EXPRESSION AND LOCALIZATION OF MULTIDRUG RESISTANCE PROTEIN 4 (Mrp4) IN THE RETINAL TISSUE OF *Danio rerio*

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ABSTRACT

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SUPERVISING PROFESSOR: DANA GARCIA

The neural retina of zebrafish (*Danio rerio*) is one of the more specialized tissues of the diencephalon, and it encompasses distinct and well defined cellular classes, which includes the photoreceptor cells. The retinal pigment epithelium (RPE) forms the distal blood-retinal barrier playing essential functions for cellular homeostasis. The zebrafish is a powerful model for functional retinal studies as it closely resembles the retina of higher vertebrates. Nonetheless, the zebrafish retina exhibits distinct mechanisms for light- and dark-adaptation termed retinomotor movements. These mechanisms for light- and dark-

adaptation occur by morphological changes of photoreceptors along with redistribution of melanosomes within the apical processes of RPE cells.

The purpose of retinomotor movements is to protect and optimally expose cones or rods to light quanta. Regulation of retinomotor movements involves cAMP as higher concentration of cAMP induces the dark-adaptive response. The multidrug resistance protein 4 (Mrp4) is the member of the Mrp family with the highest affinity for cAMP. The aim of this study is to characterize the spatial and temporal patterns of expression of Mrp4 in the retina to elucidate the role of Mrp4 in regulating retinomotor movements.

In this study, I show Mrp4 expression on dark-and light-adapted retinas, and I report subcellular location and distribution of Mrp4. I also show that Mrp4 inhibition results in decreased melanosome aggregation. The results from this study are consistent with the model I propose, in which Mrp4 regulates intracellular cAMP levels by exporting cAMP into the subretinal space as cytoplasmic levels rise under dark conditions. Once in the subretinal space, cAMP is taken up by RPE to induce and maintain the dark-adapted state.

CHAPTER I

INTRODUCTION

I. The Retinal Plan

The vision of teleost fishes is the result of millions of years of selective pressure, which has led to complex ocular features in many members of this class of ray-finned fishes. Although teleost fishes are considered organisms of modern emergence, arising first during the Triassic period and expanding in the early Cenozoic, they are the most diverse extant vertebrate group and exhibit some of the most complex and specialized retinal arrangements known (Marc and Cameron, 2001; Yazulla and Studholme, 2001).

The teleost neural retina is one of the more specialized tissues of the diencephalon, and it comprises six distinct and well defined cellular classes as shown in figure 1: [1] the rod (scotopic) and cone (photopic) photoreceptors, which contain the phototransduction machinery; [2] the bipolar cells, which are second order neurons that along with [3] the horizontal cells are attached to a corresponding photoreceptor; [4] the amacrine cells, which are retinal interneurons that together with bipolar and horizontal cells form the three distinct sub-layers of neuronal cell bodies of the internal nuclear layer; [5] the ganglion cells, which form the centermost neuronal layer separated from the vitreous by Müller cells that along with astrocytes form the group of [6] glial cells (Scholes and

Morris, 1973; Scholes, 1975; Marc, 1982; Marc, 1999; Marc and Cameron, 2001; Yazulla and Studholme, 2001).

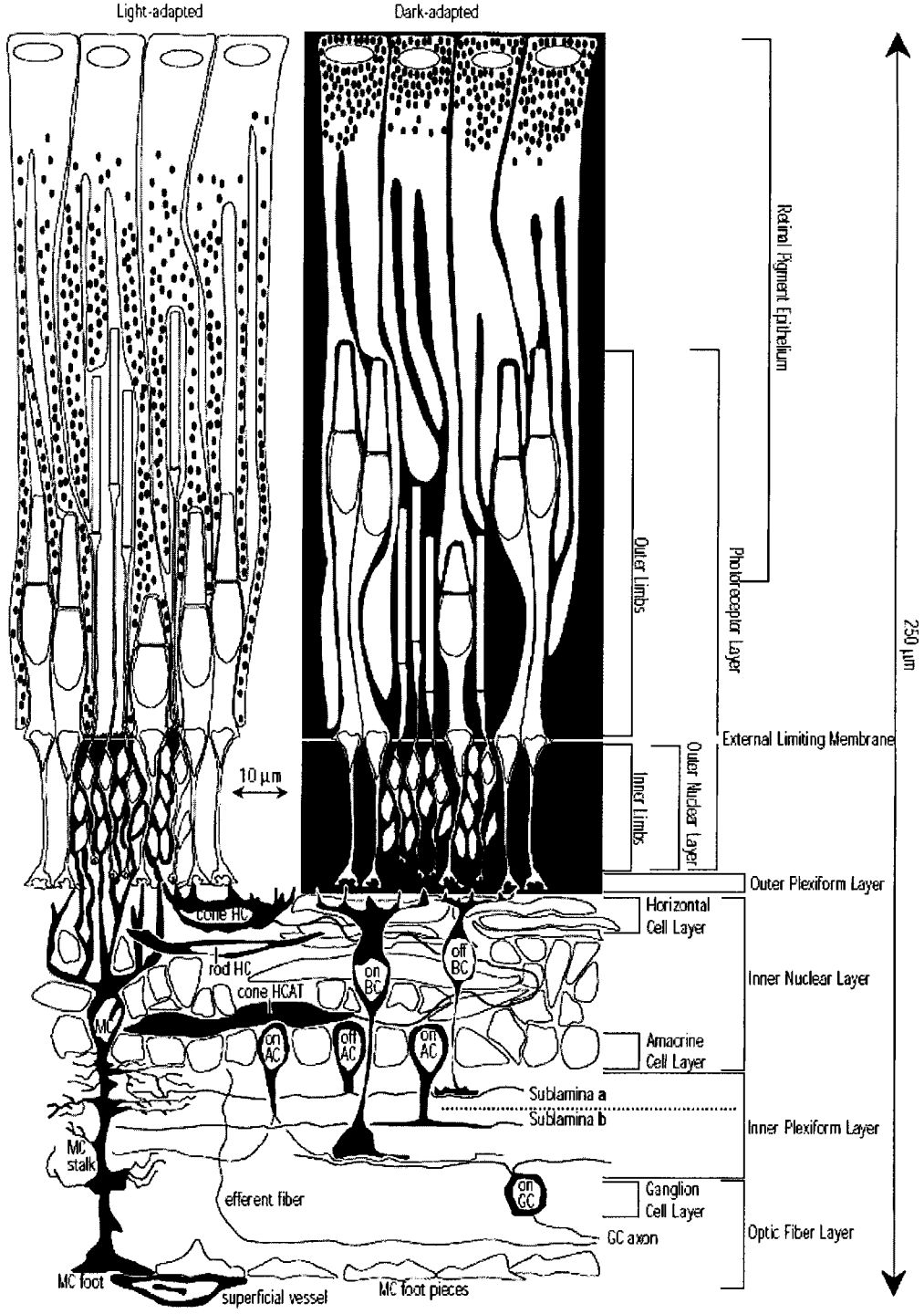

Figure 1: The Teleost Retinal Plan. Schematic representation of the three operational layers of the teleost retina: (1) photoreceptor layer, (2) intermediate neuronal layer, and ganglion cell layer. These layers include the 6 distinct histological layers: (1) the photoreceptor layer, (2) the outer plexiform layer, (3) the inner nuclear layer, (4) the inner plexiform layer, (5) the ganglion cell layer, and (6) the optic fiber layer. Figure used with permission of Dr. Robert Marc from the University of Utah (Marc, 1999).

The retinal pigment epithelium (RPE) is a monolayer of uniformly oriented and highly polarized cuboidal epithelial cells located between the choroid and the photoreceptor layer (Bruenner and Burnside, 1986; Marc 1999). The basal part of the RPE cells, the cell body containing the nucleus, rests upon the Bruch's membrane. RPE cells form the distal blood-retinal barrier, controlling the transport of oxygen, blood constituents, and other metabolites to the photoreceptor layer (Marc, 1999; Ghassemifar et al., 2006). For many species, RPE cells not only serve as a physical barrier but also play many other essential functions for the development and homeostasis of photoreceptors cells. For example, in many species the apical processes of RPE cells interdigitate with photoreceptors, providing supporting functions, which include synchronizing melanosome movements to shade outer segments (OS) and removing metabolic waste by recognizing and phagocytizing the tips of cones and rods (Bok and Young, 1979; Ghassemifar et al., 2006; Nandrot and Finnemann 2006; Xia et al., 2006).

The outer segment of photoreceptor cells consists of many stacked membranous opsin-rich disks, which are required for the phototransduction process. These disks are constantly produced and thus increase the outer segment length of photoreceptors; improper function of RPE cells leads to a rapid degeneration of rods and cones since RPE cells are active phagocytes of aged photoreceptor disks (Young, 1967; Bok and Young, 1979; Bruenner and Burnside, 1986). It has been estimated that in some vertebrates, RPE cells digest 30,000 rod outer segment disks daily, making RPE cells the most active phagocytes known (Besharse and Defoe, 1998; Nandrot and Finnemann, 2006).

II. *Danio rerio* as an Animal Model

The cyprinid *Danio rerio* (zebrafish) follows the previously described retinal plan (See Figure 1). Zebrafish has been extensively used as a model for developmental genetics, neurophysiology, biomedicine, and functional retinal studies (Spence et al., 2008). Although other cyprinids have been used to model a range of ocular processes of fishes and other vertebrate species --including mammals-- they have been deficient genetic models (Marc, 1992; Marc and Cameron, 2001) and zebrafish is fully genetically characterize.

Zebrafish have thus become very useful organisms for genetic research due to rapid development and short generation time (Spence et al., 2008). The relative ease of manipulation, accessibility, lower maintenance cost, and high rate at which mutations can be produced in zebrafish including ENU-induced mutagenesis, transgenesis, and targeted mutagenesis with TALENs have made this organism a more suitable model for developmental and behavioral studies than its mammalian counterparts (Marc, 1999; Kettleborough et al., 2011).

Other characteristics that increase the value of zebrafish as an animal model are the well defined retinal mosaic that closely resembles that of higher vertebrates and features at the cellular and molecular level that provide models for ocular processes such as membrane transport, signal transduction pathways, and response to external stimuli (Yazulla and Studholme, 2001).

Although laboratory mice and rats are suitable systems for transgenic approaches, zebrafish offer an advantage since the retina of rats and mice differ from human retina in having very few cones. The higher complexity of the teleost retina offers a wider range of neuronal classes and contains cell types, functional adaptations, and ocular mechanisms foreign to the mammalian retina (Marc, 1999; Marc and Cameron, 2001).

III. Light- and -Dark-Adaptation: Retinomotor Movements

Despite the similarities with the mammalian eye, the teleost retina –as well as the retina of other non-mammalian vertebrates- exhibits distinct mechanisms for light- and dark-adaptation (Burnside et al., 1983; Bruenner and Burnside, 1986; McCormack and McDonnell, 1994). In zebrafish, these retinal mechanisms of light- and dark-adaptation occur by morphological changes of the cone and rod cells accompanied by redistribution of melanosomes within the apical processes of the RPE cells. The purpose of these retinomotor movements is to protect and optimally expose cones or rods to light quanta by repositioning the outer segment part of the appropriate photoreceptor cell and dispersing or concentrating RPE pigment granules in response to changes in light conditions (Olla and Marchoni 1968; Burnside et al., 1982).

The contraction of the cone myoids and elongation of the rod myoids in the light and the reverse movements in the dark are regulated not only by external stimuli but also, at least partially, by endogenous circadian signals (Dearry and Burnside, 1989; McCormack and McDonnell, 1994). These cell movements and endogenous circadian signals have also been found in several animal classes including insects and amphibians, suggesting a common eukaryotic regulator origin (Wallcott, 1971; Orr et al., 1976; Young, 1998).

i. Light Adaptation

Three different types of cell movements take place during photopic states *in vivo*: [1] the myoids of rod cells elongate, increasing the length of the cell; thus their outer segments are shielded by [2] the melanosomes that migrate into the apical projections of the RPE cells, protecting the rod outer segments from light; [3] while the inner segment myoid regions of the cones contract, pulling the cone outer segments toward the direction of the incoming light quanta (Walls, 1942; Burnside et al., 1982; Burnside and Nagle,1983; Burnside et al., 1983; Dearry and Burnside, 1984-A; Dearry and Burnside, 1989; McCormack and McDonnell, 1994).

These contracting movements occur in the distal parts of cones and are restricted almost exclusively to the myoid region (Burnside, 1978). Although cone contractions take place unequally, they represent a useful tool for studying non muscle contraction phenomena since they are linear and slow (from 85 μm to 5 μm at 1-3 μm/minunte in Haemulon *sciurus* and Lutjanus *griseus*) (Burnside, 1976; Burnside, 1978).

ii. Dark-Adaptation

Aggregation of RPE cell pigment granules to the base of the cell body, rod myoid contraction, and cone myoid elongation take place under scotopic conditions. In this state, rods move in the direction of the vitreous –towards the external limiting membrane– assuming an appropriate position for optimal light reception; whereas the outer segments and ellipsoids of elongated cone cells lie approximately where the

corresponding rod outer segments were during light settings (Burnside et al., 1982; McCormack and McDonnell, 1994).

It is believed that cone elongation mechanisms are, at least partially, microtubule-dependent since longitudinally oriented microtubules are localized throughout the cone cell and disrupting them with colchicine blocks elongation (Burnside, 1978). Moreover, actin filaments are required for cone and rod contraction as well as rod elongation (Burnside, 1978). Furthermore, studies suggest that both rod and cone retinomotor movements are regulated by light whereas only cone movements are affected by circadian signals (Olla and Marchoni, 1968; McCormack and McDonnell, 1994).

Although at a glance these retinomotor movements might seem fairly simple, the mechanisms by which they take place are certainly complex. For example, melanosome dispersion can be stimulated without direct input from the central nervous system (CNS) as it has been induced in cultured enucleated eyes (Burnside and Basinger, 1983). Furthermore, it seems that melanosome movements are locally controlled *in vivo* since exposing intact dark-adapted fish retina to minute amounts of light produces dispersion only in the particular area of exposure (Easter and Macy, 1978). In contrast, dark-adaptive retinomotor movements can be achieved by elevated 3',5'-cyclic adenosine monophosphate (cAMP) (Burnside and Basinger, 1983; Koh and Chader, 1984) and maintained by low ($\leq 10^{-6}$ M) calcium levels in cultured RPE/retina (Burnside, 1982; Dearry and Burnside, 1984A). Agents that regulate cAMP levels seem to play an important role in cellular homeostasis. In addition, evidence suggests cAMP levels

modulate the renewal of the rod outer segment membrane and disk shedding (Traverso et al., 2002).

IV. Cyclic AMP as Cellular Messenger

Cyclic AMP acts as a second messenger in cells. It is formed from ATP by adenylyl cyclase in response to activation by G-protein coupled receptors (Friedman et al., 1987; Gregory et al., 1992). Regulation of retinomotor movements involves cAMP (Dearry and Burnside, 1984). Many studies have shown that agents that elevate cAMP levels in the retina induce dark-adaptive retinomotor movements, and dark-adapted retinas exhibit higher levels of cAMP than light-adapted retinas (Ferrendelli and Cohen, 1976; Orr et al., 1976; DeVries et al., 1978; DeVries et al., 1979; Farber et al., 1979; Farber et al., 1981; Burnside et al., 1982; Cohen, 1982; Burnside and Ackland, 1984; Dearry and Burnside, 1985; García and Burnside, 1994). However, improper regulation of intracellular cyclic nucleotide levels can result in neurotoxicity and retinal degeneration (Lolley et al. 1977; Bowes et al., 1990; Li et al., 1997; Montoliu et al., 1999).

When accompanied by phosphodiesterase inhibitors, cAMP induces cone elongation even in fully light-adapted retinas (Burnside et al., 1982; Brown et al., 2002). Although many compounds have been postulated as regulators of both light-adaptive and dark-adaptive retinomotor movements, it has been shown that all of them act, at least partially, by altering cAMP levels. Adenosine, ATP, melatonin, histamine, vasoactive intestinal peptide, and prostaglandins all fail to produce dark-adaptive responses in isolated RPE cells, suggesting that their effects on RPE *in vivo* must be mediated indirectly by the release of an extracellular messenger from retinal cells (García and Burnside, 1994). For

example, forskolin and prostaglandins induce the dark adaptive retinomotor movements in teleost retinas by increasing intracellular cAMP levels (Dearry and Burnside,1985; Dearry and Burnside,1989). Also, in both *in vitro* and *in vivo* conditions, melatonin induces melanosome aggregation and cone elongation in frogs (Kemali et al., 1986). Furthermore adenosine receptors have been reported in RPE cells of various species (Friedman et al., 1989; Gregory et al., 1992). Many studies indicate that dopamine inhibits dark-adaptive movements by lowering cAMP levels; other evidence suggests that dopamine may also act by alternative pathways to induce the light-adaptive response; dopamine has also been implicated with circadian control mechanisms in the retina (Watling et al. 1980; Dearry and Burnside, 1986; Dearry and Burnside, 1989; Dearry et al., 1990; McCormack and McDonnell, 1994).

V. The Cellular Transporter Multidrug Resistance Protein 4, a cAMP Regulator

The multidrug resistance proteins (MRPs) belong to the large superfamily of ATP-binding cassette (ABC) transport proteins (Lai and Tan, 2002). Functionally, all ABC transporters utilize ATP hydrolysis to energize export of xenobiotics, metabolites, and endogenous substrates that can accumulate in tissues and lead to toxicity (Lai and Tan, 2002, Maher et al., 2007).

The multidrug resistance protein 4 (Mrp4), encoded by *ABCC4*, is capable of transporting anticancer drugs, antiviral agents and endogenous molecules such as prostaglandins, steroids, bile acids, folate, and the cyclic nucleotides cAMP and cGMP (Jedlitschky et al., 2000; Chen et al., 2001; van Aubel et al., 2002; Zelcer et al., 2003; Legler et al.,

2004; Luft et al., 2004; Scandella et al., 2004; Abla et al., 2008; Lin et al., 2008; van de Ven et al., 2008). This efflux mediation of purine nucleotide analogs makes Mrp4 a key protein during cellular detoxification (Norris et al., 2005; Sassi et al., 2008). The cyclic nucleotides cAMP and cGMP are well characterized molecules with critical physiological roles in cellular communication and signaling. They mediate a wide range of cellular responses by activation of cNMP-dependent protein kinases; thus, the ability of Mrp4 to transport these cyclic nucleotides suggests that Mrp4 plays a biological role in cellular signaling and communication (Sampath et al., 2002; Lin et al., 2008). Mrp4 also plays a crucial role during the immune response as it transports inflammatory mediators involved in dendritic cell migration (Rius et al., 2008; van de Ven et al., 2008).

Mrp4 has been reported to be involved in acquisition of cytotoxicity resistance to chemeotherapeutic agents (Sampath et al., 2002). Mrp4 can lower intracellular levels of antiretroviral drugs in HIV-infected cells, leading to impaired suppression of HIV replication (Schuetz et al., 1999). Furthermore, expression of Mrp4 in cancer cells is a factor in the chemotherapy response and it has been linked with poor prognosis in neuroblastoma patients (Sampath et al., 2002).

Mrp4 is expressed ubiquitously, and high expression in prostate and hematopoietic cells has been reported. Presence in brain, kidney proximal tubules, liver, and smooth muscle has also been documented along with other members of the Mrp family (Nies et al., 2002; van Aubel et al., 2002; Dazert et al., 2003; Mitani et al., 2003; Rius et al., 2003; Leggas et al., 2004). Interestingly, Mrp4 is apically localized in most epithelia; however, basal localization has also been reported in some organs (Abla et al., 2008). Although no

disease has been directly linked to altered Mrp4 activity, knowledge of Mrp4 localization and expression may provide insights on the physiological function of this protein.

CHAPTER II

HYPOTHESIS

The aim of this study is to characterize the spatial and temporal patterns of expression of Mrp4 in zebrafish retinal tissue. As shown in Figure 2, I hypothesize that Mrp4 is expressed by photoreceptor cells, where it could regulate intracellular cAMP levels by exporting it into the subretinal space. Once in the interstitial space, cAMP can be taken up by RPE cells to induce and maintain the dark-adapted state. Elucidating retinal expression of Mrp4 will provide a broader understanding of how teleost retinomotor movements occur and how their regulation is affected by this protein

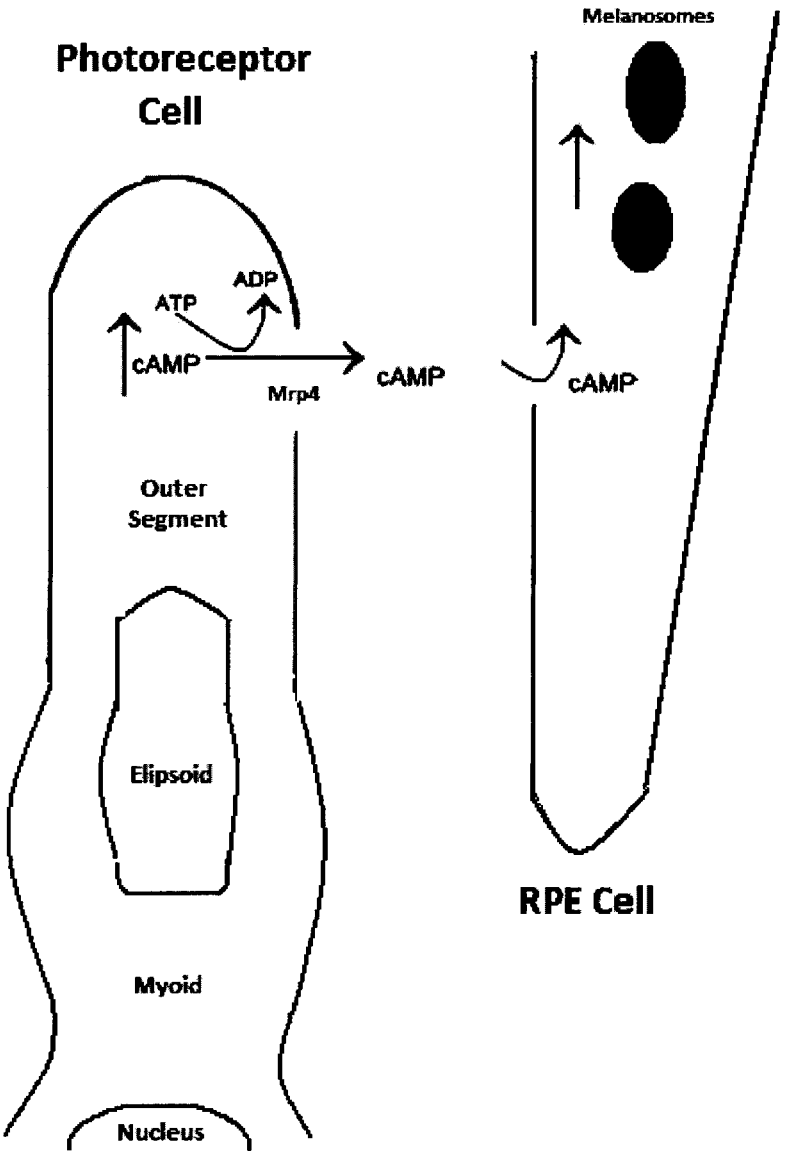

Figure 2. Proposed Model of cAMP Regulation by Mrp4. Extracellular signal activate nucleotide cyclases activity and/or decrease activity of phosphodiesterases, which lead to higher intracellular levels of cAMP in the photoreceptor cell. The accumulated cAMP is exported by Mrp4 making cAMP available for RPE cell influx. Cyclic AMP enters RPE cells and induces dark-adapted movements.

CHAPTER III

MATERIALS AND METHODS

I. Husbandry

Wild-type AB zebrafish were kindly provided by Dr. Jeff Gross from The University of Texas at Austin, and they were cared for in the laboratory of Dr. Dana García at Texas State University-San Marcos under a 12 hour light-12 hour dark diurnal cycle. All fish were acclimated for a minimum of 14 days prior to performing any experiments. This research was conducted under the standards of the Public Health Service Guide for the Care and Use of Laboratory Animals (National Research Council, 2010). The Institutional Animal Care and Use Committee (IACUC) approved the protocol to perform this study; the IACUC protocol approval number is 0931_1103_38 for vision-related research on fishes.

II. Experimental Overview

This study was divided into two phases: (i) descriptive studies and (ii) functional studies. In the descriptive part of this study, protein expression was analyzed by performing immunohistochemistry using goat anti-ABCC4/MRP4 antibody (Everest, Oxford, UK) to localize protein expression via immunofluorescence. Immunogold transmission electron

microscopy was used to determine the subcellular location and distribution of Mrp4. In the functional part of this study, Mpr4 was inhibited by sildenafil citrate (Sigma-Aldrich, St. Louis, MO) via intraocular injection and pigment indexes were measured to assess the role of Mrp4 in retinomotor movements.

III. Experimental Techniques

Descriptive Studies

Tissue Extraction

Dark-adapted eyes were obtained from fish that were placed in complete darkness 3 hours into their light cycle for 2 hours prior to sacrificing the animals. Light-adapted eyes were obtained from light-adapted fish sacrificed at midpoint of their light cycle. Fish were sacrificed by overdose of Tricaine Methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA). Dark-adapted fish were sacrificed in complete darkness, and eye extraction was performed under dim light using sterile Dumont® #5 forceps. Eye extraction for light-adapted fish was performed in a well-illuminated setting. Eyes were processed for immunofluorescence or electron microscopy.

Tissue Processing

i. Immunofluorescence

For immunofluorescence labeling, eyes were fixed overnight in 4% paraformaldehyde (PFA, Electron Microscopy Sciences) prepared in phosphate buffered saline (PBS; 137

mM NaCl, 11.9 mM Na_2HPO_4, 11.9 mM KH_2PO_4, and 2.7 mM KCl). Three PBS washes of 10minutes each were performed after fixation, and tissue was cryoprotected using 30% sucrose in PBS solution. The tissue was sectioned at 20 μm using Tissue-Tek O.C.T compound as an embedding medium at -24°C in a Zeiss HM 505N cryotome. Tissue was collected on gelatin coated microscope slides and stored at -20°C.

ii. *Electron Microscopy*

For immunogold labeling, eyes were fixed overnight at room temperature in a solution containing 3% PFA and 1% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M sodium phosphate buffer (0.4 M Na_2HPO_4, 0.4 M NaH2PO_4). After fixation, retina was removed from eye and was cut into 3 pieces and washed for 15 minutes with 0.1 M sodium phosphate buffer 3 times. Retina was transferred to 1% OSO_4 in 0.1 M sodium phosphate buffer for 4 hours at room temperature; tissue was then washed 3 times with 0.1 M sodium phosphate buffer and left overnight at 4°C. Tissue was dehydrated in 2 washes of 30 minutes each in 70% ethanol. Samples were embedded in LR white medium acrylic resin (London Resin Company, London, UK) and 70% ethanol (2:1 mixture) for 1 hour. Gentle shaking was performed and tissue was transferred to a 1:1 LR white acrylic resin-70%ethanol mixture for 15 minutes. Tissue was the transferred to 100% LR white for 30 minutes changing out the resin twice. Sample was added to a gelatin capsule, which was filled with 100% LR white and left at room temperature for 30 minutes to remove air bubbles. Polymerization was performed by placing samples at 60°C for 48 hours.

After polymerization, blocks were trimmed to a 1mm x1mm block face and tissue was cut with freshly made glass knives into 70 nm thick sections using a Leica Reichert Ultracut S Microtome. Tissue sections were collected on hexagonal 300-mesh nickel grids and stored at room temperature.

Immunofluorescence

Sections with well preserved morphology were selected for further processing. To prevent non-specific binding, sections were incubated in a 20% non-fat dry milk solution prepared in PBS for 2 hours at room temperature. After blocking, sections were washed 3 times with 0.05% Tween20 in PBS (PBST) solution. For immunodetection of Mrp4, sections were incubated with goat anti-ABCC4 / MRP4 antibody at a 1:160 dilution at 4°C overnight. Sections were washed 3 times with PBST after incubation of primary antibody, and they were treated with a secondary anti-goat IgG whole TRITC-conjugated antibody (Sigma-Aldrich, St. Louis, MO) at 1:60 dilution for 2 hours at room temperature. Secondary antibody incubation and subsequent steps were performed under red light settings to prevent photo-bleaching. After secondary antibody incubation, sections were once again washed and treated with Hoechst dye for nuclear staining at 1:2000 dilution at room temperature from 20 minutes. Coverslips were mounted using 90% glycerol. Imaging was performed using an Olympus FV1000 laser scanning confocal system and post-processing of images were enhanced using was performed using Adobe PhotoShop. Detailed information of image acquisition is given in figure legends.

Immunogold

For immunogold, nickel grids laden with samples were placed tissue-side down for 2 hours at room temperature on top of a layer of Parafilm-M containing drops of 20% non-fat dry milk solution prepared in PBS. After blocking, sections were washed 3 times with PBS solution. All transfers of grids from droplet to droplet were performed with fine forceps.

For immunodetection of Mrp4, grids were incubated with goat anti-ABCC4 / MRP4 antibody at a 1:160 dilution for 2 hours. After primary antibody incubation, grids were washed 3 times in PBS droplets for 10 minutes each, and a rabbit polyclonal antibody to goat IgG-H&L conjugated to 10nm colloidal gold (Abcam, Cambridge, MA) was used as a secondary antibody. Secondary antibody incubation was performed at 1:60 dilution for 2 hours at room temperature. Grids were then washed 3 times with PBS and analyzed using a JEOL JEM-1200EX II electron microscope. Images were captured using a Gatan SC1000 ORIUS® CCD TEM digital camera system. Images editing was performed using Gatans digital micrograph™ and adobe photoshop. Table 1 shows anibody dilutions used for immunofluorescence and immunogold techniques.

Table 1: Antibodies and stains used to evaluate Mrp4 expression in dark- and light-adapted zebrafish.

Application	Primary	Dilution	Secondary	Dilution
Immunofluorescence	Goat anti-ABCC4/MRP4. Everest Biotech LTD. Product EB06538	1:160 in PBS	Anti-goat IgG (whole molecule)–TRITC, antibody produced in rabbit. Sigma-Aldrich. Product T7028	1:60 in PBS
Immunogold		1:160 in PBS	Rabbit polyclonal to goat IgG-H-L(10nm gold). Abcam Product ab39594	1:60 in PBS

Functional Studies

To test whether Mrp4 is necessary for melanosome aggregation in zebrafish, sildenafil citrate, a well characterized Mrp4 inhibitor (Russel et al., 2008), was used to disrupt Mrp4 activity via intraocular injection. Twenty-four light-adapted zebrafish were divided into three experimental (injected) groups and 12 fish remained untreated as controls to assess normal dark-adaptive positions. The left eye of each fish from the experimental groups was injected with sildenafil citrate or PBS as vehicle control, and the right eye remained uninjected. Sildenafil citrate working solution (200 μM in PBS) was obtained from a stock solution dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO) at a concentration of 20 mg/ml as recommended by the manufacturer. Twelve adult fish from the same age were anesthetized in a 0.2% MS-222 solution and 0.3 μl of 200 μM

sildenafil citrate in PBS was intraocularly injected at approximately 11:30 AM (3.5 hours into the light cycle) in the left eye using a sterile Hamilton syringe. The volume of injection was calculated based on protocol previously described by Fimbel et al. (2007), and the sildenafil citrate dose was based on IC_{50} previously described by Reid et al. (2003) and Russel et al. (2008). To perform the intraocular injection, the anesthetized fish were wrapped in a wet paper towel leaving the head exposed and positioned under a stereoscope. After injection, fish were allowed to recover in aerated aquaria until balance was regained and normal swimming pattern was resumed. Fish were dark-adapted starting 30 minutes after injection (~12PM) and sacrificed by decapitation at different time points as explained in Table 2. To account for the effects of injection a separate group of 12 fish were injected with 0.3 µl of PBS under the same conditions as described above. The sildenafil-injected group and the PBS-injected group were kept under the same conditions after the injection.

Table 2: Time points of pharmacological disruption of Mrp4 activity in zebrafish via intraocular injections

Group	Time of injection	Time in Darkness/ Euthanized time	Time in Darkness/ Euthanized time	Time in Darkness/ Euthanized time	Time in Darkness/ Euthanized time
Sildenafil	11:30	0min /12:15	30min/12:45	75min/13:30	105min/14:00
PBS	11:30	0min /12:15	30min/12:45	75min/13:30	105min/14:00
Uninjected (Control Group)	N/A	0min /12:15	30min/12:45	75min/13:30	105min/14:00

After euthanizing fish by decapitation in complete darkness, all heads were fixed in 4% PFA in PBS overnight. Tissue was processed as previously described in the immunofluorescence section. Briefly; eyes were removed, washed, cryoprotected, and sectioned to yield 20 μm thick sections. Tissue was collected on gelatin coated microscope slides and stored at -20°C. Pigment indexes were assessed by measuring the distance from Bruch's membrane to the melanosome positioned closest to the vitreous (distance A) divided by the distance from Bruch's membrane to the outer limiting membrane (distance B) based on protocols described by Menger et al. (2005) and Levinson and Burnside (1981). Ten images were taken per eye per time point using an Olympus FV1000 laser scanning confocal system and pigment indexes were compared using analysis of variance.

The second set of intraocular injections was performed using DMSO in PBS, sildenafil in PBS, and sodium citrate in PBS. Three fish were used for each group, the left eye of each fish was injected as previously described and 3 more fish were left uninjected to be used as a control group. Fish were dark-adapted starting 30 minutes after the injection, and sacrificed by decapitation at 2PM. Pigment indexes were measured and compared for statistical differences.

Statistical Analysis

Measurements from pigment indexes were compared using R program for statistical analysis. Analysis of variance test was used to assess statistical differences among the different groups of treatment and Tukey's test was performed when statistical significant differences were found.

CHAPTER IV

RESULTS

Immunohistochemistry

Immunofluorescence was used to assess the expression of Mrp4 in the zebrafish retina. Fluorescence immunolabeling results show strong Mrp4 expression in dark-adapted experimental retinal sections with prominent labeling in the photoreceptor layer (Figure 3A). There was also intense immunolabeling observed in RPE and outer plexiform layer from dark-adapted retinal samples, but not as prominent as the Mrp4 labeling observed in the photoreceptor layer. Mrp4 immunolabeling was not detected in the dark-adapted control sections (Figure 3B).

Mrp4 labeling in light-adapted experimental samples was also detected in photoreceptor and RPE layers but to a lesser extent than the labeling observed in the dark-adapted experimental sections (Figure 4A). As in the dark-adapted control samples; no immunolabeling was detected in the light-adapted control sections (Figure 4B).

Due to the location and pattern of labeling observed in both dark- and light-adapted experimental sections, it was difficult to distinguish whether Mrp4 immunofluorescence emerged from RPE or photoreceptor cells. Thus, in order to elucidate the expression of Mrp4 at the cellular and subcellular levels immunogold technique was employed.

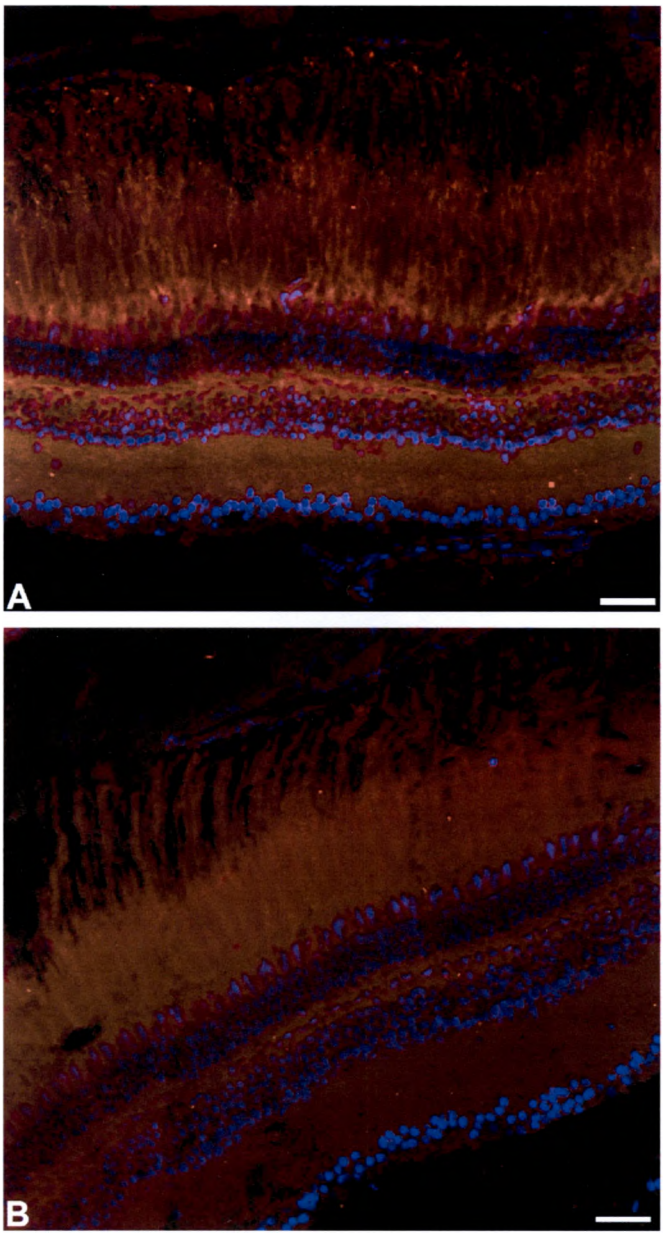

Figure 3. Dark-Adapted Confocal Images. A z-projection of 17 optical sections taken with 20X water immersion lens of a dark-adapted experimental retinal section (A), labeled with goat anti-ABCC4 as primary antibody and antigoat IgG TRITC as secondary antibody. Nuclei were identified by Hoescht DNA stain (blue). Dark-adapted control section (B) was not treated with primary antibody. Scale bar represents 30 μm for both images.

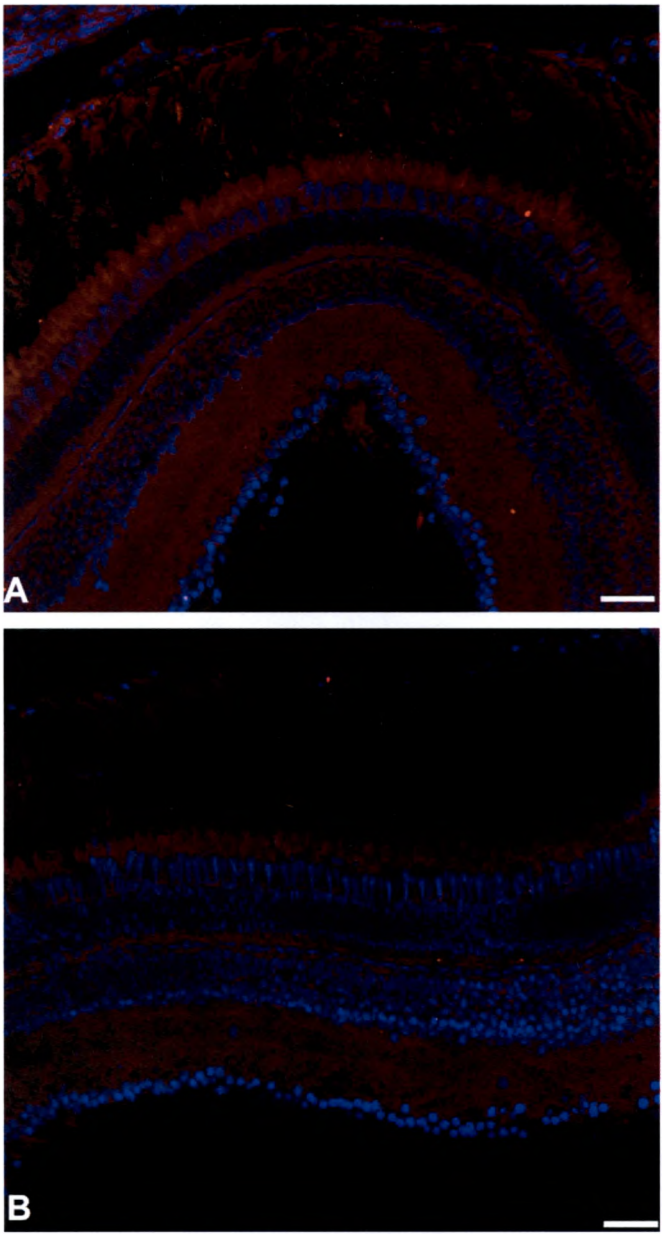

Figure 4. **Light-Adapted Confocal Images**. A z-projection of 17 optical sections taken with 20X water immersion lens of a light-adapted experimental retinal section (A), labeled with goat anti-ABCC4 as primary antibody and antigoat IgG TRITC as secondary antibody. Nuclei were identified by Hoescht DNA stain (blue). Light-adapted control section (B) was not treated with primary antibody. Scale bar represents 30 μm for both images.

Immunogold electron miscroscopy results were obtained from a retinal area of approximately 600 μm^2 from different micrographs. The number of colloidal gold particles found in a total tissue area of 290.12 μm^2 was evaluated for the light-adapted samples and an area of 291.28 μm^2 for the dark-adapted samples.

Areas evaluated were photoreceptor outer segment, photoreceptor inner segment, RPE cells excluding melanosomes, and melanosomes counted as separate entities. Colloidal gold particles found on extracellular space were taken into consideration for experimental and control groups from both dark-and light-adapted samples to ensure that labeling found on subcellular structures was specific.

Electron microscopy results show expression of Mrp4 localized to cones, rods, and RPE cells. This is consistent with the immunofluorescence findings. Subcellularly, Mrp4 labeling was found in photoreceptor inner and outer segments, as well as RPE cells including pigment granules, in both dark-adapted and light-adapted experimental retinal samples as shown Figure 5 and Figure 7 respectively.

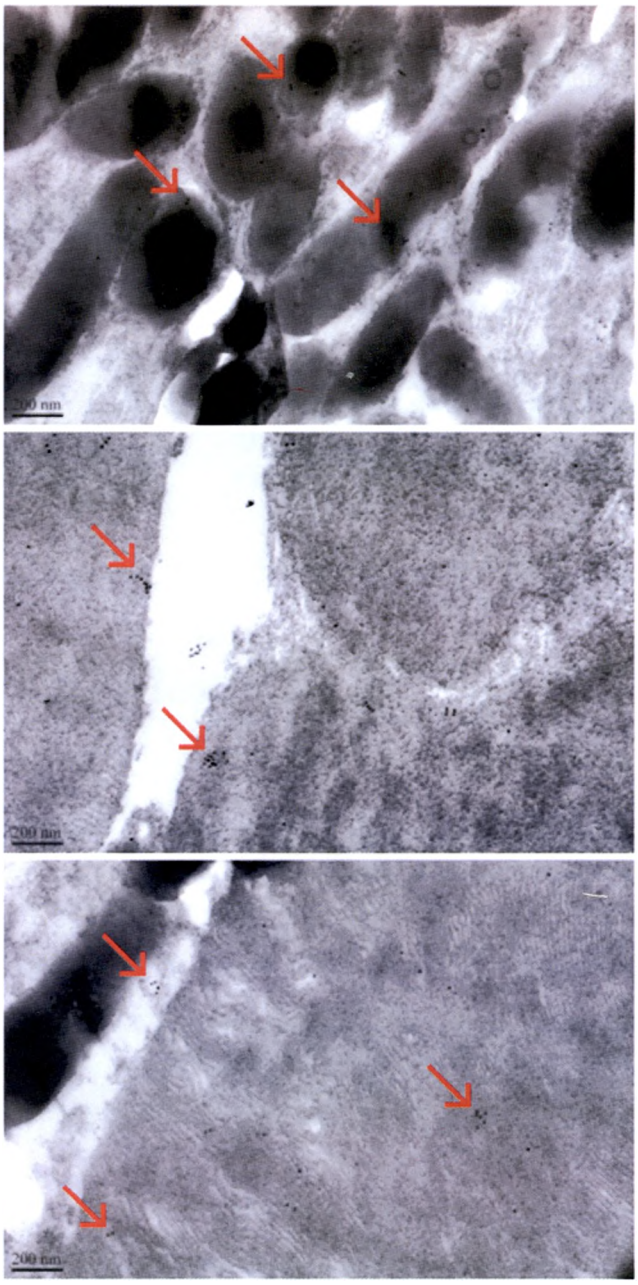

Figure 5. Dark-Adapted Electron Experimental Micrographs. Images depic immunogold labeling of Mrp4 from 2 hour dark-adapted zebrafish retinas using goat anti-ABCC4 as a primary antibody and rabbit polyclonal to goat IgG-H-L conjugated to 10 nm colloidal gold as secondary antibody. Images show immunogold labeling of Mrp4 on melanosomes (top), photoreceptor outer segment (center), and photoreceptor outer segment and RPE cells (bottom).

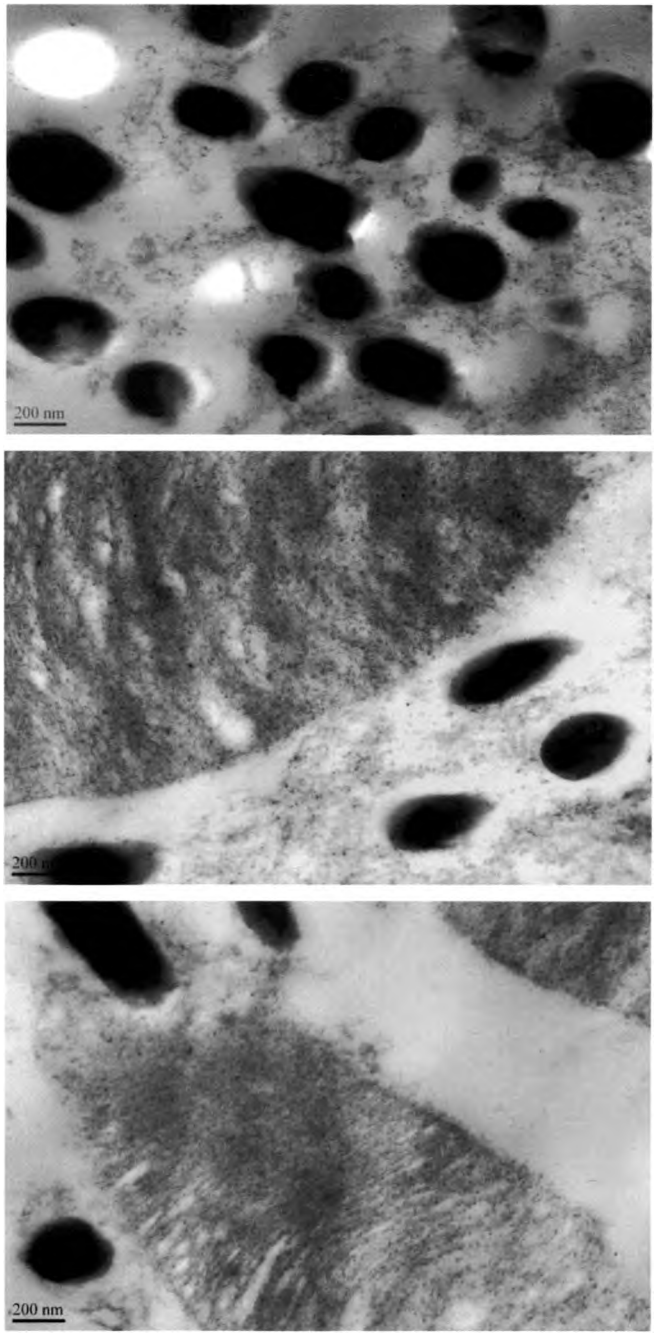

Figure 6. Dark-Adapted Negative Control Electron Micrographs. Electron micrographs from 2 hour light-adapted zebrafish retinas with no primary antibody treatment. No specific immunogold labeling of Mrp4 was detected on images.

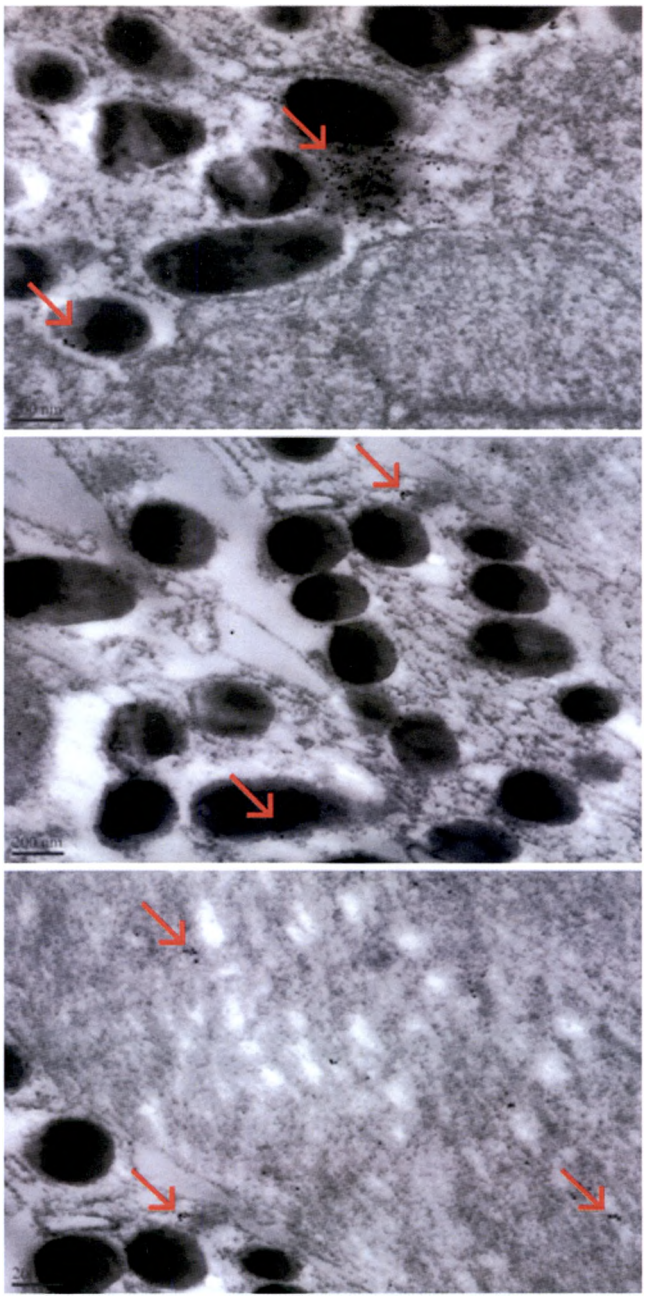

Figure 7. Light-Adapted Electron Experimental Micrographs. Images depict immunogold labeling of Mrp4 from light-adapted zebrafish retinas using goat anti-ABCC4 as a primary antibody and rabbit polyclonal to goat IgG-H-L conjugated to 10 nm colloidal gold as secondary antibody. Images show immunogold labeling of Mrp4 on photoreceptor inner segment and RPE cells (top), melanosomes (center), and photoreceptor outer segment (bottom).

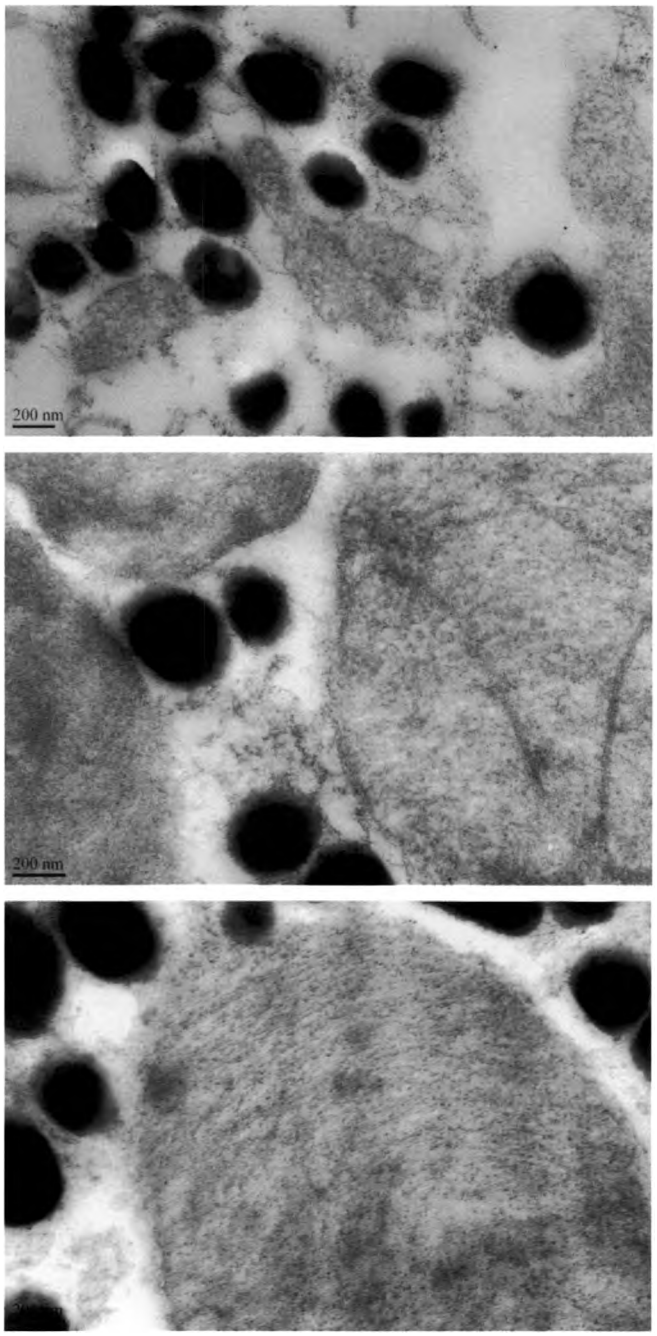

Figure 8. **Light-Adapted Negative Control Electron Micrographs**. Electron micrographs from 2 hour light-adapted zebrafish retinas with no primary antibody treatment. No specific immunogold labeling of Mrp4 was detected on images.

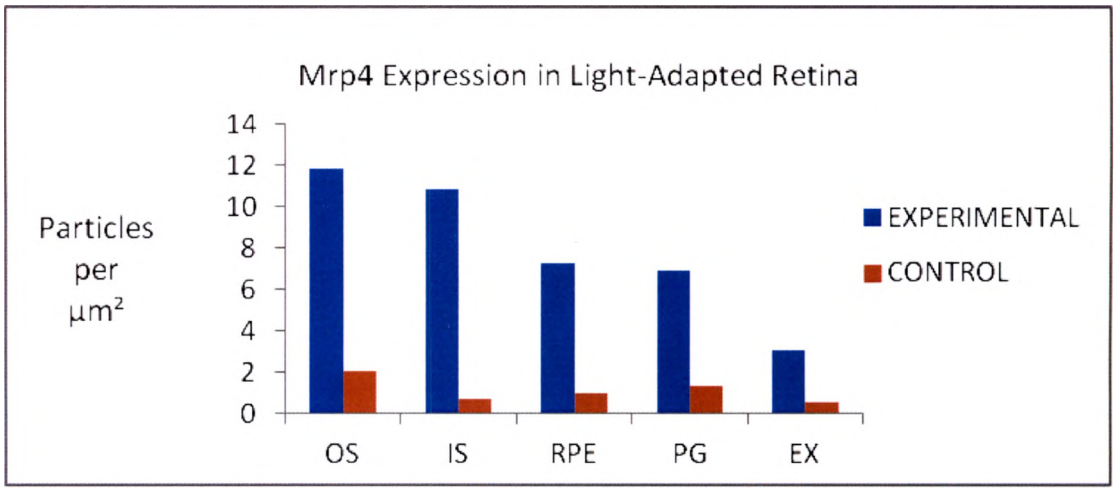

Figure 9. Mrp4 Expression in Light-Adapted Retina. Graph showing distribution of gold particles on Mrp4 labeled light-adapted zebrafish retina using goat anti-ABCC4 as a primary antibody and rabbit polyclonal anti-goat IgG- H-L (10nm gold). No primary antibody treatment was performed on control samples. The bar graph shows various degrees of Mrp4 expression depending on subcellular location. Outer segment (OS), inner segment (IS), retinal pigment epithelium (RPE) excluding pigment granules (PG), and extracellular space (EX).

Colloidal gold particle counts were markedly higher for both experimental groups when compared with their respective controls. As shown in Figure 9, immunogold labeling from light-adapted samples revealed experimental group to control group ratios were approximately 6:1 for photoreceptor outer segments, 15:1 for photoreceptor inner segment, 8:1 for RPE, and 5:1 for melanosomes. When combined, RPE group and pigment granules group together show a ratio over 6-fold higher than the one observed from the control counterparts combined. The relatively consistent and low level of labeling in the control samples with no primary antibody indicates that there was little non-specific binding of the gold-bearing secondary antibody in these experiments.

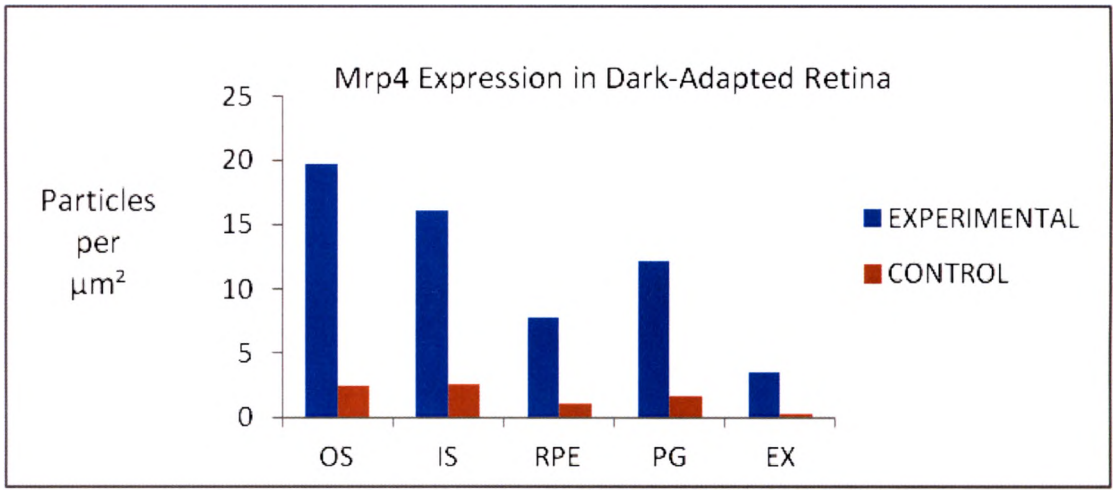

Figure 10. Mrp4 Expression in Dark-Adapted Retina. Graph showing distribution of gold particles on Mrp4 labeled 2 hour dark-adapted zebrafish retina using goat anti-ABCC4 as a primary antibody and rabbit polyclonal anti-goat IgG- H-L (10nm gold). No primary antibody treatment was performed on control samples. Images show various degrees of Mrp4 expression depending on subcellular location. Outer segment (OS), inner segment (IS), retinal pigment epithelium (RPE) excluding pigment granules (PG), extracellular space (EX).

As in the light-adapted samples, experimental groups exhibited a higher level of Mrp4 labeling in dark-adapted sections (see Figure 10). Immunogold labeling from dark-adapted samples revealed experimental group to control group ratios were approximately 8:1 for photoreceptor outer segments, 7:1 for photoreceptor inner segment, 7:1 for RPE, and 8:1 for melanosomes. When combined, RPE group and pigment granules group together show a ratio of almost 7.5-fold than the one observed from the control counterpart combined. The low level of binding of gold particles in the extracellular space confirms that there was relatively nettle non-specific binding of the primary antibody.

Dark-adapted samples showed more gold particles in every experimental group when compared with their respective light-adapted samples as shown in Figure 11. Outer and

inner segment areas from dark-adapted experimental samples exhibited the highest number of gold particles per area. Melanosomes from dark-adapted experimental sample showed higher expression of Mrp4 when compared with their light-adapted counterpart at approximately a 2:1 ratio. This tendency of higher Mrp4 expression in dark-adapted retinas is consistent with the results obtained from immunofluorescence. The similar low level of binding in the extracellular space of both light- and dark-adapted retinas further confirms that there was a relatively low level of non-specific binding by the primary antibody.

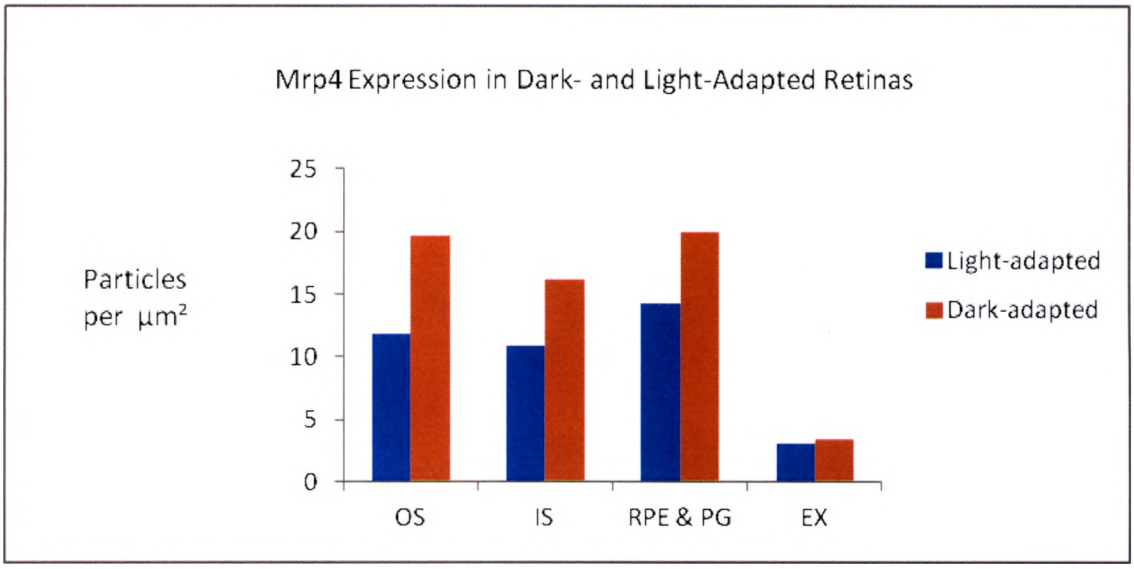

Figure 11. Mrp4 Expression in Dark- and Light-Adapted Retinas. Graph depicting immunogold labeling of Mrp4 on outer segment (OS), inner segment (IS), Retinal Pigment Epithelial (RPE) including pigment granules (PG), and extracellular space (EX) from dark-adapted and light-adapted zebrafish retina using goat anti-ABCC4 as primary antibody and rabbit polyclonal anti-goat IgG- H-L (10nm gold).

Functional Studies

In order to test whether Mrp4 is necessary for melanosome aggregation in zebrafish, sildenafil citrate was used to inhibit Mrp4 activity (Russel et al., 2008) via intraocular injection. The intraocular injections were performed using PBS, DMSO in PBS, sildenafil in PBS, and sodium citrate in PBS. Three fish were used for each group per time point; the left eye of each fish was injected as previously described in the Materials and Methods section.

In order to assess the results from the functional studies, pigment indexes from all groups were calculated as explained in the Materials and Methods section. Briefly, Pigment indexes were assessed by measuring the distance from Bruch's membrane to the most vitread positioned melanosome (distance A) divided by the distance from Bruch's membrane to the outer limiting membrane (distance B). Ten images were taken per eye per time point and pigment indexes were compared using analysis of variance.

Figure 12 shows the PBS-injected group failed to completely undergo normal pigment aggregation after 2 hours of dark adaptation but partial pigment aggregation was observed. In contrast, the sildenafil citrate injected group exhibited complete inhibition of pigment aggregation after 2 hours of dark adaptation.

After being dark-adapted for 2 hours, the PBS contralateral, sildenafil contralateral, and intact groups, all showed a low pigment index with no statistically significant differences among those groups; however, the pigment index change over time exhibits different behavior for three groups. The sildenafil-injected group showed the least change in

pigment position, and the uninjected group exhibited the greatest change as shown in Figure 12.

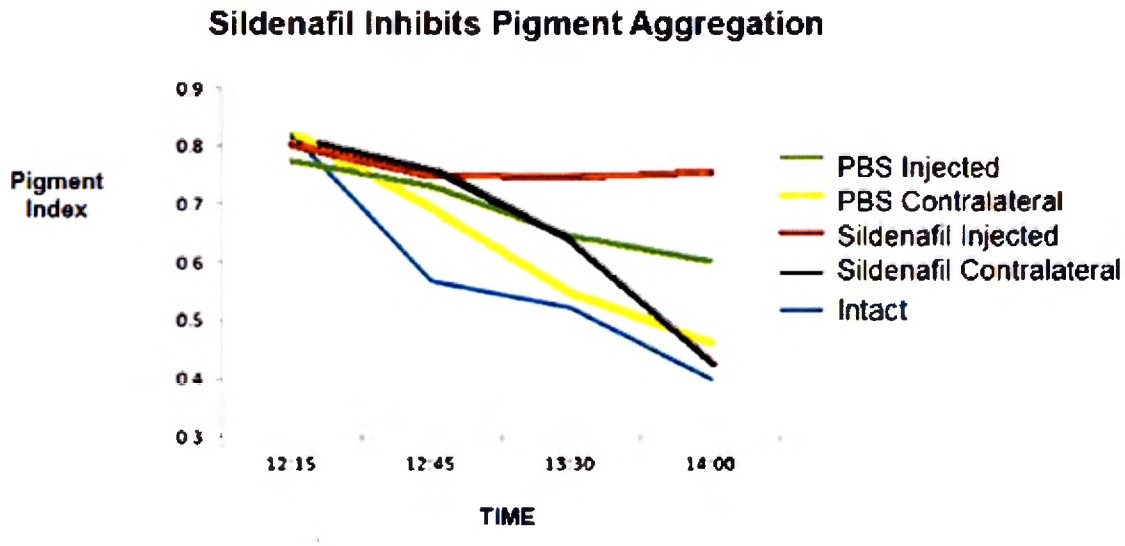

Figure 12. Sildenafil Inhibits Pigment Aggregation. Graph depicting pigment index change overtime for all the time points from PBS-injected group, PBS contralateral group, sildenafil-injected group, sildenafil contralateral group, and uninjected (intact) group. Sample size for each group n=3 per time point.

Figure 12 shows pigment aggregation is inhibited by sildenafil citrate and though there is some variability in the other groups, they all do undergo pigment aggregation. A more detail analysis of the statistical differences from all time points is provided in the Appendix section.

Pigment indexes from intraocular injections using DMSO in PBS and sodium citrate in PBS as carrier and counterion controls, respectively, were assessed. A second group of sidenafil-injected, PBS-injected, and intact eyes were also evaluated in this experiment.

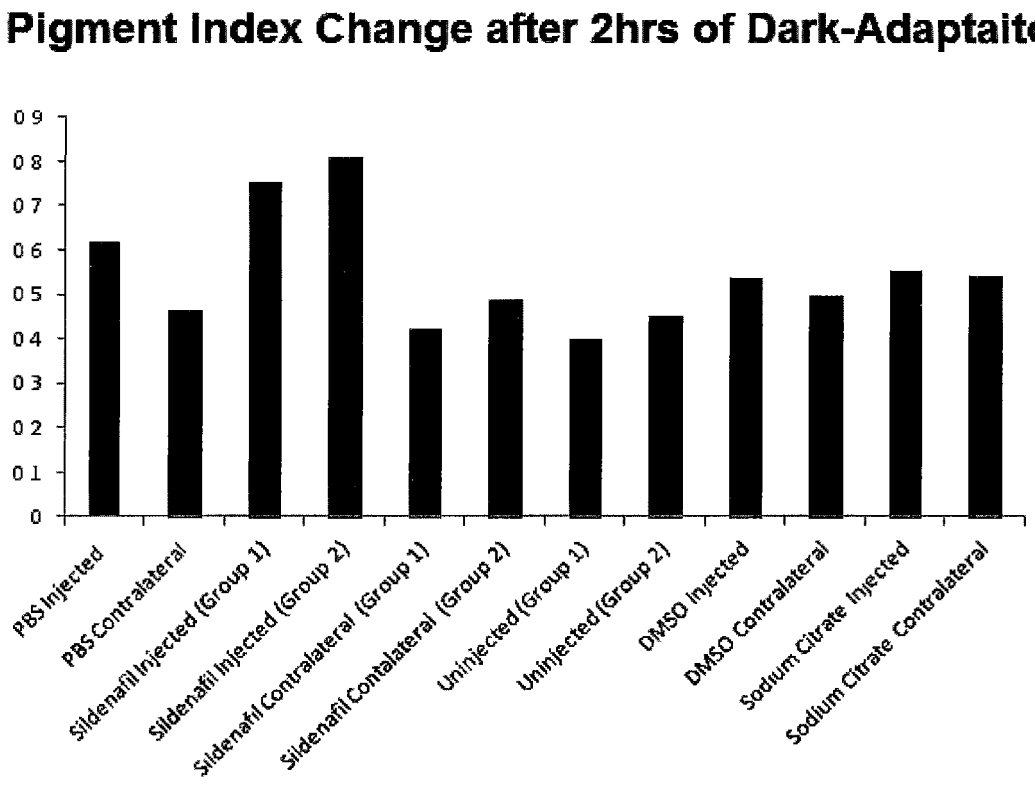

Figure 13. Pigment Index Change after 2hrs of Dark-Adaptation. Statistical differences of pigment index after 2 hours of dark adaptation for the first and second round of intraocular injections.

Figure 13 shows the sildenafil-injected groups from the later experiment and the earlier experiment show the highest pigment indexes but they were not statistically significantly different from one another (P value= 0.3882574). Also, there was no statistical differences between both uninjected groups (P value= 0.1642412). Furthermore, the sildenafil contralateral groups did not exhibit statistical sifnificant differences neither (P value=0.3443666). Thus, treatment groups from both trials were combined for subsequent statistical analysis. All the statistical analysis from the all the time points is

summarized in Table 3, which depicts P values from analysis of variance as well as P adjusted values from a Tukey's test for experimental and control groups.

Table 3. Pigment Index Change over Time. Table depicting statistical differences of pigment index change overtime for sildenafil injected group, PBS injected group, contralateral groups, and the intact group. Codes are as follows: *** highly statistically different, **statistically different, *slightly statistically different.

Group	P value	Timepoints that differ	P adjusted
Sildenafil Injected	0.02704 *	12:15 vs. 13:30	0.0373926
PBS Injected	$4.724e^{-09}$ ***	12:15 vs. 13:30	0.0000075
		12:15 vs. 14:00	0.0000001
		12:45 vs. 13:30	0.0055998
		12:45 vs. 14:00	0.0001174
Intact	$2.2e^{-16}$ ***	12:15 vs. 12:45	0.0000000
		12:15 vs. 13:30	0.0000000
		12:15 vs. 14:00	0.0000000
		12:45 vs. 13:30	0.0091405
		12:45 vs. 14:00	0.0000000
		13:30 vs. 14:00	0.0000000
Sildenafil Contralateral	$2.2e^{-16}$ ***	12:15 vs. 12:45	0.0015309
		12:15 vs. 13:30	0.0000000
		12:15 vs. 14:00	0.0000000
		12:45 vs. 13:30	0.0000000
		12:45 vs. 14:00	0.0000000
		13:30 vs. 14:00	0.0000000
PBS Contralateral	$2.2e^{-16}$ ***	12:15 vs. 12:45	0.0000078
		12:15 vs. 13:30	0.0000000
		12:15 vs. 14:00	0.0000000
		12:45 vs. 13:30	0.0000002
		12:45 vs. 14:00	0.0000000
		13:30 vs. 14:00	0.0046110

P adjusted values obtained from 2 hour-dark-adapted retinas using Tukey's test indicate that the sildenafil-injected group is statistically significantly different from the rest of all the groups. The uninjected group was statistically significantly different from most of the groups. Data from Table 4 summarize all statistical differences found in all injected groups, their contralateral counterparts, and the intact group as well. Statistical differences are noted by a numerical value and ND notes no statistical significant differences found.

Table 4. Statistical differences of pigment indexes after 2hrs of dark adaptation for sildenafil injected group, PBS injected group, sodium citrate injected group, DMSO injected group, their respective contralateral groups, and the intact group. ND stands for no difference.

DMSO Contralateral	ND	-						
Sodium Citrate Injected	ND	ND	-					
Sodium Citrate Contralateral	ND	ND	ND	-				
PBS Injected	0.0207652	0.0000116	ND	0.0473748	-			
PBS Contralateral	ND	ND	0.0068522	0.0227394	0.0000000	-		
Sildenafil Injected	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	-	
Sildenafil Contralateral	0.0022163	ND	0.0001175	0.0006587	0.0000000	ND	0.0000000	-
Uninjected	0.0000001	0.0016836	0.0000000	0.0000000	0.0000000	ND	0.0000000	ND
	DMSO Injected	DMSO Contralateral	Na Citrate Injected	Na Citrate Contralateral	PBS Injected	PBS Contralateral	Sildenafil Injected	Sildenafil Contralateral

CHAPTER V

DISCUSSION

In this study, I characterize the spatial and temporal patterns of expression of Mrp4 in the zebrafish retina to infer the possible roles that Mrp4 might play in retinomotor movements. Here, I propose a model by which retinomotor movements might take place. I hypothesize that Mrp4 is expressed by photoreceptor cells, where it regulates intracellular cAMP levels. In this model, regulation of cAMP levels by Mrp4 occurs by exporting cAMP from photoreceptors into the subretinal space. Once in the interstitial space, cAMP is taken up by RPE cells where it induces and maintains the dark-adapted retinomotor position of the pigment granules.

In the descriptive part of this study, protein expression was analyzed by immunohistochemistry. Here, I show Mrp4 expression on dark- and light-adapted retinas by immunofluorescence, and I report location and distribution of Mrp4 by immunogold electron microscopy. In the functional part of this study, Mrp4 was inhibited via intraocular injections with a specific Mrp4 inhibitor. I report pigment indexes measurements to assess whether or not Mrp4 plays an active role during retinomotor movements.

The detoxifying functions of Mrp4 are well documented. In the kidney of mammals, Mrp4 is localized on the brush border membrane of proximal tubules, where it transports endogenous substrates from cell to lumen (Van de Water et al., 2005; El-Sheikh et al., 2008); however, little is known about Mrp4 in the retina. By means of immunofluorescence, Bataille et al. showed renal expression of Mrp4 (Bataille et al. 2008). In my thesis work, immunofluorescence was used to assess the expression of Mrp4 in the zebrafish retina.

Here, I report Mrp4 immunolabeling in the zebrafish retinal tissue from both dark- and light-adapted samples. Fluorescence immunolabeling results show strong Mrp4 expression in dark-adapted experimental retinal sections with prominent labeling in the inner and outer segments part of the photoreceptor layer when compared with dark-adapted control sections (Figure 3). These findings are consistent with the model I propose, in which, Mrp4 from photoreceptor cells could be involved in inducing and maintaining the dark-adapted retinomotor state in RPE cells. This model is based in part on the observation that agents that elevate cAMP concentration in the retina induce dark-adaptive retinomotor movements (Ferrendelli and Cohen, 1976; Orr et al., 1976; DeVries et al., 1978; DeVries et al., 1979; Farber et al., 1979; Farber et al., 1981; Burnside et al., 1982; Cohen, 1982; Burnside and Ackland, 1984; Dearry and Burnside, 1985; García and Burnside, 1994) and cAMP is an endogenous substrate of Mrp4 (Chen et al., 2001).

Considering the fact that cAMP is present to a higher extent in dark-adapted retinas and that Mrp4 is the member of the Mrp family with the highest affinity for cAMP, an Mrp4-cAMP relationship that mediates dark-adaptive retinomotor movements could be

proposed. This is supported by immunofluorescence data in which Mrp4 immunolabeling from dark-adapted experimental samples was also detected to a higher extent in photoreceptor and RPE layers when compared to the light-adapted samples.

There are 48 ABC genes in the human genome and the majority of them are conserved in all vertebrates, suggesting that their functions have been retained (Dean and Annilo, 2005). Some ABC transporters have been localized in the human retina (Aukunuru et al., 2001; Stojic et al, 2007). Mrp4 has been shown to be expressed in isolated mouse retinal endothelial cells and human retinal vascular endothelial cells, suggesting vascular functions of Mrp4 (Tachikawa et al., 2008; Tagami et al., 2010). Mrp4 is exclusively expressed in retinal endothelial cells in normal mice, but an oxygen-induced retinopathy mouse study showed that Mrp4 expression is diminished in pathological retinal vessels (Tagami et al., 2009). In this study, I am the first to report Mrp4 expression and localization in the retina of zebrafish. A distinctly higher Mrp4 immunogold labeling was observed in dark-adapted retinal samples when compared to light-adapted retinal samples. Furthermore, the patterns of subcellular localization of Mrp4 were elucidated for both types of samples.

Photoreceptor outer and inner segments from dark-adapted experimental samples exhibited the highest number of colloidal gold particles per area, suggesting that Mrp4 could contribute to the modulation of photoreceptor intracellular cAMP levels. If this is true, Mrp4 accomplishes a dual function in the retina; it not only maintains cellular homeostasis and avoids retinal degeneration by exporting cAMP from photoreceptors when cytoplasmic levels rise in the dark; but it also establishes an extracellular cell to cell

communication signaling pathway between photoreceptors and RPE cells by making cAMP available from photoreceptor cells to the RPE cells.

Melanosomes from dark-adapted experimental samples showed higher density of Mrp4 labeling when compared with their light-adapted counterparts, approximately a 2:1 ratio. Immunogold labeling in RPE cells, not including melanosomes, was also greater in the dark-adapted samples. This could suggest that Mrp4 plays regulatory functions not only in photoreceptor cells but in RPE cells as well. These data raise the question of whether Mrp4 regulates intracellular cyclic nucleotide levels within localized compartments of the retinal cells. Furthermore, my findings that sildenafil inhibits melanosome aggregation imply that cAMP in RPE cells is derived exogenously as inhibiting Mrp4 on RPE cells would be expected to lead to accumulation of RPE derived cAMP and enhancing melanosome aggregation; instead, inhibition of melanosome aggregation was observed. The data from immunogold experiments is in agreement with the findings from the immunofluorescence studies, and both results suggest Mrp4 could play a major role in regulation retinomotor movements.

In the descriptive part of this study, I was able to show Mrp4 expression and identify its subcellular location in zebrafish retina. In the functional part of this study, sildenafil citrate, an Mrp4 inhibitor (Reid et al., 2003), was used to disrupt Mrp4 activity to assess its putative contribution to the regulation of retinomotor movements. It has previously been shown that extracellular cAMP can induce dark-adaptive retinomotor pigment aggregation in RPE cells (García and Burnside, 1994; Keith et al., 2006). The premise of the present experiment is that if Mrp4 activity is required to export cAMP to the

subretinal space so that cAMP can be available for RPE cells, then animals lacking functional Mrp4 should fail to undergo dark-adaptive response.

This study shows that RPE-pigment granule aggregation following 2 hours of dark-adaptation was significantly inhibited in retinas treated with sildenafil. The sildenafil-injected group showed no statistical differences in pigment position when compared to the light-adapted retina. Since Mrp4 function was presumably inhibited in the sildenafil-injected animals and these animals failed to achieve pigment aggregation, our studies strongly suggest that Mrp4 plays an active role during retinomotor movements, via elevating cAMP in the subretinal space. In 2008, Sassi et al. provided evidence that, in human and rat arterial smooth muscle cells, Mrp4 functions as an endogenous regulator of intracellular cAMP levels and as a consequent mediator of related signaling pathways (Sassi et al., 2008).

The results described above are supported by the pigment aggregation data observed from the PBS-injected group, the DMSO-injected group, and the sodium citrate-injected group. All these groups exhibited a negative slope during the 2 hours of dark-adaptation, which indicates that pigment granules became increasingly aggregated as dark-adaptation time increased. The premise of this conclusion is that if sildenafil citrate, which was dissolved in DMSO to prepare a stock solution, and it was subsequently dissolved in PBS to reach a working solution impeded pigment granule aggregation; then it was possible that citrate, DMSO, PBS, or factors altered by the injection (e.g., intraocular pressure) could have been the active agents for inhibiting pigment aggregation. However, since sodium citrate, DMSO or PBS groups failed to prevent pigment aggregation; it follows that sildenafil

itself mediated the aggregation-inhibiting response of zebrafish retina during scotopic conditions.

All the above observations support the idea that Mrp4 play a major role in regulating retinomotor movements. The data are consistent with a role for Mrp4 of exporting cAMP into the subretinal space when photoreceptor cytoplasmic levels rise under dark conditions. These results support the model that Mrp4 is expressed by photoreceptors cells where it regulates intracellular cAMP levels by exporting cAMP into the subretinal space; this is in accordance with reported functions of Mrp4 as a modulator of cyclic nucleotide-mediated signal transduction (Sassi et al., 2008). Once cAMP is in the subretinal space, it could be taken up by RPE cells via organic anion transporter to induce pigment aggregation and maintain the dark-adapted state as proposed by García and Burnside (1994).

In conclusion, using zebrafish as an animal model, I have demonstrated expression and distribution of Mrp4 in the retina. My findings strongly suggest functional relevance of Mrp4 in the regulation of retinomotor movements. These findings suggests that Mrp4 inhibition in the retina is able to modulate cAMP levels that then translate into significant activation of cell to cell communication related signaling pathways.

APPENDIX

This section shows a detailed statistical analysis of all the interim time points of groups from the functional studies. Groups were analyzed by time points and treatment using analysis of variance test and Tukey's test; all data are summarized in Table 5.

The 12:15PM sildenafil-injected group and the 2PM sildenafil-injected group were not statistically significantly different from each other. The sildenafil-injected group exhibited a slightly significant difference in pigment index between the 12:15PM and 1:30PM time points; the rest of the time points for the sildenafil injected group showed no statistical significant differences among the groups.

The PBS injected group showed highly significant differences in pigment indexes between the time points that underwent longer dark-adaptation (1:30PM and 2:00PM) when compared with the short-dark-adapted group (12:45PM) and the non-dark-adapted group (12:15PM).

The intact group showed highly significant differences for all time points. Pigment indexes decreased as dark-adaptation time increased. The same tendency was observed in the PBS contralateral and sildenafil contralateral groups; however they both exhibited different slopes for pigment aggregation. Although, all the time points from sidenafil contralateral group and PBS contralateral group exhibited statistically significant

differences among them for the interim time points, they exhibited a similar tendency as the one observed for the intact group.

Table 5 depicts all the P adjusted values obtained from the 12:15PM, 12:45PM, and the 1:30PM groups using Tukey's test. For the 12:15PM groups, the PBS-injected group exhibited differences from the PBS contralateral group, uninjected group, and sildenafil contralateral group. After 30 minutes of dark-adaptation, the intact group of eyes was statistically significantly different from the rest of all the groups. The sildenafil contralateral group and the PBS contralateral group were statistically significantly different from one another. There were no statistically significant differences for the rest of the groups from this time point. For the 1:30PM time point, the PBS contralateral group was statistically significantly different from the sildenafil-injected group and sildenafil contralateral group. In addition, the sildenafil-injected group was statistically significantly different from the rest of all the groups. The PBS injected group was statistically significantly different from the uninjected group, sildenafil-injected group, and PBS contralateral group. The uninjected group was statistically significantly different from the PBS-injected group, sildenafil-injected group, and sildenafil contralateral group.

Table 5. Statistical Differences of Pigment Indexes for Interim Time Points.

12:15PM GROUPS				
PBS Contralateral	0.0013431	–		
Sildenafil Injected	ND	ND	–	
Sildenafil Contralateral	0.0074018	ND	ND	–
Uninjected	0.0004584	ND	ND	ND
	PBS Injected	**PBS Contralateral**	**Sildenafil Injected**	**Sildenafil Contralateral**
12:45PM GROUPS				
PBS Contralateral	ND	–		
Sildenafil Injected	ND	ND	–	
Sildenafil Contralateral	ND	0.0170005	ND	–
Uninjected	0.0000000	0.0000000	0.0000000	0.0000000
	PBS Injected	**PBS Contralateral**	**Sildenafil Injected**	**Sildenafil Contralateral**
1:30PM GROUPS				
PBS Contralateral	0.0005035	–		
Sildenafil Injected	0.0004505	0.0000000	–	
Sildenafil Contralateral	ND	0.0025012	0.0000744	–
Uninjected	0.0000001	ND	0.0000000	0.0000012
	PBS Injected	**PBS Contralateral**	**Sildenafil Injected**	**Sildenafil Contralateral**

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