE-000329-3	51

Results

Transcription factor binding site analysis

In order to identify possible factors that might control regulation of *apoc1* expression, we performed multiple *in silico* analyses on the sequence of 5 kb upstream of the transcription start site of *apoc1*. Within the upstream sequence, alignment/match was found within regions annotated by Ensembl to be "promoter" sequences upstream of human *APOC1*, suggesting that this 5 kb sequence is likely to contain regulator sequences for *APOC1* expression, which, due to the orthology of *APOC1* and *apoc1* (discussed in Chapter 1), could likely be present also in zebrafish. In addition, the online sequence analysis tool PROMO[49,50] indicated the presence of several predicted nuclear hormone transcription factor binding sites in this upstream region of *apoc1* in zebrafish (Figure 3.1A). Predicted binding sites that interested us included those that involved retinoid and rexinoid receptors (RAR, RXR, RAR:RXR). In addition, a predicted binding site for PPARα:RXR was identified within the 5 kb sequence (Figure 3.1A). Further, *in vivo* real-time imaging data from our group suggests that when microglia colonize the retina, they migrate through a zone of active RA signaling, suggesting they may be influenced by it (not shown). We therefore hypothesized that retinoids and/or RXR ligands could regulate *apoc1* expression.

An RXR-specific agonist increases apoc1 transcripts

To investigate if RAR or RXR ligands may regulate *apoc1* expression, zebrafish embryos were treated with three concentrations of the RAR:RXR ligand, 9-cis retinoic acid (9-cisRA), or the RXR specific agonist, bexarotene. RT-qPCR was performed in order to quantify the changes of *apoc1* transcript level resulting from these treatments. Treatments began at around 52 hpf to allow for normal development and lasted 24 hours. This timepoint was also chosen because it is known that microglia begin retinal colonization around 2 dpf in zebrafish embryos[4], and (as discussed previously in Chapter 1) microglia begin expressing *apoc1* by 3 dpf. After treatment, embryos were collected and anesthetized, and the heads were surgically separated from the body using dissecting scissors to ensure transcript measurements were from the eyes and brain, and not the developing liver (as described in methods). RNA was isolated from the heads and cDNA was synthesized. We then used qPCR to quantify changes in *apoc1* transcript levels in treated versus control groups.

In order to ensure 9-cisRA and bexarotene treatments were effective, *lws1* expression was used as a positive control, as it has been previously shown to be upregulated in the retina upon 9-cisRA and bexarotene treatments[51]. An increase of *lws1* transcript was observed at 300 nM of both treatments (p value=0.0032, bexarotene; p value= 0.0715, 9-cisRA) (Figure 3.1B and 3.1C). Expression of *mpeg1*, a gene expressed by zebrafish microglia[1,29,48], was also included as a control to ensure treatments were not affecting all genes expressed by microglia. The *mpeg1* transcript levels showed no significant difference in expression for all treatments (Figure 3.1B and 3.1C).

Interestingly, qPCR results indicated no significant change in *apoc1* transcript levels following 9-cisRA treatment (Figure 3.1B). However, when embryos were treated with 300 nM bexarotene, *apoc1* transcript levels had a 2-6 fold increase (p value=0.0059) (Figure 3.1C). We also included measurements of *apoeb* transcripts because microglia also express *apoeb*[48] and coordinate regulation of the apolipoprotein gene cluster has been reported[35,36,54], meaning that expression of the apolipoprotein genes tend to follow the same trend (upregulated or downregulated). It has also been shown previously that retinoids (including 9-cisRA) increased *apoE* expression in astrocytes *in vitro* [55]. Interestingly, the bexarotene and 9-cisRA treatments in zebrafish embryos resulted in no significant change in *apoeb* expression (Figure 3.1C). Collectively, these results suggest that *apoc1* expression during development is modulated via RXR transcription factors but not retinoid receptors.

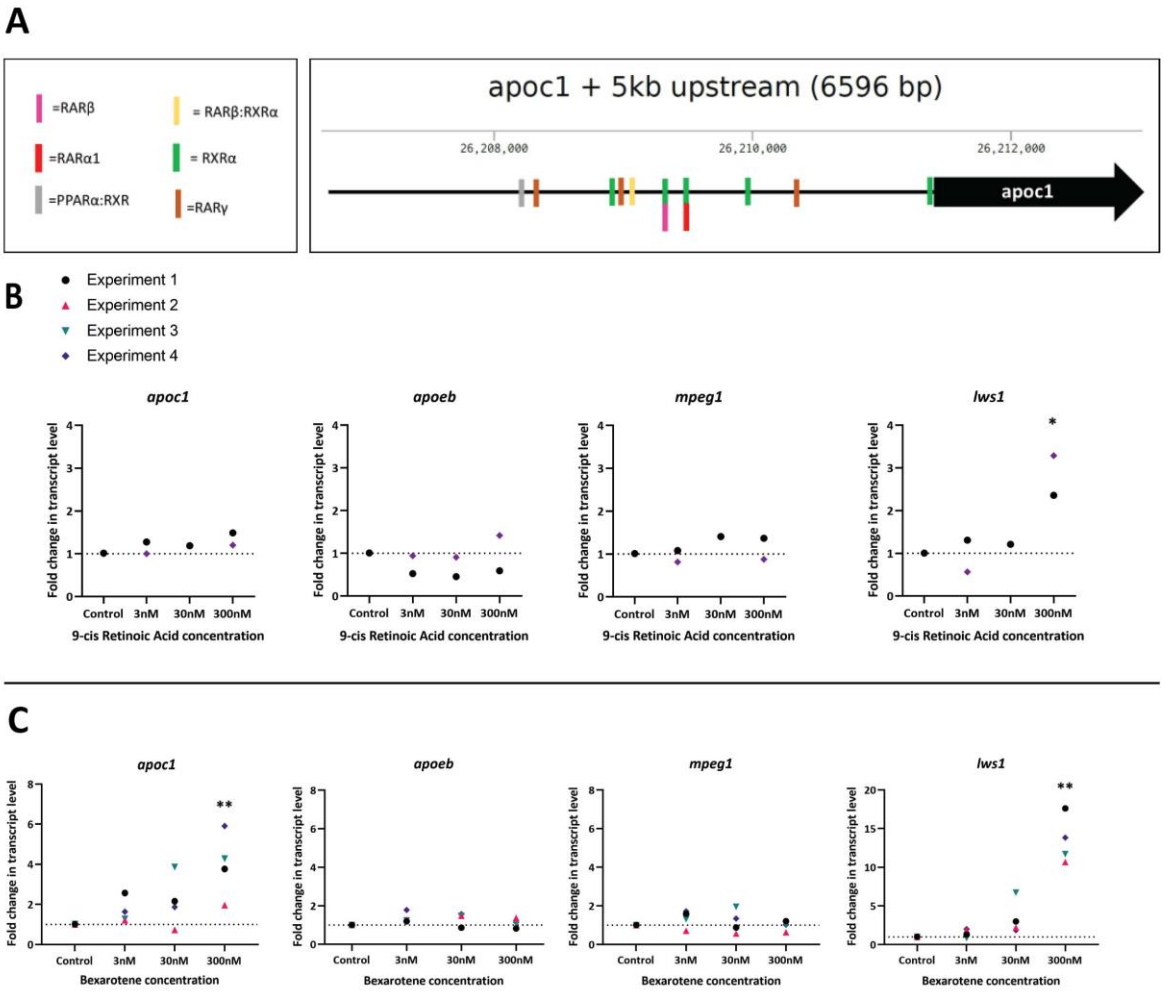

Figure 3.1. *Apoc1* transcripts are upregulated by an RXR agonist. Transcription factor analysis (via PROMO ALGGEN[49,50]) revealed multiple possible putative transcription factor binding sites that involved retinoid and rexinoid receptors. **(A)** Schematic diagram of 5 kb region upstream of the *apoc1* transcription start site with predicted RAR and RXR binding sites shown at their approximate location relative to the transcription start site. RT-qPCR was performed on RNA extracted from the heads of zebrafish embryos treated for 24 hours, beginning at approximately 52 hpf, with 3nM, 30nM, and 300nM 9-cis retinoic acid **(B)** or bexarotene **(C)**. Each symbol represents results from separate experiments. In each experiment, approximately 7-20 embryos were pooled for RNA extraction. DMSO (solvent) was used as a control for all treated groups. *Lws1* was used as a positive control for 9-cis retinoic acid and bexarotene treatments, and *mpeg1* was used to ensure treatments were not affecting all microglial genes. (* indicates p values < 0.1, ** indicates p values < 0.05, One-way ANOVA (Kruskal-Wallis) was performed, then a post-hoc Dunnett's multiple comparisons test to analyze statistically significant differences).

Bexarotene treatment does not affect the number of retinal microglia

The increase in *apoc1* transcripts with bexarotene treatment could be due an increase in individual cell expression, an increase in the overall number of microglia present in the retina, or the onset of *apoc1* expression in other cell types. To determine which was the case, we used i*n situ* hybridization to visualize microglia in combination with *apoc1* expression in embryos, using probe sets complimentary to *mpeg1* (to label microglia) and *apoc1*. For these *in situs* the Hybridization Change Reaction (HCR) protocol was used[56]. We decided to use this protocol instead of our previous protocol

(described in Chapter 2) due to its ability to multiplex probe sets to visualize two different transcripts in combination, and the ease of protocol compared to traditional methods for probe generation and *in situ* hybridization.

Treatments with bexarotene were performed at approximately 52 hpf for 24 hours, as done for qRT-PCR discussed above. Control (DMSO) treatments showed *apoc1* and *mpeg1* transcripts co-localizing in microglia (Figure 3.2A'-A"). Transcripts *mpeg1* and *apoc1* also were found to co-localize in bexarotene treated embryos (Figure 3.2B'-B"). Few instances of cells expressing only *mpeg1* or only *apoc1* transcripts were observed in both control and bexarotene treated embryos.

We quantified the number of cells in the retina expressing both *mpeg1* and *apoc1* transcripts, only *mpeg1* transcripts, and expressing only *apoc1* transcripts, in control and bexarotene treated embryos (Figure 3.2C-E). We did not detect differences in any of these categories, or in the total of all these types (Figure 3.2F). Further, the rarely observed cells expressing only *apoc1* and not *mpeg1*, displayed morphology representative of microglia, indicating that bexarotene does not induce apoc1 expression in other retinal cell types. These results indicate that bexarotene treatment does not change the number of microglia in the developing retina. Therefore, the increase in *apoc1* transcript levels from bexarotene treatment is likely explained by increased *apoc1* expression on a per-cell basis in microglia.

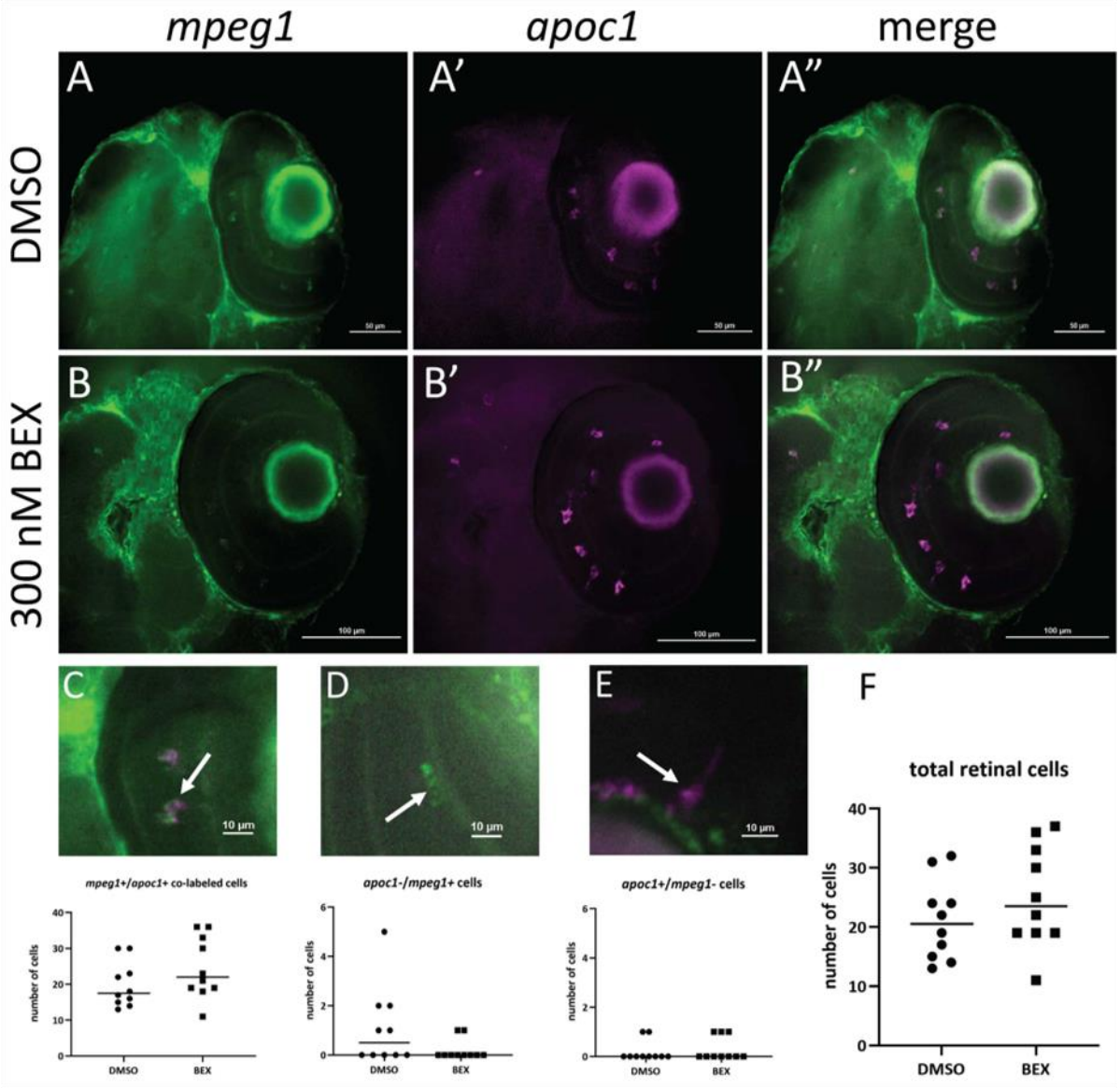

Figure 3.2. Bexarotene treatment does not affect the number of retinal microglia. HCR *in situ* hybridization was performed on wild type zebrafish embryos treated with control (DMSO) or 300nM bexarotene. **(A-A")** In control embryos (n=10), *apoc1* transcripts co-localize with *mpeg1* transcripts, indicating that they are microglia (Scale bars represent 50µm). **(B-B")** In 300nM treated embryos (n=10) *apoc1* transcripts are also co-localized with *mpeg1* transcripts (Scale bars represent 100µm). **(C)** Representative image of a microglia that expresses *apoc1* and *mpeg1*, and the cell counts of the treated and control groups (Scale bars represent 10µm). **(D)** Representative image of a microglia that only expresses *mpeg1*, and cell counts in the retina (Scale bars represent 10µm). **(E)** Representative image of a microglia that only expresses *apoc1*, and cell counts. **(F)** Total cell (counts all *mpeg1* and *apoc1* expressing cells) for both treated and control groups (Scale bars represent 10µm). Cell counts were performed on images of the embryos, and cells were considered microglia and counted based on their morphology (Unpaired t-test used to compare cell counts). Images shown **(A-E)** are flattened z projections.

Conclusions and Discussion

Through transcription factor analysis, we were able to identify predicted binding sites for multiple nuclear hormone receptors, including RARs and RXRs. Due to the predicted presence of these binding sites, we performed experiments to determine if ligands for either of these receptors affected expression of *apoc1* in developing zebrafish embryos. Treatments with the RAR:RXR ligand 9-cisRA and the RXR agonists bexarotene, and subsequent qRT-PCR suggested that during zebrafish development, *apoc1* can be regulated via RXR mechanisms, but not RAR. Known binding partners for RXRs include RARs, TRs, PPARs, VDRs, and LXR[57], though which of these are also expressed in microglia during development is not well established. We found a predicted PPAR:RXR transcription factor binding site in the *apoc1* promoter region. Current studies have varied results as to how PPAR:RXR factors influence *apoc1* expression[58–61], thus further experiments are needed to investigate if PPAR ligands regulate *apoc1* expression *in vivo*. It remains to be determined if RXR mediated transcription of *apoc1* is required for microglia expression of this gene, or if these transcription factors function in a cell-autonomous manner to regulate *apoc1* expression in microglia.

Multiple studies have been done on transcriptional regulation of *APOC1* and *APOE*. Some studies suggest there are regions downstream of *apoc1* that may regulate macrophage expression of the gene in mice and humans. ME.2 appears to be a regulatory element, found downstream of *APCO1* and *APOE*, that acts as an enhancer for all genes in the apolipoprotein clusters, including *APOC1* and *APOE*, specifically in macrophages[62]. Hepatic control regions (HCRs) also appear to control hepatocyte expression of genes in the apolipoprotein cluster [63–65]. These studies show that there are multiple regulatory elements that are needed for apolipoprotein gene regulation and some elements may determine cell type/tissue specific expression. The studies mentioned are done in macrophages *ex vivo*, from adult mice, and in cell lines *in vitro*. More studies *in vivo* are needed to understand the regulation of *apoc1*, and other apolipoprotein, transcriptional regulation.

Our data also suggests that certain factors may regulate *apoc1* expression separate from *apoeb*. This is an interesting result, due to the regulation of *APOE* and *APOC1* seeming to coincide in other studies[33,34]. These two apolipoproteins share similar roles in the periphery and possibly similar roles in the CNS as well. *APOE* is highly expressed in the liver, is known to be produced by astrocytes in the brain, and is found primarily in HDLs[33] in humans. *APOC1* is also highly expressed in the liver, and is also known to be associated mainly with HDLs in the periphery tissue[35]. However, less is known about the homeostatic function of *APOC1* in the CNS. Both genes are disease associated and seem to be coordinately regulated[33,34], however our results indicate that there may be separate mechanisms of regulation for *apoeb* and *apoc1*. This provides evidence that more studies on *apoc1*, in

the context of microglial biology, are needed to understand its function in the CNS. This could lead to a better understanding of homeostatic functions that are disrupted in neurodegenerative diseases.

Chapter 4: Tools and Transgenics

Introduction

In this chapter, we describe molecular biology methods used to create a DNA construct used for transgenesis. The transgenic reporter lines *mpeg1*:GFP and *mpeg1*:mCherry, which allow for visualization of macrophages[38], have been used to visualize microglia in the zebrafish CNS[1,4,28,29,66]. While these lines are useful, fluorescence in microglia is relatively weak. Given that *apoc1* transcript abundance is significantly higher than that of *mpeg1* in zebrafish microglia (Figure 4.1A), we considered that a transgenic reporter line driven by the *apoc1* promoter would provide a more robust tool to track zebrafish microglia *in vivo*. In addition, given our interest in regulation of this gene, such a reporter line would allow for simpler visualization of microglial expression of *apoc1* in fixed tissues compared to *in situ* hybridization which would save time and resources. In addition, such a reporter line would allow for *in vivo* real time imaging experiments. While we successfully cloned the DNA transgenesis construct and achieved stable transgenesis, the resulting zebrafish line did not display the expected expression in microglia.

Methods and Materials

Cloning of apoc1 transgenic reporter line

We used a BAC (Bacterial Artificial Chromosome) clone as a template to amplify and clone the putative zebrafish *apco1* promoter region. BAC clone DKEY-7F3 (Source Bioscience) contains a region of Danio rerio chromosome 16. The *apoc1* gene and 5kb upstream sequence are included in this BAC clone. Chromosomal sequence match was confirmed using NCBI blast of the BAC sequence to *Danio rerio* (Tax ID 7955) genome, with an outcome of 100% alignment. A blast of the BAC sequence to danio rerio RNA transcripts returns 100% alignment to *apoc1* mRNA (variants 1 and 2), as well as several other genes included in the BAC. Another blast of the BAC sequence to the *Danio rerio* genomic sequence corresponding to 5,400 bp upstream of apoc1, TSS, and the 5'UTR sequence of *apoc1* returned 100% alignment.

BAC DKEY-7F3 was used as a PCR template to amplify the genomic region corresponding to nucleotide #16:23967126 to nucleotide 16:23972953, which includes the *apoc1* 5'UTR sequence and 5000 bp upstream from the first 5'UTR nucleotide, the transcription start site. Primers used in this reaction are as follows:

Forward: 5’ ggtggtGGCCGGCCGCGTTCTCATAACTCACATTTTG 3’

Reverse: 5’ ggtggtGGCGCGCCCTTGTCGTTTTTTTCCCTGTAAC 3’

*Red highlighted nucleotides correspond to FseI and AscI RE sites for cloning the PCR product into the transgenesis vector. The uppercase unhighlighted nucleotides represent the sequence that corresponds to the PCR product being produced for cloning. The lower case unhighlighted nucleotides are random sequences that ensure the restriction enzyme cleaves the DNA in the correct place.

We created a “destination vector” pDESTTol2CG2 with Fse/Asc restriction sites upstream of an enhanced green fluorescent protein (eGFP) sequence by inserting three entry clones into the destination vector. The three sequences were a 5’ clone that included the Fse/Asc sites (p5E-Fse-Asc), a middle clone that included the eGFP sequence (pME-EGFP), and a 3’ clone that included a polyA signal sequence (p3E-polyA) [67]. The three vectors were combined into the destination vector using recombination reactions [67].

The resulting 5828 bp PCR product containing the *apoc1* upstream region was inserted into the pDESTTol2CG2-Fse-Asc-eGFP-PA destination vector [67] through restriction enzyme (RE) digests, followed by ligation reactions, and transformed into NEB® competent *E. coli* cells. This vector also included a *cmlc2*:eGFP heart marker, used to identify successful transgenic embryos. The newly created construct was renamed *apoc1p:*eGFP and orientation of the desired inserts was validated by restriction digest, analysis of resulting DNA fragments, and sequencing of the portion of the plasmid and inserted *apoc1* upstream sequence. The results confirmed that *apoc1p* was successfully inserted upstream of eGFP.

Transgenesis

The final transgenesis vector carrying the *apoc1p*:eGFP (25 ng) expression construct was injected with transposase mRNA (25 ng) into zebrafish embryos in the single cell stage of development. After microinjection, embryos were screened for green fluorescence of hearts at 1 and 2 dpf. Embryos with GFP+ hearts (F0) were reared to adults and outcrossed to wildtype fish in order to identify transgenics with germline integration, by screening F0 x wildtype offspring for GFP+ hearts (see Figure 4.1D). The resulting F1 were then raised to adults and in-crossed to generate a stable line.

Results

Generation of transgenic apoc1p:eGFP reporter line

Towards generating the *apoc1p*:eGFP transgenic reporter line, we chose 5kb upstream of the *apoc1* transcription start site and the 5' UTR of *apoc1* to drive GFP expression. We chose this region because it contained putative regulatory sequences as indicated by Ensembl (discussed previously in Chapter 2). In addition to predicted RAR:RXR and PPAR:RXR binding sites (described previously in Chapter 2), this region also contained predicted transcription factor binding sites for myeloid lineage factors (CEBP, PU.1, and c-Myb), as indicated by *in silico* analysis using the PROMO virtual laboratory[49,50] (Figure 4.1B).

The chosen 5.8kb upstream sequence (putative promoter region) was amplified by PCR using a BAC clone of zebrafish chromosome 16, which contained the region of interest, as the template. Using procedures based on cloning of transgenesis constructs based on Gateway cloning methods and Tol2-mediated transgenesis (described in Kwan et al., 2007), we created a DNA transgenesis vector with the putative *apoc1* promoter region (*apoc1p*) immediately upstream of eGFP and a downstream poly-adenylation (polyA) sequence (Figure 4.1C). The transgenesis vector also contains a *cmlc2*:GFP sequence in the opposite orientation, which results in green fluorescent hearts in zebrafish that express the transgenesis construct (Figure 4.1D). Tol2 transposon insertion sites flank the beginning of the putative promoter of *apoc1* and the end of the *cmlc2*:GFP sequence in the vector (Figure 4.1C). This construct (renamed *apoc1p:*eGFP) and orientation of the desired inserts was validated via restriction digest, analysis of resulting DNA fragments, and sequencing of the portion of the plasmid and inserted *apoc1p* upstream sequence.

The final transgenesis vector carrying the *apoc1p*:eGFP expression construct was mixed with Tol2 transposase mRNA and injected into zebrafish embryos at the single cell stage (Figure 4.1E). The resulting fish were screened for expression of green fluorescent hearts (Figure 4.1D), to indicate expression of the transgenesis construct, and dubbed the F0 generation. Eleven F0 fish were positively selected for expression of the transgenesis construct following microinjection. These eleven F0 fish were reared to reproductive age and were then outcrossed to wild type zebrafish in order to determine whether there was germline integration, by identifying those that produced F1 offspring with GFP+ hearts. Out of the eleven F0 fish, one F0 fish had successful germline integration and produced three F1 with GFP+ hearts. The F1 offspring with GFP+ hearts were raised to breeding age, then outcrossed to wild type zebrafish to produce an F2 generation. At this point, F2 zebrafish that had green fluorescent hearts were considered a stable transgenic line.

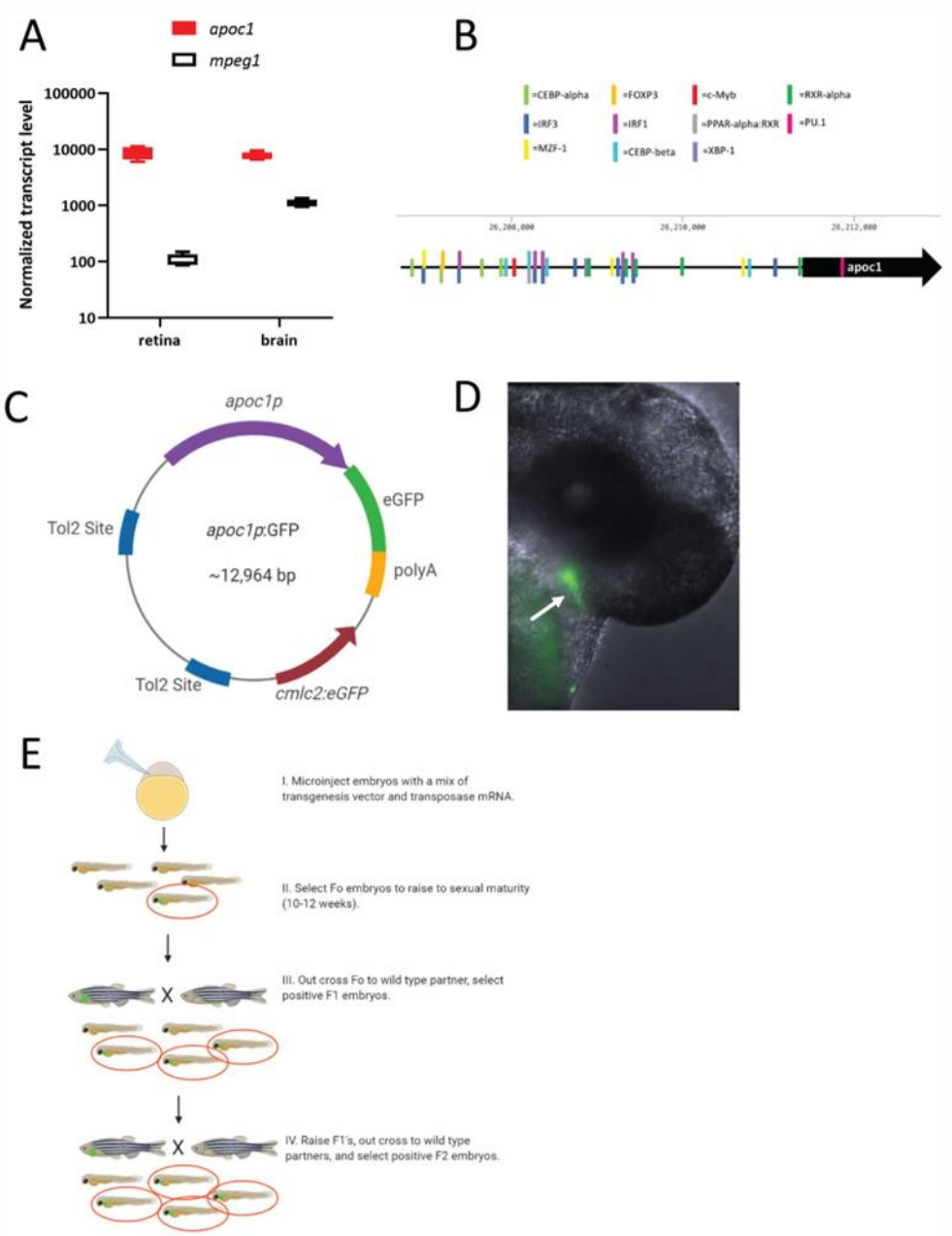

Figure 4.1. Strategy to create a transgenic *apoc1p*:eGFP reporter line. (A) Graphs show the normalized transcript levels of apoc1 and mpeg1 detected in microglia in the zebrafish retina and brain by RNA-seq (RNAseq data provided for retinal[1] and brain[25] microglia). The *apoc1* transcript abundance is significantly higher than that of *mpeg1* in zebrafish microglia. **(B)** The suspected promoter region of *apoc1* was identified as 5kb region upstream of the *apoc1* transcription start site and the 5'UTR which contained possible regulatory sequences as indicated by Ensembl and several predicted transcription factor binding sites, including those for myeloid lineage transcription factors predicted by the PROMO virtual laboratory[49,50]. **(C)** Representative image of the DNA transgenesis vector we created using cloning procedures based on the Tol2kit[67] and Gateway based cloning. It included the putative *apoc1* promoter region (*apoc1p*) immediately upstream of an eGFP sequence and a downstream poly-adenylation (polyA) sequence. In addition a *cmcl2*:GFP sequence which leads to GFP expression in the heart (to be used as a transgenesis marker), which are all flanked by Tol2 transposon insertion sites. **(D)** Representative image of a GFP+ heart (white arrow) in a zebrafish embryo due to expression of the DNA construct. **(E)** Outline of the basic protocol followed to produce a stable transgenic line of zebrafish (made in BioRender) beginning with microinjection to create the F0, screening of F0 embryos, selecting those with GFP+ hearts, raising them to breeding age and outcrossing them to wild type zebrafish.

While the breedings were taking place, we initially screened some of the F1 generation embryos at 2 dpf and 5 dpf for GFP+ fluorescence in the microglia, which was expected to be visible as a reporter of *apoc1* expression. We then later examined F2 embryos for GFP+ signal in microglia. We know from our *in situ* hybridization experiments (Figures 3 and 6 shown previously in Chapter 1), that *apoc1* is highly expressed in microglia in embryonic zebrafish. However, there was no detectable GFP fluorescence in microglia in the transgenic F1 or F2 generation embryos. These embryos did display green fluorescent hearts, indicating that the DNA construct successfully integrated into the genome. In the end, our resulting stable line did not result in the anticipated reporter.

Conclusions and Discussion

We consider that there could be several reasons that the transgenic fish did not show *apoc1p*:GFP fluorescence in the microglia. One possibility is that the sequence that we predicted to contain a sufficient promoter region to drive *apoc1* expression (*apoc1p*) is in fact not sufficient to drive expression of *apoc1* and therefore did not drive strong expression of GFP *in vivo*. There could be other regulatory elements required for strong *apoc1* expression, that were not included in our DNA construct. These regulatory elements could lie further upstream than the 5kb region we chose or could possibly lie downstream within the *apoc1* gene itself or could lie outside of the *apoc1* coding region. Another possibility is that the transgenesis construct containing the *apoc1p*:GFP sequence could have integrated into an area in the genome that is transcriptionally silent. The *cmlc2* promoter is very strong[68] and so may still be expressed even if the construct is integrated in such a region, but the *apoc1* promoter may not be sufficiently active. One other possibility (although unlikely) is that the portion of the transgenesis construct containing *apoc1p*:eGFP was not stably integrated, but the portion containing *cmcl2*:GFP was integrated.

In retrospect, if we again attempted to produce an *apoc1p*:GFP transgenic reporter line, we would make several changes to our approach. We only had one F0 with successful germline integration and this number may not be sufficient to identify a founder that has integrated the construct into the genome and also faithfully expresses the *apoc1p*:GFP sequence. Therefore, we would screen a larger number of F0s (30 or more, based on personal correspondence with other researchers) to try and identify F1 fish with the desired reporter expression. It is also important to learn more about the regulatory regions controlling *apoc1* expression. For example, some studies suggest there are regions downstream of *apoc1* that may regulate macrophage expression of the gene in mice and humans[62–65]. It could be worthwhile to create transgenesis constructs that also contain similar regions of the

zebrafish genome. Further, future directions resulting from the work presented in Chapter 3 could provide critical information in this regard.

Chapter 5: Discussion

In this body of work, we provide evidence that *apoc1* is expressed exclusively by microglia in the zebrafish central nervous system. This microglia-specific expression begins during early CNS development and appears to persist in the adult animal. We identified putative transcription factor binding sites in the 5 kb region upstream of the *apoc1* transcription start site, which lead us to investigate possible mechanisms that could regulate *apoc1* transcription. We provide evidence that *apoc1* expression in microglia may be regulated by RXR ligands, though the RXR biding partners involved remain to be determined. Known binding partners for RXRs include RARs, TRs, PPARs, VDRs, and LXRs[57], though which of these are also expressed in microglia during development is not well established. We also provide evidence that RA is not likely a major factor in regulating *apoc1* expression in microglia during CNS development. When treated with the RAR agonist (9-cisRA), zebrafish embryos showed no significant change in *apoc1* transcript levels. There are several possible explanations for why RARs (and their ligands) may not be a major factor in *apoc1* regulation in microglia. Microglia may not express RA transporters required for uptake and response to RA. Alternatively, or in addition, microglia may not express significant levels of RARs during development. It is also possible that microglia degrade RA upon uptake during development.

Along these lines, we found a predicted PPAR:RXR transcription factor binding site in the *apoc1* promoter region (Chapter 3). Current studies have varied results with PPAR:RXR factors influence on *apoc1*. Some studies show that *apoc1* and *apoe* expression was induced via PPAR:RXR and effected microglial phagocytic activity[58–60]. Whereas in another study using PPAR shRNA and alternative PPAR agonists, both *APOE* and *APOC1* expression was reduced[61]. Studies mentioned were performed *in vitro*. Thus, further experiments are appropriate to investigate if PPAR ligands regulate *apoc1* in zebrafish microglia *in vivo*.

While we did not find any LXR factor binding sites in our analysis of the 5 kb region upstream of the *apoc1* transcription start site, other studies have also shown that LXR ligands affect *APOC1* transcription in macrophages[69,70]. We might not have found LXR binding sites due to the fact that we performed our analysis based on human transcription factors when analyzing the zebrafish *apoc1* promoter region using the PROMO tool. The sequences for LXRs could be different between humans and zebrafish, and although an LXRα ortholog exists, there is no known LXRβ ortholog in zebrafish[57], which reinforces this theory. Therefore, it would be pertinent to investigate LXR agonists effects on *apoc1* transcription level.

While we have made some necessary beginning steps to understand *apoc1*, more investigation is necessary for true comprehension of its function and regulation. In particular, loss of function experiments are required to understand the factors that establish *apoc1* expression in microglia versus those that modulate expression levels. For example, it could be that myeloid lineage transcription factors initiate *apoc1* expression upon microglial colonization of the CNS, while ligands for nuclear hormone receptors such as PPAR:RXR or LXR:RXR further modulate levels of *apoc1* transcripts. We have also speculated that when microglia digest apoptotic cells, the molecules derived from ingested lipids/sterols could be ligands for a receptor (such as a PPAR:RXR or LXR:RXR receptors) that then modulates the expression of *apoc1*.

The function of *APOC1* in liver tissue[35] has been described. In contrast, its function as a microglia specific factor in the CNS is not understood. Given the association of both *APOC1* and microglia with human neurodegenerative disease, this is of great interest. In the periphery, *APOC1* is known to aid in lipid metabolism, and it could have a similar role in microglia[35]. Macrophages are well appreciated for their function in peripheral lipid/cholesterol homeostasis. The gene allows the macrophages to ingest certain lipids/sterols which in turn controls extracellular lipid and sterol levels and can also act as signals for inhibiting immune responses[35]. We therefore consider that such a function in the CNS is likely, but specialized or unknown functions may also exist. Synapses demand high levels of cholesterol/lipid turnover, so *apoc1* may have a role on maintaining synaptic integrity and function.

Future experiments will aim to reveal the function of *apoc1* expression by microglia in the CNS. In loss of function of approaches (where *apoc1* is 'knocked out" for example), we hypothesize that CNS lipid homeostasis would be altered. We think that lack of *apoc1* would render microglia deficient in uptake of certain lipid particles, which may also inhibit their ability to clear cellular debris in CNS tissue. In retinal regeneration, the lipids microglia consume, facilitated by *apoc1*, could also be used as signals to initiate glial cell differentiation to replace damaged retinal cells via an indirect mechanism in which genes expressed by microglia would influence Müller cell differentiation. Therefore, if *apoc1* were to be silenced in a regenerating retina, it is possible that there would be inhibition of glial cell differentiation. While this is all speculation, more studies are needed in order to understand the role of *apoc1* in CNS tissue microglia. In addition, since loss of function of *apoc1* appears to be embryonic lethal in zebrafish[42] (and our own preliminary data, not shown), novel approaches for *apoc1* loss of function are required.

Apoc1 should also be further studied due to its demonstrated, but not understood, association with Alzheimer's disease (AD) and other neurodegenerative diseases[33,34]. As it was mentioned previously

(Chapter 3), the regulation of *APOE* and *APOC1* seem to coincide with each other. In addition, both *APOE* and *APOC1* are associated with AD in humans[33,34], though this genetic association is not well understood. Our data indicates that specific factors may regulate *APOC1* and *APOE in vivo* (Chapter 3). In order to determine what role these apolipoproteins play in AD, more studies must be done. Understanding this gene may lead to further discoveries in its role in the CNS and may lead to treatments for neurodegenerative diseases.

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