M_{odd} MUSCARINIC RECEPTOR FUNCTION AND EXPRESSION IN BLUEGILL RETINAL PIGMENT EPITHELIUM

THESIS

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ABSTRACT

Light adaptation in most vertebrates occurs through adjustments in pupillary diameter. Teleosts, however, have a fixed pupil size so they rely on retinomotor movements to regulate the amount of light that reaches the photoreceptors. Retinomotor movements include photoreceptor contraction and elongation as well as the aggregation and dispersion of membrane bound pigment granules located in the retinal pigment epithelium (RPE). Pigment granule dispersion can be induced by using the acetylcholine analog carbachol, and carbachol-induced dispersion can be inhibited by M_{odd} muscarinic receptor specific antagonists. In this study I demonstrate that the native ligand, acetylcholine, also induces pigment granule dispersion but only in the presence of the acetylcholinesterase inhibitor huperzine-A. Previous pharmacological studies have shown that Modd agonists and antagonists are able to mediate carbachol induced dispersion while Meven agents do not. To investigate the involvement of the M_5 receptor in light adaptation, isolated bluegill RPE cells were treated with the venom of the Malayan spitting cobra, *Naja naja sputatrix*. The venom contains an M_5-specific antagonist that inhibited carbachol-induced dispersion. Furthermore, my studies using snake venom revealed possible involvement of phospholipase A_2 in carbachol-induced pigment granule dispersion. I also sought to demonstrate M_{odd} receptor expression in RPE by immunolabeling with an anti-human M_3 antibody and by using *in situ* hybridization targeting the M_5 receptor transcripts. Labeling with the M_3 antibody was

seen in the inner and outer nuclear layers and the inner plexiform layer of the bluegill retina. *In situ* hybridization with the M_5 probe indicated a similar distribution of the receptor as that seen with the antibody and also appeared to label structures in the ganglion cell layer. These results suggest that multiple muscarinic receptor subtypes are expressed in the fish retina or that the labels failed to adequately discriminate among M_{odd} receptors.

CHAPTER I

INTRODUCTION

Light adaptation in most vertebrates occurs in part through adjustments in pupillary diameter. Increasing light intensity causes the constriction of the pupil via the pupillary light reflex (Heller et al., 1990). Light falling on the retina initiates a signal that is propagated from the pretectum to the Edinger-Westphal nucleus in the brain. The Edinger-Westphal nucleus has a group of pre-ganglionic parasympathetic neurons that innervate the sphincter muscle of the iris (Chen et al., 2004). Parasympathetic signaling can induce constriction of the pupil by causing the contraction of the sphincter muscle.

The parasympathetic nervous system relies on the neurotransmitter acetylcholine acting on cholinergic receptors identified as either nicotinic or muscarinic. Previous studies have shown that smooth muscle contractions, including those of the iris, are controlled in part by muscarinic receptors, of which there are 5 known subtypes in human (Bonner et al., 1998). In 2002, Matsui et al. produced a mutant mouse lacking two muscarinic receptor subtypes, M_2 and M_3. They concluded that different receptor subtypes can be involved in pupillary constriction and dilation resulting from acetylcholine signaling and that their absence results in unusually small pupils.

Unlike many other vertebrates, teleost fish have fixed pupil diameters so they must employ mechanisms other than pupillary constriction to adjust the amount of light

that reaches the photoreceptors. The mechanisms they employ are called retinomotor movements, and these retinomotor movements (fig.1) include the aggregation and dispersion of membrane-bound pigment granules in retinal pigment epithelium (RPE) as well as photoreceptor contraction and elongation (Burnside and Nagle, 1983).

Located between the choroid and the neural retina, RPE is a thin layer of simple cuboidal cells that is important in nutrient transport and in phagocytosis of shed photoreceptor discs (Zinn and Marmor, 1979). In fish, RPE cells have long apical processes that interdigitate with the photoreceptors. Aggregation of the pigment granules into the cell body occurs in response to low light or darkness while dispersion of the granules into the apical processes occurs in intense light (fig 1). This transport of pigment granules allows the appropriate conditions for rod outer segment function (Douglas et al., 1992).

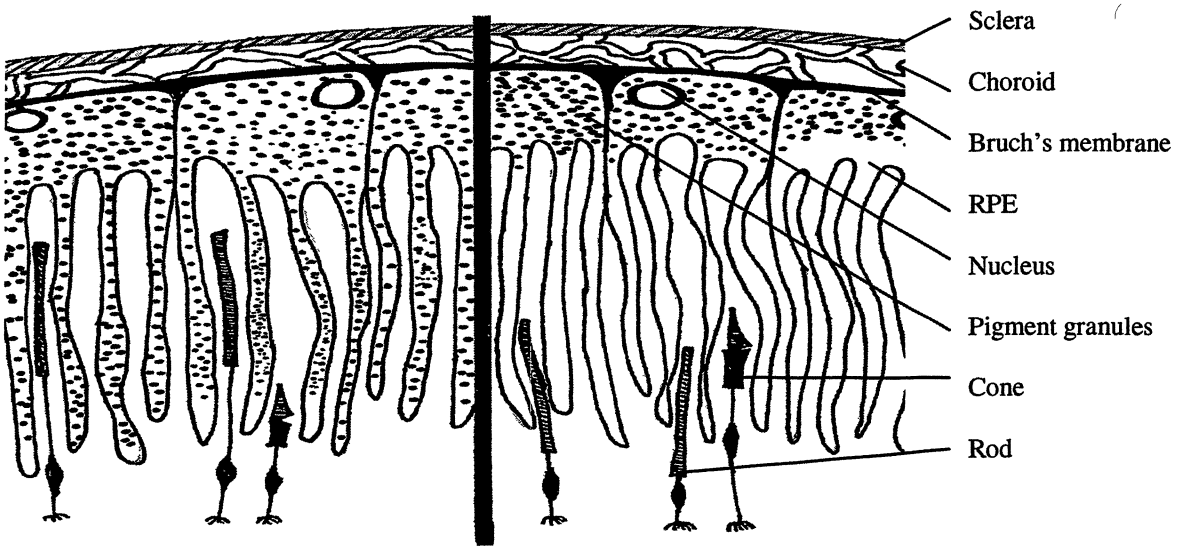

Figure 1. Diagram illustrating retinomotor movements involving RPE and photoreceptors in the teleost eye. In bright light rods elongate, cones contract and membrane-bound pigment granules disperse into the apical processes. In darkness the rods contract and cones elongate while the pigment granules migrate to the cell body.

Previous work has shown that pigment granule dispersion can be induced by carbachol, an analog of the neurotransmitter acetylcholine, in RPE isolated from green sunfish (Garcia, 1998) and bluegill (González et al., 2004). González et al. (2004) showed further that carbachol-induced pigment granule dispersion inhibited by atropine, indicating that carbachol induced pigment granule dispersion by activating muscarinic receptors. Muscarinic receptors are members of the G-protein coupled receptor family. G-protein coupled receptors are characterized by seven membrane-spanning domains. Muscarinic receptors can be further subdivided into subtypes based on their association with particular G-proteins (Caulfield et al., 1998).

G-proteins are heterotrimeric proteins that associate with the intra-cytoplasmic loops of the receptor. When the extracellular portion of the receptor binds a ligand, a conformational change in the receptor's structure causes a change in the G- protein itself leading to decreased affinity of the alpha subunit of the G-protein for its bound GDP molecule. GTP replaces GDP as the bound guanine nucleotide, and the protein complex dissociates and initiates the intracellular signal cascade. M_{odd} receptors (M_1, M_3, and M_5) are associated with G_q proteins which activate phospholipase C causing the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). They have the downstream effect of increasing intracellular calcium by opening calcium channels on the endoplasmic reticulum. M_{even} receptors (M_2 and M_4) are coupled to $G_{inhibitory}$ proteins. Upon activation $G_{inhibitory}$ proteins inhibit adenylyl cyclase activity and ultimately decrease intracellular cAMP (Hann and Chazot, 2004).

Muscarinic receptors are of interest to the biomedical community because of their distribution throughout the body and involvement in numerous signaling pathways.

Among these are glucose homeostasis, resulting from M_3 receptor activity in the beta cells of the Islets of Langerhan (Gautam et al., 2007), and stomach motility resulting from both M_2 and M_3 receptor activity (Kitazawa et al., 2007). These receptors are also targets of emerging therapeutics for the treatment of Alzheimer's disease and other neurodegenerative disorders. In 2002, Messer reported increased memory in animal models for Alzheimer's treated with M_1 selective agonists. Thus muscarinic receptor pharmacology is of interest to the pharmaceutical industry and a number of subtype-specific cholinergic agonists and antagonists have been developed (Caulfield et al., 1998).

Pharmacological studies using subtype specific agonists and antagonists suggest that light adaptive pigment granule movement is mediated by M_{odd} receptors (González et al., 2004; Phatarpekar et al., 2005). This hypothesis required further scrutiny since the pharmacological agents used were characterized in mammals and differences in receptor affinity for some antagonists have been observed in other species (Tietje and Nathanson, 1991; Hsieh and Liao, 2002). Phatarpekar et al. (2005) extended the pharmacological evidence and used molecular characterization to develop a model for muscarinic receptor involvement in pigment granule dispersion. Specifically, they showed that bluegill RPE expressed M_5 muscarinic receptors but were unable to demonstrate expression of M_2 receptors.

For my thesis research, I set about to accomplish four objectives, oriented toward testing the hypothesis that **pigment granule dispersion is induced by acetylcholine and is mediated by the M_5 muscarinic receptor**. First, I tested whether the native muscarinic receptor ligand acetylcholine activated pigment granule dispersion in

isolated RPE. Although the relationship between the endogenous ligand acetylcholine and pigment granule dispersion in RPE had been implied by previous work, it had not been established experimentally.

My second objective was to test further whether the M_5 receptor mediates carbachol-induced pigment granule dispersion. Until now only M_5 has been found to be expressed in bluegill RPE using RT-PCR (Phatarpekar et al., 2005). However, Phatarpekar et al. (2005) did not test for muscarinic receptor subtypes other than the M_2 receptor. Thus, he did not rule out the presence of other muscarinic receptors or establish that the M_5 receptor was directly involved in pigment granule dispersion. To demonstrate functional M_5 involvement I used *Naja naja sputatrix* venom. According to Miyoshi and Tu (1999), the venom from this snake species has a muscarinic receptor inhibitor that has been shown to have an affinity for the human M_5 receptor an order of magnitude greater than for the other human muscarinic receptors.

My third objective was to verify that the M_5 receptor gene product detected by Phatarpekar et al. (2005) was indeed expressed in the RPE. Reverse-transcriptase-PCR is susceptible to contamination, therefore, the possibility remained that the M_5 gene product detected arose from cells other than the RPE. I hoped to verify M_5 expression using *in situ* hybridization in retina by targeting a portion of the third intra-cytoplasmic loop of the receptor.

Finally, Nuckels (2006) showed antibody labeling in zebrafish RPE using an anti-M_3 polyclonal. However his analysis of the epitope sequence revealed a high degree of similarity among M_{odd} receptor subtypes. Therefore I sought to demonstrate labeling in

bluegill retina and to determine which receptor(s) was recognized by the antibody using western blot analysis.

CHAPTER II

MATERIALS AND METHODS

Fish maintenance

Bluegill (*Lepomis macrochirus*) were purchased from Johnson Lake Management San Marcos, Texas. The fish were acclimated in 55-gallon aquaria in a 12-hour light–12-hour dark cycle room for a minimum of two weeks before use in accordance with IACUC approved protocol (069744F82).

Pharmacology

All chemicals, unless otherwise noted were purchased from Sigma, St. Louis, MO. To isolate RPE, the fish were dark adapted for 30 minutes in a light-tight box six hours into their light cycle. The fish were killed by double pithing following severing of the spinal cord in lighting of 2 lux or less. The eyes were then removed and hemisected, and the lens, vitreous humor and neural retina were removed. A low-calcium modified Ringer's solution (24 mM $NaHCO_3$, 3 mM HEPES free acid, 116 mM NaCl, 5 mM KCl, 1 mM $NaH_2PO_4 \cdot H_2O$, 26 mM dextrose, 1 mM ascorbic acid, 0.8 mM $MgSO_4$, 1 mM EGTA, 0.9 mM $CaCl_2$, gassed with a 5% CO_2/95% air mixture 15 minutes before and during the experiment to maintain pH at 7.2) was used to flush out RPE sheets. The isolated RPE was incubated forty-five minutes in a solution containing 100μM forskolin (LC Laboratories, Woburn, MA) to induce pigment granule aggregation.

To determine the effects of acetylcholine on pigment granule position, the RPE was incubated in 100 nM acetylcholine or carbachol (Chemicon, Pittsburg, PA) with and

without 100μM huperzine-A (LC Laboratories,) for 45 minutes. RPE was also treated with 90μg/ml venom from the Malayan spitting cobra (*Naja naja sputatrix,* (Sigma) alone and with 50 μM 4-bromophenacyl bromide (MP, Irvine, CA) in the presence and absence of 100 nM carbachol (Acros, Geel, Belgium). The RPE samples were then fixed overnight at 4° in 0.5% gluteraldehyde, 0.5% paraformaldehyde and 0.8% $K_3Fe(CN)_6$ in PBS (137 mM NaCl, 10 mM $NaPO_4$, 2.7 mM KCl, pH 7.4).

The fixed RPE samples were chopped into smaller pieces using a glass cover slip, mounted and viewed using a phase contrast microscope (Zeiss, Jena, Germany). To measure the effects of each treatment, pigment indices (PI) were recorded for 30 cells from a minimum of 3 replicates by calculating the ratio of the length of the cell containing pigment granules to the total cell length (Fig. 2; Bruenner and Burnside, 1986). The collected results were analyzed using a single factor ANOVA (Microsoft Excel, Redmond, WA) and a Tukey's post hoc test for multiple comparisons (S-plus, Chicago, Il).

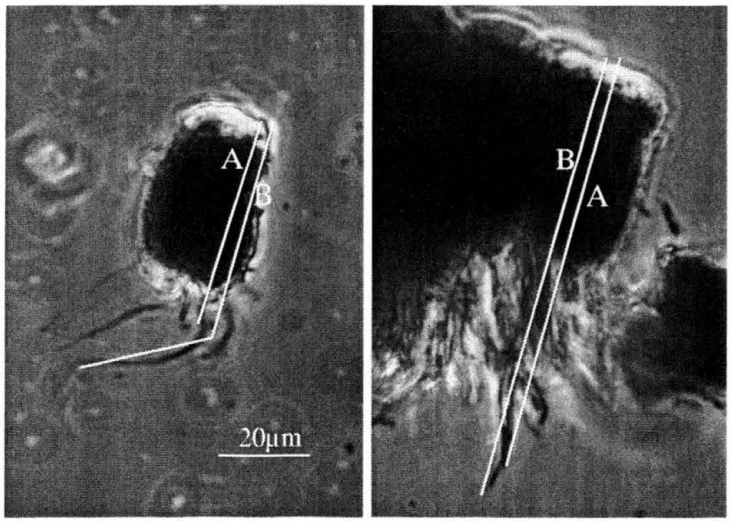

Figure 2. Phase contrast images of aggregated and dispersed RPE cells. The cell on the left was treated with 10 μM forskolin (FSK) for 45 minutes to induce aggregation. The cell on the right was treated with 100 nM carbachol after the FSK treatment to

induce dispersion. Pigment indices (PI) are measured by recording the length of the cell containing pigment (A) divided by the total length of the cell (B).

Preparation of sections for immunolabeling and *in situ* hybridization

Bluegill eyes were removed 6 hrs after light onset in the light or after a 30 minute dark adaptation. The eyes were punctured and fixed overnight in 4% paraformaldehyde in PBS at room temperature followed by an overnight incubation in 30% sucrose/PBS at room temperature. The eyes were frozen in hexane at -70°C and sectioned in a Zeiss Microm cryostat at a thickness of 10 μm. Sections were collected on slides coated with 0.1% gelatin and 0.05% K_2SO_4, allowed to dry at room temperature and stored at 4°C until used.

Immunolabeling

Previously prepared slides were rinsed in PBTD (PBS, 0.1% Tween, 1% DMSO) and incubated one hour at room temperature in PBTD containing 5% normal goat serum. The primary antibody, polyclonal rabbit anti human M3 I2 loop (Abcam, catalog number 13063, Cambridge, MA), was prepared at a 1:150 dilution in PBTD with 5% normal goat serum and applied to the slides overnight at 4°C. Following three 10-minute washes in PBTD, the slides were treated for 1 hour at room temperature with the secondary antibody, goat anti rabbit IgG conjugated to Alexafluor 488 (Invitrogen, Carlsbad, CA), diluted 1:200 in PBTD containing 5% normal goat serum. After the slides were washed a final time in PBTD, coverslips were mounted using Citifluor as a mounting medium (Ted Pella, Redding, CA). Images were obtained using a Nikon Eclipse 80i epifluoresence light microscope with ACT-1 image acquisition software (Nikon, Melville, NY) using identical settings and processed using Photoshop.

Western Blotting

Six bluegill were dark adapted and killed six hours after light onset. Their neural retinas, hearts, RPE and brains were collected and homogenized in a protein extraction buffer (0.1% Triton X-100, 1 mM EDTA, 35 µg/ml phenylmethylsulphonyl fluoride and 0.5 µg/ml leupeptin in PBS). The homogenates were left on ice for 30 minutes and then centrifuged at top speed for 8 minutes, discarding the pellet. Protein concentrations were estimated with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) using the A280 module (table 1). The homogenates were mixed with 5X sample buffer (50 mM Tris-HCl, 25% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.1% bromophenol blue). The samples were boiled for 10 minutes and centrifuged for 3 minutes at 10,000 rpm to remove debris.

Table 1. Bluegill protein concentrations used in SDS-PAGE. Six bluegill were used to prepare pooled protein samples of heart, brain, retina and RPE.

Tissue	Extract concentration (mg/ml)	Quantity used in SDS-PAGE (µg)
Heart	**8.56**	**29.10**
Brain	**10.78**	**32.34**
Retina	**5.33**	**29.84**
RPE	**3.31**	**26.48**

Approximately 30 µg of protein from each sample (table 1) and a protein standard (Bio-Rad, Hercules, CA. catalog number 161-0373) were loaded onto two 10% acrylamide gels prepared with a 4% stacking gel. The proteins were separated electrophoretically using a Mini Protean II apparatus (Bio-Rad, Hercules, CA) running at 200V for 30 minutes containing electrophoresis buffer (20 mM Tris-HCl, 0.192 M

glycine, and 0.1% SDS). One of the sister gels was stained using Coomassie blue for 45 minutes and de-stained overnight in 10% glacial acetic acid and 5% methanol. The proteins on the duplicate gel were transferred onto nitrocellulose membrane (Bio-Rad) in the apparatus run at 100V for 1 hour in transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol).

After the transfer, the nitrocellulose was washed for 10 minutes in TBST (0.05% Tween-20, 20 mM Tris and 0.9% NaCl), 10 minutes in TBS (20mM Tris, 0.9% NaCl, pH 7.5), and 45 minutes in blocking solution (3% non-fat dry milk, 0.1% BSA and 0.01% NaN_3 in TBS). The nitrocellulose was incubated in the primary antibody, polyclonal rabbit anti human M_3 I2 loop (Abcam 13063), at a 1:900 dilution in 0.1% BSA/TBST overnight at 4°C. After a rinse in TBST, the nitrocellulose was washed twice in TBST for 10 minutes per wash, twice for 10 minutes per wash in TBS and for 30 minutes in blocking solution. The secondary antibody, goat anti rabbit IgG conjugated to alkaline phosphatase, was diluted 1:30,000 in blocking solution and applied for 1 hour at room temperature. The secondary was rinsed off with TBST and the nitrocellulose was washed 3 times (10 minutes each) in TBST.

A SigmaFast NBT/BCIP tablet was dissolved in 10 ml de-ionized water and applied for 10 minutes. After the bands became visible the nitrocellulose was rinsed with de-ionized water, dried and scanned.

***In situ* hybridization**

The oligonucleotide probe sequences were designed using the known bluegill M5 sequence (accession # AY834251) and using Primer 3 software (http://frodo.wi.mit.edu/) to make sure that the selected sequences had at least 20 base pairs of which 50% were G

or C. Probes were made by Thermo Fisher Scientific using a portion of the I3 loop of the M_5 receptor as the template (table 2). Each probe was labeled at the 5' end with a digoxigenin hapten.

Table 2. M_5 probe sequences synthesized by Thermo Fisher Scientific for use in *in situ* hybridization. The anti-sense probe was complementary to the target mRNA sequence, and the sense probe was used as a negative control.

Probe name	Sequence
M5 sense	CAC CCA GTC TTC ATG GTC CT
M5 anti-sense	AGA CCA TGA AGA CTG GGG TG

The previously prepared sections were dehydrated for 3 minutes each in 50%, 70%, and 95% ethanol. Hybridization chambers were prepared by placing a Kimwipe moistened with hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 7, 0.1% SDS) in 50 ml centrifuge tubes and incubating tubes at 46°C for a few minutes before use. The sections were hybridized at 46°C for one hour using 5ng/µl of probe prepared in a warm 50% hybridization buffer/ 50% formamide mix. The slides were washed in hybridization buffer at 46°C for 20 minutes, rinsed with deionized water, and allowed to dry at room temperature. The digoxigenin hapten is detected using an HRP conjugated Fab antibody (Roche, Basel, Switzerland) at a 1:100 dilution in a buffer containing 0.5% blocking agent (Roche), 0.1 M maleic acid at a pH of 7.5, and 0.15 M NaCl at room temperature for one hour. After a rinse with deionized water the slides were washed for 15 minutes in a buffer containing 0.1 M maleic acid at a pH of 7.5, and 0.15 M NaCl, and again in a buffer containing 0.1 M Tris/HCl at a pH of 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$ followed by another rinse with deionized water and air-drying. The CARD (catalyzed reporter deposition) method was performed using tyramide signal

amplification reagents (Perkin Elmer, Waltham, MA) to improve the detectability of hybridization. The sections were incubated for 15 minutes in a buffer containing 0.1 M Tris/HCl, at a pH of 7.5, 0.15 M NaCl, and 0.05% Tween 20. Fresh tyramide solution was prepared by diluting the tyramide reagent 1:50 using the amplification dilutant. The tyramide solution was then applied to each slide for 5 minutes at room temperature followed by a rinse and wash in a buffer containing 0.1 M Tris/HCl, at a pH of 7.5, 0.15 M NaCl, and 0.05% Tween 20. Citifluor (Pella) was used to mount coverslips on the air-dried slides. Images were obtained using an Nikon Eclipse 80i epi-fluoresence equipped light microscope with ACT-1 image acquisition software. The captured images were processed using Photoshop (San Jose, CA).

CHAPTER III

RESULTS

When the isolated RPE cells were treated with forskolin (fig. 3), the pigment indices (PIs) ranged between 0.59 and 0.71 with a mean 0.64 ± 0.06 (n=3). Treatment with 0.1 nM acetylcholine resulted in PIs between 0.66 and 0.77 with a mean of 0.73 ± 0.07 (n=3). Treatment with 0.1 nM and 10 nM acetylcholine resulted in PIs ranging between 0.64 to 0.68 with a mean of 0.66 ± 0.02 (n=3) and 0.72 to 0.80 with a mean of 0.75 ± 0.04 (n=3), respectively. This range of indices indicated no effect of increasing acetylcholine concentrations ($p = 0.079$; $n = 3$ at each concentration) (Fig. 3).

When huperzine–A, an acetylcholinesterase inhibitor, was used in conjunction with acetylcholine (fig. 4), PI's ranged from 0.87 to 0.92 with a mean of 0.90 ± 0.02 (n=3), similar to what was seen when cells were treated with carbachol alone (0.87 ± 0.01; n=35) or with carbachol with huperzine–A (0.87 ± 0.04; n=3). Treatment with forskolin (0.67 ± 0.02), acetylcholine (0.61 ± 0.01) or huperzine – A (0.68 ± 0.02) alone, yielded PI values indicating similarly aggregated cells as opposed to the more robust dispersal of pigment granules caused by the other treatments ($p < 0.0001$) (Fig. 4).

The pharmacological studies were continued using venom isolated from the spitting cobra (*Naja naja sputatrix*) to determine M_5 muscarinic receptor involvement in

pigment granule dispersion (Fig.7). As seen in previous experiments, treatment with forskolin resulted in PI's ranging from 0.59 to 0.66 with a mean of 0.63 ±0.01 (n=3) while cells treated with 100 nM carbachol had PIs ranging from 0.78 to 0.92 with a mean of 0.84 ± 0.02 (n=3). Because the venom is known to have phospholipase A-type activity (Miyoshi and Tu, 1999), I tested whether the inhibition of carbachol-induced pigment granule dispersion was a consequence of the phospholipase activity. Treatment with the PLA_2-inhibitor 4-bromophenacyl bromide alone had no effect on pigment granule dispersion; PIs for treated cells ranged from 0.64 to 0.75 with a mean of 0.72 ± 0.03 (n=3). Cells treated with both 4-bromophenacyl bromide and carbachol or with 4-bromophenacyl bromide, snake venom and carbachol had PIs ranging from 0.70 to 0.84 with a mean of 0.77 ± 0.03 (n=3) in the former case and from 0.80 to 0.88 with a mean of 0.83 ± 0.03 (n=3) in the latter case. Carbachol and snake venom together resulted in a PI range of 0.62 to 0.70 (0.67 ± 0.02) indicating that carbachol induced dispersion was inhibited ($p < 0.001$)(Fig. 5).

To demonstrate the location of M_5 receptor in the bluegill retina, light- and dark-adapted retinas were sectioned, prepared and labeled with an oligonucleotide probe targeting a portion of the I3 loop of the receptor. Labeling in the light-adapted sections (Fig. 5) indicates that mRNA encoding the receptor is present in the inner and outer nuclear layer, the outer plexiform layer and the ganglion cell layer. The dark-adapted sections (Fig. 6) appear to have labeling in the RPE/photoreceptor layer in addition to the layers labeled in the light-adapted sections.

Immunolabeling using the M_3 muscarinic receptor antibody on both light and dark adapted retinal sections indicated the presence of antigen in multiple layers of the neural

retina and more pronounced labeling was visible in the dark-adapted sections (Fig. 8). In the dark-adapted sections the outer nuclear layer, outer plexiform layer, inner nuclear layer and the ganglion cell layer appear to be labeled. The light-adapted sections have similar labeling with the exception of the ganglion cell layer. Western blot analysis indicates that there are multiple proteins recognized by the antibody (Fig. 9). The brain and retina extracts displayed bands at 70, 35, 20 kD and 70, 40, 35, 30 kD, respectively. Heart extract had a single band at approximately 90 kD. No bands were labeled in the RPE extract.

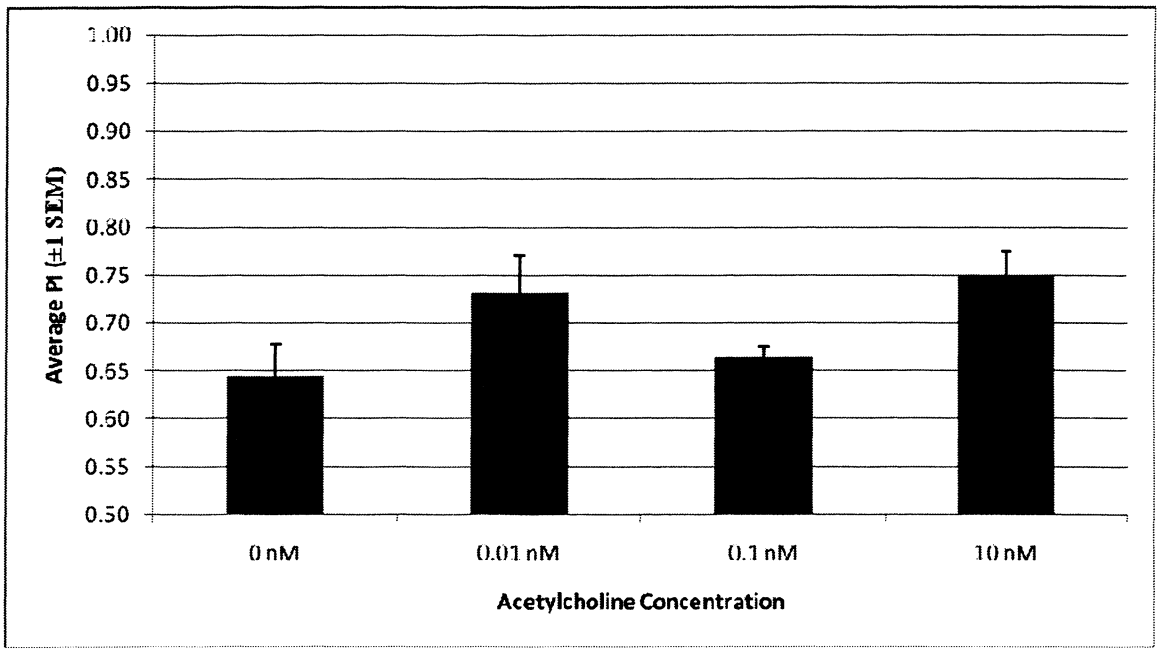

Figure 3. Acetylcholine alone does not induce pigment granule dispersion in isolated RPE. RPE was isolated from bluegill and first treated with 10 μM forskolin for 45 minutes to induce pigment granule aggregation. Forskolin was washed out, and RPE was then treated with acetylcholine for an additional 45 minutes. For each sample, n = 3. Acetylcholine did not induce pigment granule dispersion at any concentration used yielded a PI significantly different from that of the forskolin-treated cells ($p = 0.079$).

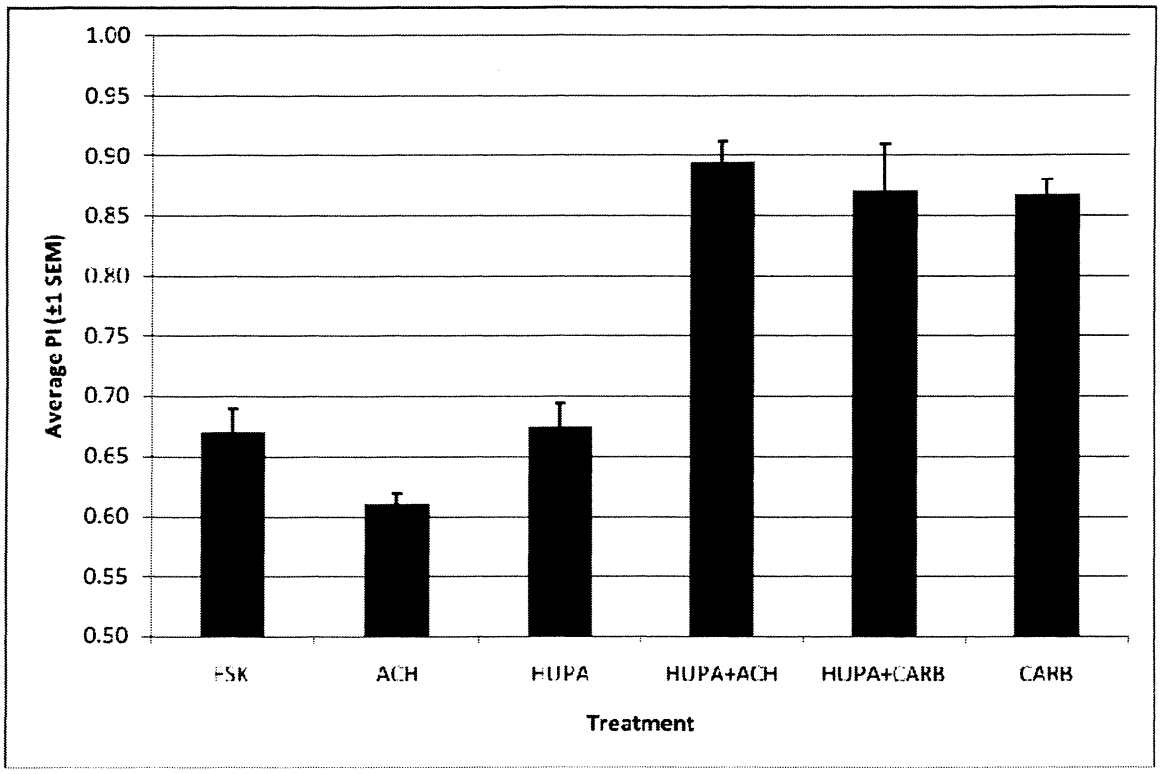

Figure 4. Acetylcholine induces pigment granule dispersion in isolated RPE in the presence of huperzine-A. RPE cells were isolated from bluegill and treated with 10 μM forskolin (FSK) to induce pigment granule aggregation. Following pigment aggregation, tissue was treated with 0.1 μM acetylcholine (ACH) or 0.1 μM carbachol (CARB) in the presence or absence of the acetylcholinesterase inhibitor huperzine-A (HUP). For each sample, n = 3. Although neither huperzine nor acetylcholine had an effect on pigment position, acetylcholine in the presence of huperzine induced pigment granule dispersion as robustly as did carbachol, with pigment indices being significantly greater ($p < 0.0001$) than those for forskolin-treated tissues or tissues treated with either drug alone.

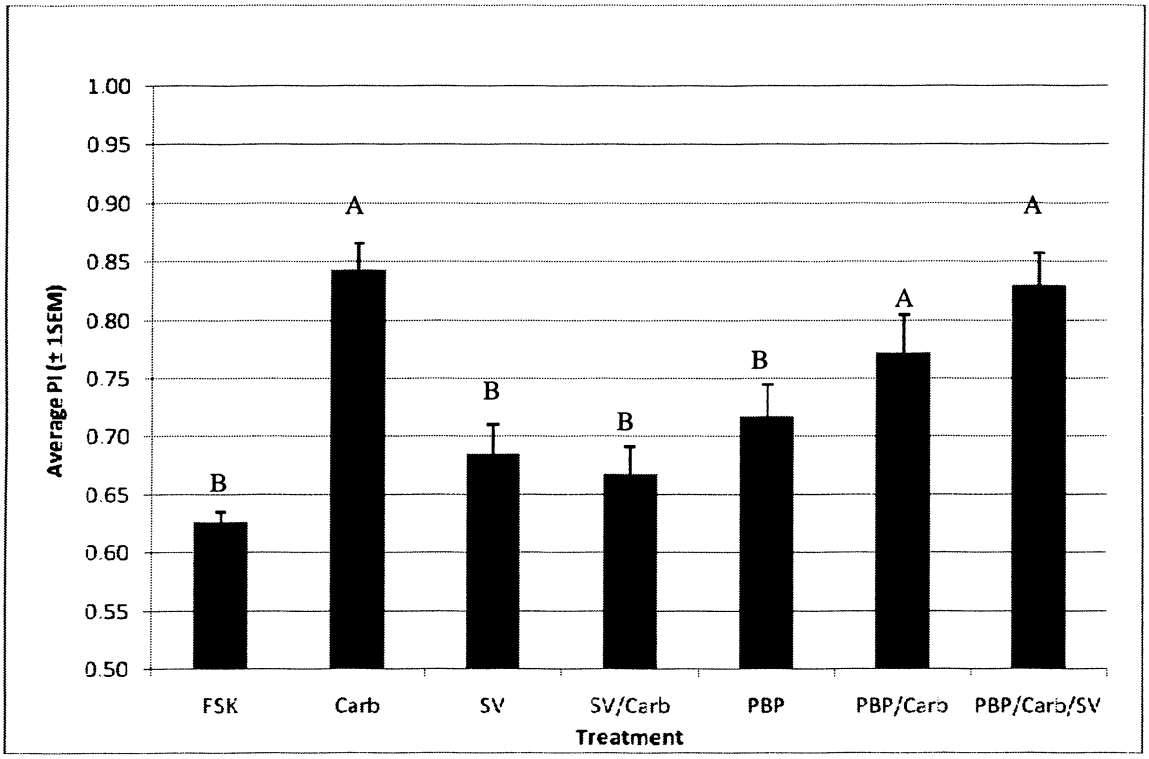

Figure 5. Carbachol-induced pigment granule dispersion is inhibited by *Naja naja sputatrix* venom even in the presence 4-bromophenacyl bromide (PBP) a PLA_2 inhibitor. RPE cells were isolated from bluegill and treated with 10 μM forskolin (FSK) to induce pigment granule aggregation. Following pigment aggregation, tissue was treated with 0.1 μM carbachol (Carb) in the presence or absence of 90 μg/ml venom (SV) and/or 50 μM PBP. Carbachol-induced dispersion was inhibited by the venom but when the cells were treated with carbachol and PBP, there was also a less robust dispersion than that seen with carbachol alone ($p < 0.0001$, numbers on each bar indicate sample size). Treatments noted with the letter A have means that are significantly different than those noted with the letter B.

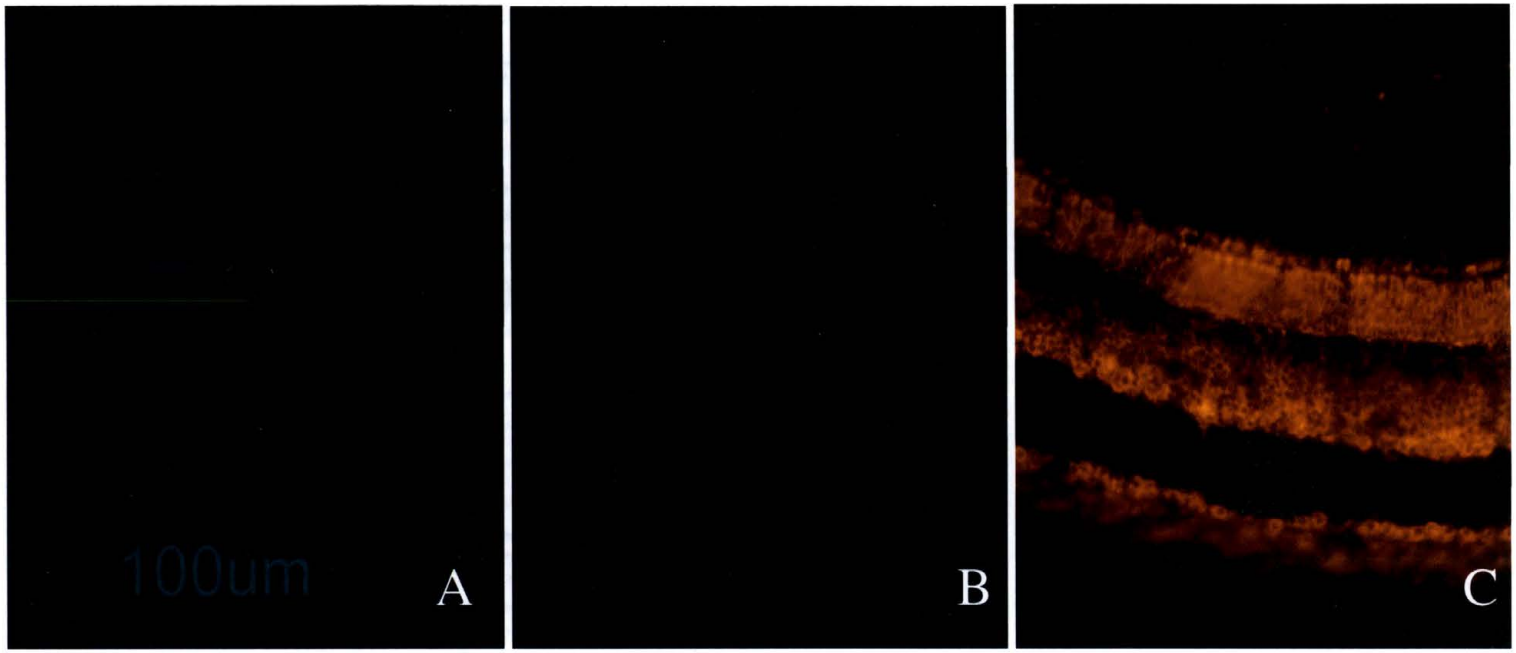

Figure 6. *In situ* hybridization of M_5 muscarinic receptor message in light-adapted bluegill retinal sections. Bluegill eyes were fixed 6 hours into the light cycle and sectioned at a 10 μm thickness. The signal amplification procedure alone (A) yielded no labeling, nor did treatment with the sense probe (B) followed by signal amplification. Treatment with the anti-sense probe and amplification (C) produced labeling in the inner nuclear layer, outer nuclear layer and the bipolar cell layer.

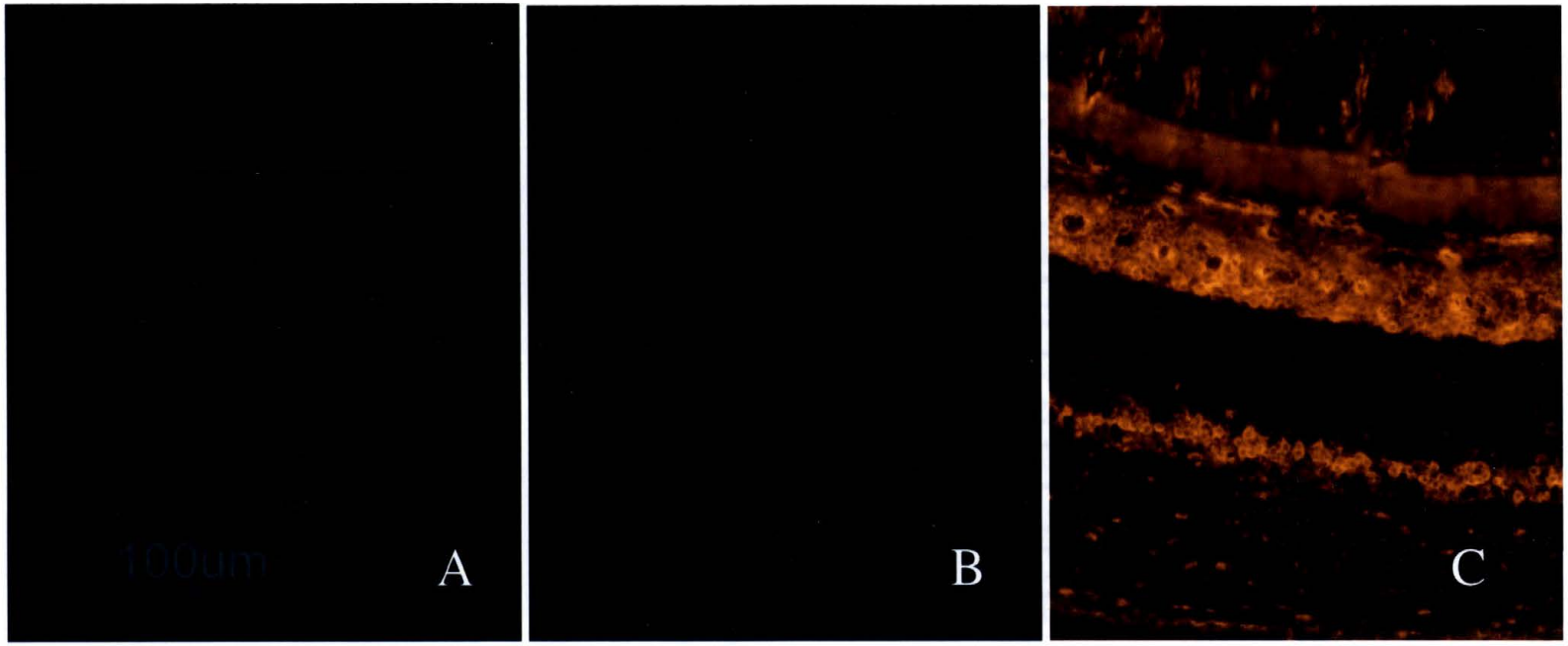

Figure 7. *In situ* hybridization of M_5 muscarinic receptor message in dark-adapted bluegill retinal sections. Bluegill eyes were fixed 6 hours into the light cycle after a thirty-minute dark adaptation and sectioned at a 10μm thickness. Treatment with tyramide CARD method alone (A) yielded no labeling as did treatment with the sense probe (B) and CARD. Treatment with the anti-sense probe and CARD (C) produced labeling in the inner nuclear layer, outer nuclear layer, the bipolar cell layer and in the RPE/photoreceptor layer.

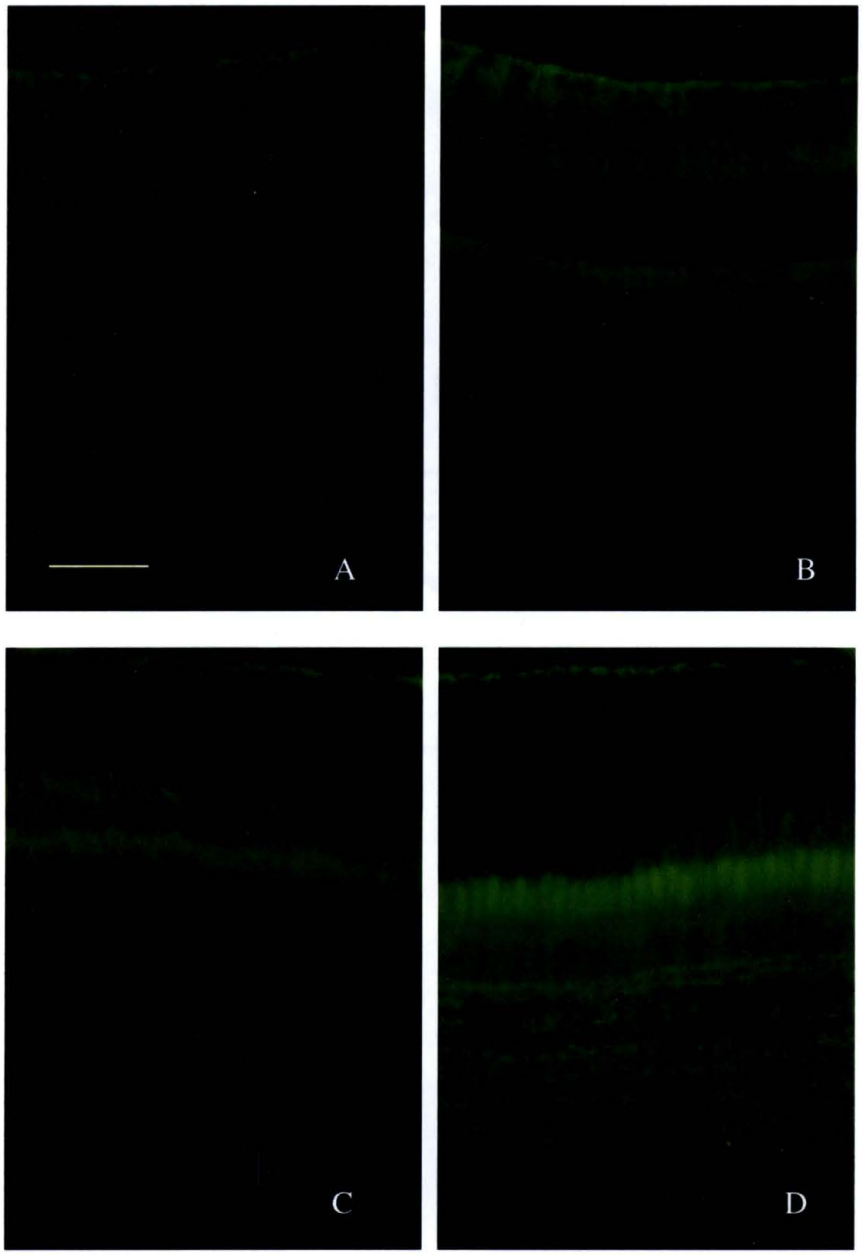

Figure 8. M_3 antibody labeling of bluegill retinal sections. Light (A and B) and dark (C and D) adapted bluegill eyes were sectioned at a 10μm thickness where A and C are the controls. The sections were labeled with polyclonal rabbit anti-human M_3 IgG antibody and goat anti-rabbit IgG conjugated to Alexa 488. The scale bar represents 50 μm. Labeling is present in several layers of the neural retina.

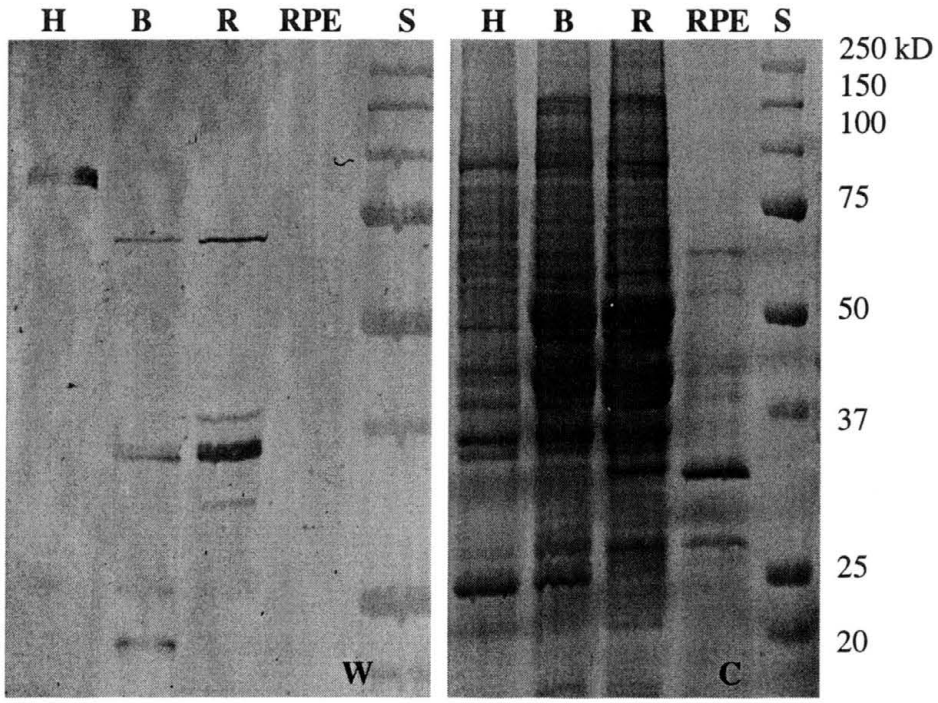

Figure 9. Western blot analysis of bluegill proteins recognized by the M_3 antibody. Protein extracts from heart (H), brain (B) retina (R), and RPE were loaded on sister SDS-PAGE gels, stained with Coomassie blue or transferred onto nitrocellulose for western blot analysis. The M_3 antibody recognizes proteins of various molecular weights in all but the RPE extracts. Comparison of the protein bands with the molecular weight standard (S) indicates the presence of an antigen of about 70kD in brain and retina and another about 35kD. The heart extract had a single band at about 90 kD.

CHAPTER IV

DISCUSSION

The effects of acetylcholine on RPE

I show here that the physiological ligand acetylcholine induces dispersion in RPE isolated from bluegill retina. However this result is only observed when RPE are simultaneously treated with an acetylcholinesterase inhibitor. This result, published in Phatarpekar et al. (2005) is the first demonstration of the native ligand acetylcholine eliciting pigment granule dispersion in isolated RPE. Previous pharmacological experiments relied on the use of the acetylcholine analog carbachol to induce pigment granule dispersion as was first described in green sunfish RPE by García in 1998. Among its many roles in the retina, acetylcholine is involved in object edge detection in the rabbit retina (Masland et al., 1984) and acts via muscarinic and nicotinic receptors to detect motion (Jardon et al., 1992). It is also interesting to note that the retina of the teleost *Eugerres plumieri* releases 52% more acetylcholine when it is light adapted as compared to the dark adapted retina (Vivas et al., 1980). Taken together these results are consistent with acetylcholine being a modulator of light adaptive responses in the retina. The observation that acetylcholine also induces pupillary constriction (Chen et al., 2004) adds an interesting additional role for acetylcholine in light adaptation.

M_5 receptor pharmacology

Previous work from García's group suggests that carbachol induced pigment granule dispersion is mediated via M_{odd} receptors since M_1 (González et al., 2004) and M_3 (Phatarpekar et al., 2005) receptor antagonists inhibit dispersion, but M_{even} receptor antagonists do not (González et al., 2004). Furthermore, dispersion is induced by M_{odd} agonists but not M_{even} agonists (González et al., 2004). The ability of González et al. (2004) and Phatarpekar et al. (2005) to conclude which muscarinic receptor subtype(s) could be involved in carbachol induced dispersion was limited by both the commercial availability of M5 specific antagonists and questions about the specificity of antagonists for non-mammalian receptors.

The only reported M_5 antagonist is a component of the Malayan spitting cobra venom. Miyoshi and Tu (1999) reported that this inhibitor has a higher affinity for the M_5 receptor, especially in the presence of calcium. Miyoshi et al. (1999) later found by calculating binding constants using cloned human muscarinic receptors that the inhibitor has the highest affinity for the M_1 and the M_5 receptors with binding constants of 160 μM^{-1} and 1,100 μM^{-1} respectively. Isolated RPE cells treated with carbachol and venom simultaneously appeared as aggregated as the forskolin treated cells, indicating that snake venom inhibited dispersion (Fig. 5).

However, the protein sequence of the M_5- inhibitor isolated from cobra venom bears a great similarity to the cobra's PLA_2 sequence (Miyoshi et al., 1999), raising the possibility that the inhibition of pigment granule dispersion was an effect not of M_{odd} antagonism, but of PLA_2 activity. Therefore, I repeated this experiment but including the PLA_2 inhibitor, 4-bromophenacyl bromide (PBP), in the incubation mixture. PBP

prevented inhibition of carbachol-induced pigment granule dispersion by snake venom. Treatment with PBP and carbachol simultaneously did not produce a significantly different pigment index than that seen with carbachol alone. These results support the hypothesis that M_5 activation is involved in carbachol induced pigment granule dispersion since the venom inhibited carbachol induced dispersion but the question of PLA2 involvement requires further scrutiny.

Prior to this study, a role for PLA_2 in carbachol-mediated pigment granule dispersion had not been investigated. However, one can envision its involvement. M_{odd} signaling begins with binding of the ligand acetylcholine to the extracellular domain of the muscarinic receptor, causing the activation of the G_q-protein that is in the cytoplasm. The G-protein activates PLC, causing the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) from phosphatidylinositol-4, 5-biphosphate (PIP_2). IP_3 binds to its receptor on the endoplasmic reticulum causing the release of stored calcium. Protein kinase C (PKC) can now be activated by DAG with the aid of calcium.

In the presence of calcium, PKC can activate PLA_2 (Felder et al., 1990). PLA_2 breaks down phospholipids to produce arachidonic acid, a precursor for a variety of eicosanoids. M_{odd} receptor stimulation using carbachol has been shown to increase arachidonic acid production in mouse A9L cells, while M_{even} receptors do not (Conklin et al., 1988). These studies were limited to M_1 and M_3 receptors, but in 1990, Felder et al. used hamster ovary cells expressing the M_5 receptor to demonstrate a rise in IP_3 and an increase in arachidonic acid when the cells were treated with carbachol. In my experiment, treatment with PBP and carbachol yielded a pigment index that falls between those of forskolin- and carbachol-treated cells, implying inhibited dispersion. If this is

the case, then it can be inferred that the M_5 receptor induces pigment dispersion by increasing both calcium and arachidonic acid.

In situ hybridization and immunohistochemistry

My results indicate that the M_5 receptor is expressed in several layers of the retina including the photoreceptor/RPE layer. This finding is consistent with the results of Phatarpekar et al. (2005) where an RT-PCR analysis demonstrated the expression of M_5 receptors in bluegill RPE and neural retina. Unfortunately, I was unable to resolve whether the RPE or the photoreceptors were labeled. Labeling of the photoreceptor/RPE layer appeared more intense in dark-adapted sections rather than the light-adapted ones. This variation could be a result of different pigment granule positions but it could also be due to up regulation of the receptors, a question that could be addressed using quantitative RT-PCR.

Recent immunohistochemical studies in the García laboratory have shown labeling of the zebrafish retina using an anti-human M_3 antibody (Nuckels, 2006). Although this work described changes in labeling during zebrafish development, a very similar pattern was visible in bluegill sections using this antibody. It is interesting to note the anti-human M_3 antibody labeling was more intense in the dark-adapted retinas, paralleling the results of the *in situ* hybridization. In addition to possible differences in receptor expression this result may be complicated by the similarity that exists between the antigen in both the bluegill M_2 and M_5 sequences (Fig. 10).

Human M3	DRYFSITRPLTYRAKRTTKR	gi|4502819
Bluegill M5	DRYFSITRPLTYRAKRTPKR	gb|AAW73155.1
Bluegill M2	DRYFCVTKPLSYPVKRTTKM	gb|AAY66420.1
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Figure 10. Muscarinic receptor i2 loop comparison. Muscarinic receptors are highly similar in the i2 loop. The amino acid alignment shows the high degree of identity between the bluegill M_5 receptor i2 loop and the human M_3 i2 loop, which is the target of the anti-M_3 antibody used in this study. The human M_3 receptor i2 loop is 95% identical to the bluegill M_5 sequence over the same region; the substituted proline is shown in red. In contrast, the bluegill M_2 receptor is only 65% identical to the human M_3 receptor and only 60% identical to the bluegill M_5 receptor. Asterisks denote the amino acids shared by all three receptors. An M_3 receptor for bluegill has not yet been elucidated.

Western analysis of proteins extracted from bluegill RPE using the anti-M_3 antibody revealed no labeling. Although efforts were made to quantify the amount of protein loaded on the gels spectrophotometrically, the amount of RPE protein on the Coomassie blue-stained gel appears to be much less than the amount of proteins in the other lanes. Therefore, it is possible that the failure to visualize a protein band is due to insufficient sensitivity of the assay for this sample. In contrast, samples from brain, heart and retina revealed multiple bands. The heart extract had a single band of about 90 kD. This 90kD protein is much larger than any previously described muscarinic receptor (Venter et al., 1985). Both the brain and neural retina had several smaller bands which may have been a result of protein degradation. The predicted molecular weight for the bluegill M5 receptor is 59 kD (table 3). An M3 receptor has not been described for bluegill, but the average molecular weight for known M3 receptors is 68 kD. The brain and neural retina extracts had a band at approximately 75 kD similar to the chicken M3 receptor of 72 kD. These results indicate further analysis of these proteins is warranted in order to determine their true identity.

Table 3. Molecular weights of known muscarinic receptor subtypes.

Species	Accession #	Receptor	Molecular weight (kD)
Mus musculus	NM007698	M1	51.39
Homo sapiens	AF498915	M1	51.43
Lepomis macrochirus	DQ066619	M2	55.25
Danio rerio	AY039653	M2	54.51
Homo sapiens	NM000741	M3	66.14
Gallus gallus	L10617	M3	71.99
Rattus norvegicus	AB017656	M3	66.16
Bos taurus	BTU08286	M3	66.11
Gallus gallus	NM001031191	M4	54.95
Xenopus laevis	X65865	M4	53.90
Lepomis macrochirus	AY834251	M5	59.31
Danio rerio	AY039654	M5	55.12
Gallus gallus	AF201960	M5	60.06
Homo sapiens	NM012125	M5	60.08

Future studies

The most novel result from this study is the finding that arachidonic acid may be a link in the signaling cascade for muscarinic receptor activated pigment granule dispersion. To explore the role of arachidonic acid in pigment granule dispersion it may be useful to identify the eicosanoids present in the tissue, treat isolated RPE cells and measure changes in pigment position. Previous studies have shown that prostaglandin receptors are expressed in the human neural retina but not in RPE (Schlotzer-Schrehardt

et al., 2002). Immunolabeling with prostaglandin receptor antibodies would show if this expression pattern is conserved in the teleost retina.

Another interesting result was the parallel between the M_3 and M_5 labeling. The question of whether multiple muscarinic receptors are expressed could be resolved using other muscarinic receptor probes for *in situ* hybridization. Clear labeling in the RPE-photoreceptor layer may also be possible by using isolated cell preparations and bleaching the membrane bound melanin pigment granules in the RPE. Similar attempts have been made using potassium permanganate and oxalic acid to bleach melanocytic skin lesions while retaining antigens for immunolabeling (Orchard et al., 1998). Identification of labeled cells would be simplified if cell type specific markers were used in conjunction with *in situ* hybridization and immunolabeling to be used as points of reference.

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Vita

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