ONTOGENY OF MUSCARINIC ACETYLCHOLINE RECEPTOR EXPRESSION IN THE EYES OF ZEBRAFISH

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Richard J. Nuckels, B. S.

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ONTOGENY OF MUSCARINIC ACETYLCHOLINE

RECEPTOR EXPRESSION IN THE

EYES OF ZEBRAFISH

Committee Members Approved:

Dana M. García, Chair

Joseph R. Koke

Jeffrey Gross

J. Michael Willoughby
Dean of the Graduate College

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ABSTRACT

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Richard J. Nuckels, B. S.

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December 2006

SUPERVISING PROFESSOR: DANA M. GARCIA

The vertebrate eye exhibits a stage-dependent array of gene expression and physiological activity, making it an amenable model to further our understanding of development of the central nervous system and the associated signaling cascades. Muscarinic acetylcholine receptors are important neurotransmitter receptors, and their localization in the eyes of higher vertebrates has been well documented as has their role in normal ocular development and numerous eye diseases and disorders. Only limited work has been done to show the existence, localization, or functionality of these receptors

in lower vertebrates. Zebrafish are a popular model for genetics, development, and evolutionary adaptations. Although zebrafish are widely divergent from mammals, I hypothesize that zebrafish have genes for the 5 muscarinic receptors previously identified. Here, I identify the putative M-odd subtypes of muscarinic receptors and show that they are expressed at specific developmental ages in the eyes of the zebrafish *Danio rerio.* In addition, I identify two possibly duplicated subtypes of muscarinic receptors. My results suggest that all the receptors examined are expressed in the eyes of developing zebrafish, but with different time courses. Differences between the expressions of ostensibly duplicated genes raise the possibility that subtle differences between the duplicates may enable refined regulation of specific developmental events. This manuscript was submitted on November 14, 2006.

CHAPTER 1

INTRODUCTION

Muscarinic acetylcholine receptors are important cell signaling proteins, and they are present in many tissues including the central nervous system (CNS), heart, muscle, bladder, and eye (for review, see Abrams *et al*. 2006). Muscarinic receptors are members of the G-protein coupled receptor (GPCR) family, and currently 5 subtypes of muscarinic receptors have been described that share common structural domains and sequence homology but differ in their signaling functionality (Caulfield and Birdsall, 1998).

The five known muscarinic acetylcholine receptor subtypes (M1-M5) can be divided into two subgroups, the M-odd receptors (M1, M3, and M5) and the M-even receptors (M2 and M4). All subtypes possess the same basic structural domains, including seven transmembrane domains connected by three intracellular loops and three extracellular loops. Acetylcholine is the neurotransmitter that binds to one of the extracellular regions of the third transmembrane domain (Caulfield and Birdsall, 1998) of the muscarinic receptor, initiating a conformational change that sets off an intracellular signaling cascade. The activated muscarinic receptor signals a G-protein, which in turn initiates one of two signaling paths. Initiation of the M-even pathway activates a G_i protein which inhibits adenylyl cyclase and consequently inhibits the production of cAMP. Initiation of the M-odd pathway activates phospholipase C, which hydrolyzes phosphatidyl inositol bisphosphate (PIP_2) to generate inositol 1,4,5-triphosphate,

resulting in release of intracellular calcium from the endoplasmic reticulum. A second product of PIP_2 hydrolysis, diacylglycerol acts in concert with elevated calcium to activate protein kinase C (Berridge, 1997).

Muscarinic receptors are involved in numerous organ systems throughout the body (Collison *et al.* 2000). In the central nervous system (CNS), they are involved in motor control, temperature regulation, cardiovascular regulation, behavior, memory, and learning (Caulfield and Birdsall, 1998; Collison *et al*., 2000). Muscarinic receptors are currently targets for further understanding the events that underlie disorders such as Parkinson's disease, Alzheimer's disease, schizophrenia, drug addiction (Felder *et al*., 2001; Fink-Jensen *et al*., 2003; Volpicelli, 2004) and overactive bladders (Abrams *et al*., 2006).

Muscarinic Receptor Expression in the Retina

Recently, pharmacological agents and molecular techniques have been used to show the presence of muscarinic receptors in the retinal pigment epithelium (RPE) of bluegill fish (Gonzalez *et al*., 2004; Phatarpekar *et al*., 2005). In fish, which have a fixed pupil, retinomotor movements of the photoreceptors and the RPE are used to adapt the eye to bright and dim light conditions. Under conditions of bright light, melanin pigment granules disperse into the apical processes of the RPE, which interdigitate with the photoreceptors. Consequently, pigment granule dispersion reduces the amount of light absorbed by the photoreceptors (reviewed in Nagle and Burnside, 1984). A variety of muscarinic receptor agonists and antagonists were used on RPE cells isolated from the bluegill fish, *Lepomis macrochirus,* to show that M-odd receptors are likely involved in light-adaptive pigment granule dispersion. Furthermore, Phatarpekar *et al*. (2005)

showed using reverse transcriptase-polymerase chain reaction (RT-PCR) that M5, but not M2, is expressed in the RPE. In addition to expression of an M-odd type receptor in fish RPE, the presence of M1 and M3 type muscarinic receptors in the RPE recently has been shown in human cultured RPE cells by immunoblot (Narayan *et al*., 2003).

Interestingly, light-adaptive pupillary constriction is mediated by muscarinic receptors in higher vertebrates. Muscarinic receptors have been identified in the iris sphincter of mice, and M2/M3 knockout mice have shown decreased pupillary constriction (Matsui *et al*., 2000; Matsui *et al*., 2002; Bymaster *et al*., 2003). Thus, muscarinic receptors may be involved in light adaptation using two completely independent and novel pathways when comparing mammals to non-mammalian vertebrates.

Muscarinic receptors have been localized in the neural retinal by immunohistochemistry. Previously, M3-immunoreactivity has been identified in developing chick eyes in 3 distinct strata in the inner plexiform layer (IPL), in the inner nuclear layer (INL), and in the outer plexiform layer (OPL) (Belmonte *et al*., 2000). Additionally in 7 to 14 days old chick eyes, M3-immunoreactivity was seen in the choroid, ciliary bodies near the lens, RPE, 3 strata of the IPL, bipolar cells, amacrine cells, amacrine cells displaced to the ganglion cell layer (GCL), and the OPL (Fischer *et al*., 1998a). In adult primate eyes, M3-labeling is shown in the IPL, bipolar cells, horizontal cells, and the OPL (Yamada *et al*., 2003). In human retinas M3 is the predominant subtype of muscarinic receptors located in the eye (Collison *et al*., 2000). Furthermore, using quantitative real-time polymerase chain reaction (QRT-PCR),

Collison *et al*. show, in order of decreasing amounts, M4, M5, M1, and M2 are also present in the retina.

In chick embryos, muscarinic receptors have been identified pharmacologically in the ventricular zone, a layer of neural retinal stem cells that are directly adjacent to the RPE in prenatal higher vertebrates (Pearson *et al*., 2004). It has been suggested that muscarinic receptors regulate the rate of mitosis in this stem cell population (Pearson *et al*., 2002). The Müller glia have been identified as a type of progenitor/stem cell that can differentiate into specific retinal cell types after injury (Yurco *et al*., 2004). Although the Müller glia have not directly been shown to possess muscarinic receptors, they have been identified as producing a muscarinic receptor inducing agent (Belmonte *et al*., 2000). In fish and some amphibians, progenitor retinal stem cells are found in the marginal zones and continue dividing and contributing differentiated cells to the retina throughout the life of the organism (Johns, 1977). This observation raises the question of whether muscarinic receptor expression can be observed in the marginal zone of fishes.

Why Study Muscarinic Receptors in the Developing Zebrafish Eye?

Due to the diversity of function and expression of muscarinic receptors, there is a growing interest in further understanding their expression and the signaling events that occur upon their activation. Muscarinic receptors have been identified in most parts of the developing eye of vertebrates including retinal neurons, epithelial cells, choriocapillaries, the lens, the iris sphincter, ciliary processes and the retinal stem cells. Although muscarinic receptors have been localized to specific cell types in numerous mammals, relatively little is known about the subtype specific expression and retinal localization in fishes. The zebrafish provides a powerful genetic, developmental, and

evolutionary vertebrate model that has been neglected in studies of the expression, localization, and function of cholinergic receptors, particularly in the retina.

In many developmental studies, the zebrafish is used to answer fundamental questions that are not as easily addressed in chick and mammals. Starting at fertilization, all cellular events can be visualized since zebrafish develop externally and the chorion of the embryo is transparent. Yazulla and Studholme (2001) specifically selected the zebrafish retina as a model for characterizing CNS development and function. At 3 days post-fertilization (dpf), zebrafish eyes are functionally developed (Easter and Nicola, 1996); although, they are not fully adult-like even at 28 dpf with regards to retinomotor movement (Hodel *et al*., 2006).

Currently, limited work has been done to evaluate expression or function of muscarinic receptors in zebrafish, and no one has examined the presence of these receptors in zebrafish eyes. Muscarinic receptors have been identified in the brains of zebrafish using radioactive ligand binding assays (Williams and Messer, 2004), but the specific subtypes were not characterized. Hsieh and Liao (2002) have characterized the M2 receptor in zebrafish, with an emphasis on the role of the M2 receptor in bradycardia. Hsieh and Liao show that as early as 30 hours post-fertilization, the M2 receptor is expressed and can be identified using *in situ* hybridization.

Although specific subtypes of muscarinic acetylcholine receptors have not been identified in the zebrafish retina, choline acetyltransferase (ChAT), an enzyme involved in acetylcholine synthesis, has recently been studied in the developing zebrafish brain and retina. Using immunolabeling, Arenzana *et al*. (2005) showed that ChAT is localized to specific regions of the brain and retina as early as sixty hours post-fertilization. They

specifically show that this enzyme can be detected in the retina from 66 hours post-fertilization to 90 dpf. Furthermore, ChAT-immunoreactivity can be seen in both plexiform layers, amacrine cells, and displaced amacrine cells in juvenile zebrafish. However, expression is restricted to two bands in the inner plexiform layer in the adult zebrafish retina. In mammalian retina, amacrine cells are thought to be the only source of acetylcholine synthesis (O'Malley *et al*., 1992; Cuenca *et al*., 2003; Stacy and Wong, 2003).

Previous studies have shown that muscarinic receptors are expressed differentially in a spatial and a temporal manner in the zebrafish nervous system (Hsieh and Liao, 2002). However, none of these studies focus on identifying the expression in the developing eye. Therefore, I set out to study the spatial and temporal expression pattern of M-odd receptors in the developing zebrafish retina after first identifying putative M-odd receptor genes. The results presented here show that there are additional muscarinic receptor isoforms in zebrafish that have not been previously identified in other species and that these isoforms along with the other M-odd receptor subtypes are present at specific developmental ages in ocular tissue

CHAPTER II

MATERIALS AND METHODS

Tissue Isolation

Fish were kept and maintained as described (Westerfield, 2000). Fish were euthanized by over anesthetizing them in Tricaine-S (Western Chemical Inc., Scottsdale, AZ) (IACUC protocol #05092601). For RNA isolation, eyes were removed from the fish with fine forceps and a scalpel. The eyes were placed immediately in cold Trizol (Invitrogen, Carlsbad, CA). For immunohistochemistry, eyes were removed from juvenile (28 dpf) and adult (90 dpf) fish and the corneas of the eyes were punctured with a 30 gauge needle to allow for infiltration of fixative, 4% paraformaldehyde in phosphate buffered saline (PBS). For the 7 and 14 dpf fish, whole fish were placed in fixative.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Oligonucleotide primers (Table 1 and Appendix) were designed using putative muscarinic receptor sequences available from the Ensembl genome browser database, http://www.ensembl.org/Danio_rerio/index.html, and were purchased from BioSynthesis (Lewisville, Texas). Putative sequences were identified initially by searching for the keyword "muscarinic." After obtaining putative sequences, BLAST algorithms (http://www.ncbi.nlm.nih.gov/BLAST/; http://www.ensembl.org/Multi/blastview) were used to verify each sequence was homologous with other identified muscarinic receptors. The Ensembl database identifies the chromosomal locations of these sequences, and these

loci are referred to later (Table 3) to distinguish among the receptors studied. Following tissue extraction, RNA was isolated with Trizol using 25 and 30 gauge needles to homogenize the tissue. The samples were centrifuged for 15 minutes at 12,000 relative centrifugal force (RCF) at 4°C. The aqueous layer was removed and isopropanol was added to precipitate the RNA. The samples were incubated at room temperature for 10 minutes followed by centrifugation for 10 minutes at 12,000 RCF at 4°C. The supernatant was removed and the RNA pellet was air dried for 5 minutes. After washing the RNA pellet with 70% ethanol, the pellet was resuspended in 15µl water. The RNA was then quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), and subsequently 500 ng of RNA was used for cDNA synthesis, which was carried out using an iScript cDNA synthesis kit (BioRad, Hercules, CA). Each reaction contained 500ng RNA in 15 µl of purified water, 4 µl of 5X iScript Reaction Mix, and 1 µl of iScript Reverse Transcriptase. The reaction was incubated for 5 minutes at room temperature followed by 30 minutes at 42°C and 5 minutes at 85°C. Oligonucleotide primers used for PCR reactions are listed in Table 1. Each reaction contained 1 µl of template DNA, 0.25µl Taq polymerase (New England BioLabs Inc., Ipswich, MA), 5 µl NEB 10X ThermoPol Reaction Buffer, 1 µl of 10 mM dNTP, 1 µl forward primer (100 µM), 1µl reverse primer (100 µM), and 40.75 µl of purified water.

Cloning and Sequencing

Targeted sequences were ligated into the pCR II TOPO cloning vector and cloned into One Shot TOP10 chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's instructions. All clones were verified with restriction digests followed by sequencing (DNA Sequencing Facility, ICMB, UT-Austin). Sequence data were aligned

with Ensembl's sequence data using SeqMan II sequence analysis software (DNASTAR Inc., Madison, WI). See Table 2 for a general description of the clones. Sequence data along with Ensembl's predicted transcript were copied into NCBI's ORF finder link, http://www.ncbi.nlm.nih.gov/gorf/gorf.html and the longest open reading frame (ORF) was selected for further analysis. Using the BLAST option on this website with my longest ORF, I was able to obtain percentages of similarity to other organisms and other receptor subtypes (see Tables 3-7).

Table 1. Primers used for cloning and sequencing.

Target Gene	Forward Primer Sequence	Primer Designation
M1	ATGAACACCAATTGTACTTCG	M1F1 ch5 5' end
M1	CCAACTGTTTGTGTCCCAC	M1R1 ch5 3' end
M1/M3	GTTCTCGCAGTAATGAAGATG	m1f 2rn *
M1/M3	CGGAAGTGTTTGTTACAGAG	m1r 2rn *
M3	AATGGGAGTAATGCTGAGGCTG	CH17M3F1RN
M3	CCAACCTGGTCATAAAAAAGCC	M3F i3loop
M3	TCCCAGCGACATAGCAGGATC	CH17M3R1RN
M5	AACATCACGGACGTCCAGCTG	CH17M5F1RN
M5	CGAACTTCAAGGCATCAACTCG	m5F i3loop
M5	CCACACCAATACAGCTTCTCCTCC	Ch17M5R1RN
M5v	GACGGGACTCAAGCATCATGG	JSM3F2
M5v	CATGATGTTGTATGGTGTCCAAG	JSM3R2
M5v	GGGAAATGTATCTGCCATCC	m5vrn1f
M5v	GGGGAACGTTTTAGTGATGC	m5vrn2f
M5v	TTAATGGTTTTGACCACCCC	m5vrn1r

*These primers were originally designated as M1 based on the Ensembl prediction. They target the putative M1/M3 gene on chromosome 14.

Table 2. Clones of muscarinic receptor fragments.

Target Gene	Clone	Insert Size (base pairs)	Sequence Obtained (bidirectional/total)	Predicted Transcript Length*
M1	M1 D	1416	855/1416	1416
M1/3	M1/3	595	590/595	1392
M3	M3	1574	0/1500	1752
M5	M5	1409	0/215	1473
M5v	M5vlongA	1486	940/1394	1374

*Predicted transcript length is based on sequences predicted by Ensembl (M1-M5v) or sequence data published on NCBI for the M5 gene (Accession# NP_001018639.1).

Immunohistochemistry

Following enucleation, eyes were placed immediately in 4% paraformaldehyde in 0.01M phosphate buffered saline (PBS), pH 7.4 (Sigma. St. Louis, MO) overnight at 4°C. For 28 dpf and 90 dpf fish, eyes were fixed for 3 days at 4°C. Following fixation, eyes were washed in PBS and transferred to 25% sucrose in PBS for 1 to 4 hours at room temperature and then to 35% sucrose in PBS overnight at 4°C. The eyes were embedded in OCT Tissue Tek and sectioned on a cryotome at -25°C to 10 µm thickness. Sections were collected on 1% gelatin coated slides. Sections were allowed to thaw and dry on the slide for 1 hour at room temperature and either were processed immediately or stored at -20°C. For immunostaining, sections were rinsed briefly in PBTD (PBS, 0.1% Tween 20, 1% DMSO) and then incubated for 1-2 hours in blocking solution (5% normal goat serum in PBTD) at room temperature. A polyclonal M3 antibody (Abcam) developed in rabbit against synthetic human M3 i2 loop was diluted 1:150 in blocking solution and incubated

overnight at 4°C. Following incubation, the primary antibody was removed and the sections were washed 3 times in PBTD. Secondary antibodies, Cy2-conjugated goat anti-rabbit or Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted 1:300 in blocking solution, added to the sections, and incubated for 1 hour at room temperature. The secondary antibody was removed and the sections were washed briefly in PBTD. SYTOX (Invitrogen) was diluted 1:10,000 in blocking solution, added to the sections to visualize nuclei, and incubated for 15 minutes at room temperature. Sections were washed 3 times for 15 minutes each in PBTD followed by mounting in Vectashield Hard Set Mounting Medium (Vector Laboratories, Burlingame, CA). All images were obtained with a Zeiss LSM 5 PASCAL Laser Scanning Module fitted to an Axiovert 200M microscope. The images were processed using Adobe Photoshop software (San Jose, CA).

CHAPTER III

RESULTS

Zebrafish have a multitude of M-odd receptor genes.

Five M-odd receptor genes were cloned and sequenced (Table 2). The sequences obtained were compared with muscarinic receptor sequence data available from Ensembl (http://www.ensembl.org/index.html), and the comparisons indicated that zebrafish express at last five different M-odd receptor genes, M1, M1/3, M3, M5 and M5v. Each sequence encoded a single open reading frame. Sequences obtained can be found in the appendix. Comparison with the sequences available from Ensembl revealed that the M1/3, M3, M5 and M5v sequences aligned with the initially targeted Ensembl sequence data and were on average 99% identical to the named genes (Table 3). Further analysis is necessary for M1.

At the amino acid level (based on virtual translation), M3, M5 and M5v were 99.5% ± 0.3% (average ± standard error) identical to the predicted gene available from NCBI, while averaging 55% ± 2% identity with paralogous muscarinic receptors (Table 4). In contrast, M1 and M1/3 shared much less similarity at the amino acid level to any of the muscarinic receptors predicted on NCBI, averaging 52% ± 1% identity. The muscarinic receptor with the highest level of similarity to the M1 receptor identified herein was M3 (56% identical over the 500 amino acids compared), which also showed the greatest similarity to the M1/3 receptor identified herein (60% identical over 400

amino acids compared). Therefore, the designation of M1 and M1/3 is considered preliminary.

Table 3. Zebrafish M-odd receptor genes uncovered in this study.

Gene	Amount Sequenced*	Ensembl Gene	Chromosomal Location	% Identity**
M1	1416	ENSDARG00000037292	5	***
M1/3	595	ENSDARG00000055451	14	99
M3	1500	ENSDARG00000056049	17	99
M5[1]	216	ENSDARG00000004026	17	99
M5v	1394	ENSDARG00000042524	20	99

[1]M5 has not been described in a publication, but the sequence itself was not uncovered in this study. Rather, it had already been posted on NCBI (Accession # NP_001018639.1).
*Amount sequenced unidirectionally.
**Percent identity at the nucleotide level when compared with aligned portion of Ensembl gene fragment or with the NCBI gene fragment (M5).
***More sequence analysis is necessary.

Table 4. **Comparison of zebrafish M-odd amino acid sequence.***

	M1 XP_700752.1	M3 XP_700381.1	M5 NP_001018639.1	M5v NP_001025331.1
M1	49(564)	56(500)	52(474)	52(446)
M1/3	50(460)	60**(400)	52(470)	47(456)
M3	56(500)	100(495)	51(488)	51(486)
M5	52(474)	51(488)	99(490)	67(460)
M5v	52(446)	51(486)	67(460)	99.6(457)

*Numbers in parenthesis indicate length of amino acid sequence over which comparison was made.
**Compared to Ensembl sequence

Comparison of the zebrafish M-odd receptor amino acid sequences identified herein to putative mammalian and chick orthologues revealed no clear orthology for the zebrafish M1 or M1/3 receptor sequences (Tables 5-7). However, the M3 receptor sequence averaged 62.7% ± 0.5% identity compared to mammalian and chick M3 receptors, but only 52% ± 1% identity when compared to M1 and M5 receptors. Similarly, the M5 and M5v were 60.1% ± 0.5% identical to M5 orthologues, while averaging 51.1% ± 0.5% identity to M1 and M3 paralogues.

Table 5. Comparison of zebrafish M-odd to mammalian M1 genes. Here is a comparison among zebrafish muscarinic receptor sequences and mammalian M1 receptor sequences at the amino acid level[1].

Zebrafish	Human M1 NP_000729.2	Mouse M1 NP_031724.2	Rat M1 NP_542951.1	Zebrafish ORF
M1	48(460)	48(460)	48(460)	471
M1/3	53(457)	53(457)	53(457)	460
M3	*	57(427)**	49(496)	506
M5	54(451)	55(454)	54(452)	490
M5v	51(445)	49(449)	49(449)	457

[1] The numbers of amino acids compared in the percent identity statistic are given in parentheses. This information is based on a BLAST search of the open reading frames (ORF) of sequence data.

*A significant portion of the sequence did not align.

**A significant portion of the sequence did not align. This percentage is likely lower if the remaining sequence were included.

Table 6. Comparison of zebrafish M-odd to other vertebrate M3 genes. Here is a comparison among zebrafish muscarinic receptor sequences and other vertebrate M3 receptor sequences at the amino acid level[1].

Zebrafish	Human M3 NP_000731.1	Mouse M3 NP_150372.1	Rat M3 NP_036659.1	Chick M3 NP_990730.1
M1	45(529)	46(533)	46(527)	47(534)
M1/3	*	47(521)	47(521)	47(521)
M3	63(509)	63	62(512)	63(520)
M5	52(518)	50(511)	50(511)	51(533)
M5v	50(488)	50(487)	50(487)	51(489)

[1] The numbers of amino acids compared in the percent identity statistic are given in parentheses. This information is based on a BLAST search of the open reading frames (ORF) of sequence data.

*A significant portion of the sequence did not align.

Table 7. Comparison of zebrafish M-odd to other vertebrate M5 genes. Here is a comparison among zebrafish muscarinic receptor sequences and other vertebrate M5 receptor sequences at the amino acid level[1].

Zebrafish	Human M5 NP_036257.1	Mouse M5 NP_991352.1	Rat M5 NP_059058.1|	Chick M5 NP_001026721.1
M3	52(487)	52(484)	52(484)	51(488)
M5	59(511)	59(513)	59(513)	63(517)
M5v	60(484)	60(484)	60(481)	61(479)

[1] The numbers of amino acids compared in the percent identity statistic are given in parentheses. This information is based on a BLAST search of the open reading frames (ORF) of sequence data.

All muscarinic receptors tested are expressed in the eye, but expression occurs at different developmental stages.

To assess which putative M-odd receptor subtypes were expressed in zebrafish ocular tissue at specific developmental ages, RT-PCR was performed on total RNA isolated from zebrafish eyes 7, 14, 28, and 90 dpf, representing larval, metamorphic,

juvenile, and adult fish, respectively. Figure 1 shows that at 7 dpf, two of the receptor subtypes are expressed, the putative M1/M3 and the putative M5 variant. At 14 dpf, the representative metamorphic stage, all five M-odd receptors tested are expressed. By 28 dpf, the representative juvenile fish stage, M5 expression is no longer detectable. Interestingly, M1/M3 is expressed at this stage but seems to drop off at 90 dpf, the representative adult stage. The M5 variant is expressed consistently at all 4 stages. At 90 dpf, all five M-odd receptors tested are expressed to some degree in the zebrafish eyes.

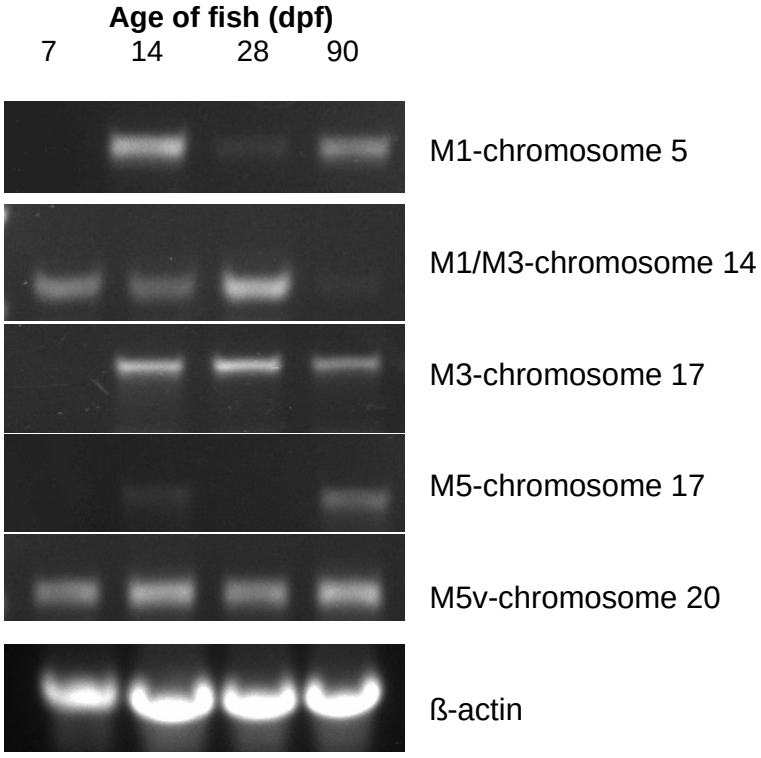

Figure 1. RT-PCR gel image showing temporal expression of M-odd receptors. M-odd type muscarinic receptors are expressed in zebrafish eyes temporally. The RT-PCR products for M1, M1/M3, M3, M5 and M5v were 866, 595, 807, 771, and 543 bp, respectively. M1/M3 and M5v shows the earliest expression, with M5v expression persisting at all time points examined. M1 expression appears at only two time points, 14 and 90 dpf, and M5 expression is most evident in the adult fish. M3 expression is absent at 7 dpf, but present at all other time points examined.

M-odd receptor proteins are expressed in a variety of ocular cells.

To determine whether gene products suggested by the RT-PCR results could be detected and localized immunohistochemically, immunolabeling using an anti-M3 antibody raised against the conserved i2 loop (Figure 2) of the human M3 receptor was performed. At all stages studied, the M3 antibody shows significant labeling in the neural retina, especially in the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), and the outer plexiform layer (OPL). Additionally, there appears to be labeling in the interface between the outer retina and RPE. At 7 dpf, labeling appears to be in two distinct strata in the IPL and in the INL (Figures 3 and 7A). Also, in the RPE in the ventral part of the retina there appears to be strong labeling, while in the central RPE there seems to be some weak labeling.

By 14 dpf, or a metamorphic stage, labeling with the anti-M3 antibody is no longer apparent in distinct strata of the IPL, although the IPL is strongly labeled throughout (Figures 4 and 7B). Also at this stage, there is an obvious layer of labeling near the outer limiting membrane and near the horizontal cell layer. Other layers that appear to express M3 include the RPE, INL, and the GCL, which may represent displaced amacrine cells.

At 28 dpf, or a juvenile stage, labeling is similar to the metamorphic stage; that is, it appears throughout the retina and RPE with a distinct band at the outer limiting membrane (Figures 5, 7C and 7G). However, in contrast to what was observed in the metamorphic fish, the INL appears to be more strongly labeled where the bipolar cells would be found compared to the labeling where the amacrine cells are located.

At 90 dpf, or an adult stage, there appears to be a more distinctive band of labeling at the horizontal cell layer (Figures 6, 7D and 7H). Also, the outer limiting membrane seems to be strongly labeled. This labeling could be on the RPE apical processes, the photoreceptor outer segments, or the tips of the Müller glia.

```
rerio M2                DRYFCVTKPLSYPVNRTTKM
rerio M1 ch5            DRYFSVTRPLTYRTKRTPKT
rerio M1/M3 chromo14    DRYLSVMRPLTYRAKRTPKR
HUMAN M3 gi|4502819     DRYFSITRPLTYRAKRTTKR
rerio m3 ch17           DRYFSITRPLTYRAKRTTKR
rerio M5 ch17           DRYFSITRPLTYRAKRTPKR
rerio M5v ch20          DRYFSITRPLTYRAKRTPKR
                         *** *   ***** *** *
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Figure 2. M-odd i2 loop comparison. Zebrafish M-odd receptors are highly identical in the i2 loop compared to each other and to the i2 loop of the human M3 receptor. A ClustalW alignment shows the high degree of homology (up to 100%) between the M-odd receptor subtypes, and in particular between the human M3 i2 loop and the zebrafish sequences determined in this study. The M3 antibody used for immunolabeling in this study targets the i2 loop and was made against a synthetic human sequence. Zebrafish sequences are in bold. Asterisks denote the identical M-odd amino acids.

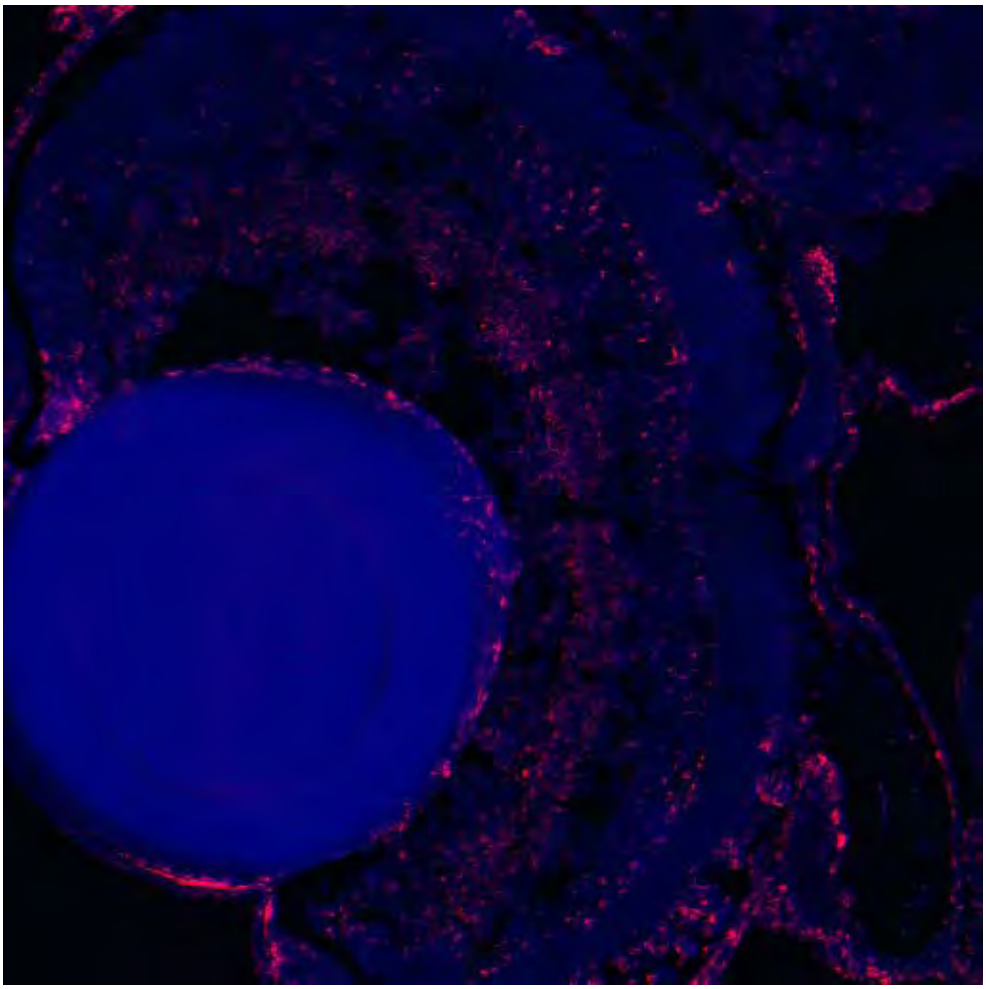

Figure 3. Immunolabeling of M-odd receptors on 7 day old zebrafish eye section. M-odd receptors are spatially expressed in the 7 dpf zebrafish eye. Especially noteworthy is labeling in the marginal zone, which may correspond to M-odd receptor expression by the retinal stem cells.

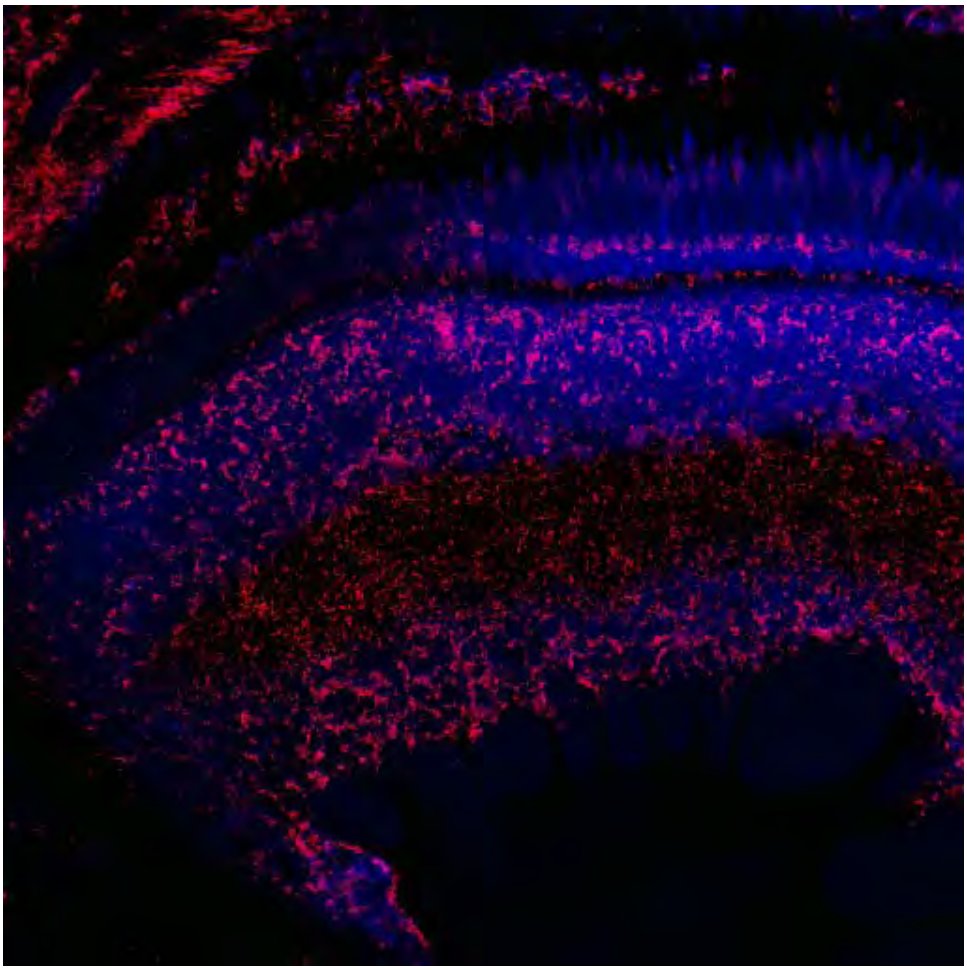

Figure 4. Immunolabeling of M-odd receptors on 14 day old zebrafish eye section. M-odd receptor expression in the 14 dpf zebrafish eye intensifies in the IPL and RPE layers. Also noteworthy is the labeling in the GCL, which may correspond to M-odd receptor expression by the ganglion cells.

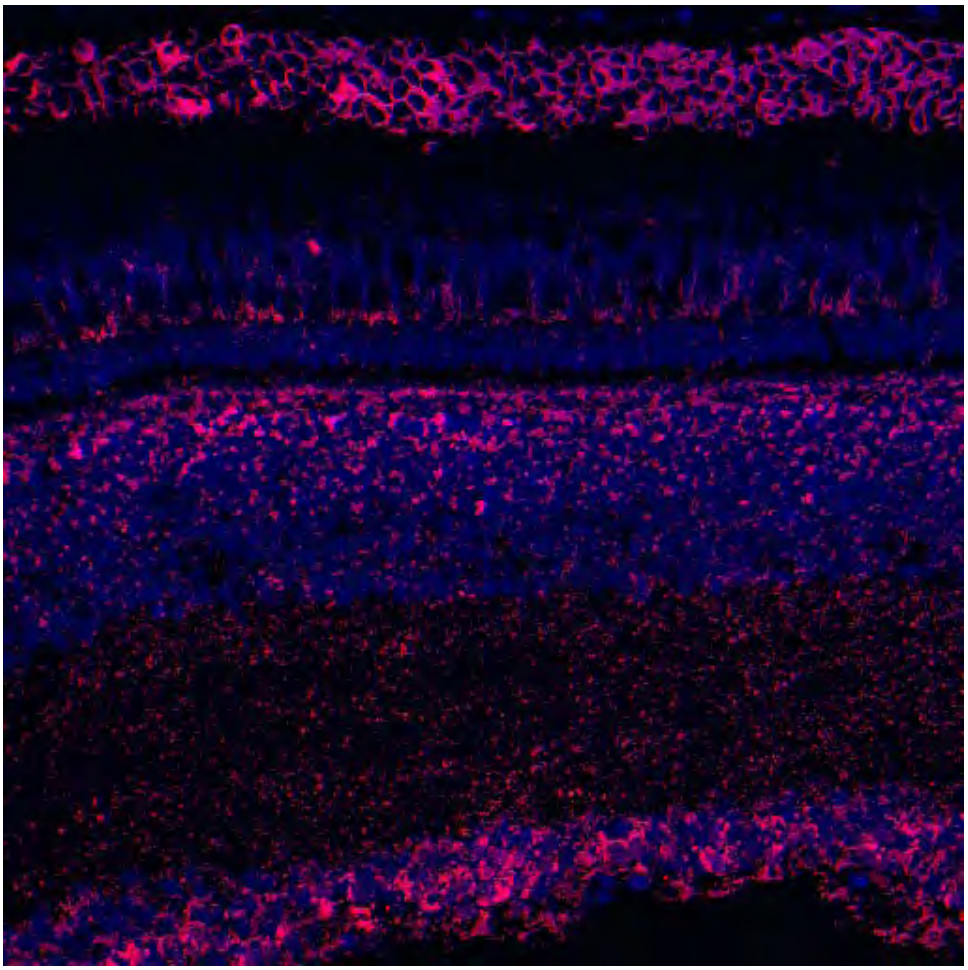

Figure 5. Immunolabeling of M-odd receptors on 28 day old zebrafish eye section. Expression of M-odd receptors intensifies at the base of the RPE in the 28 day old zebrafish retina as well as at the interface between the RPE and the photoreceptors. A line of staining can also be observed in the INL, possibly representing labeling of horizontal cells. Labeling also appears intense in the GCL.

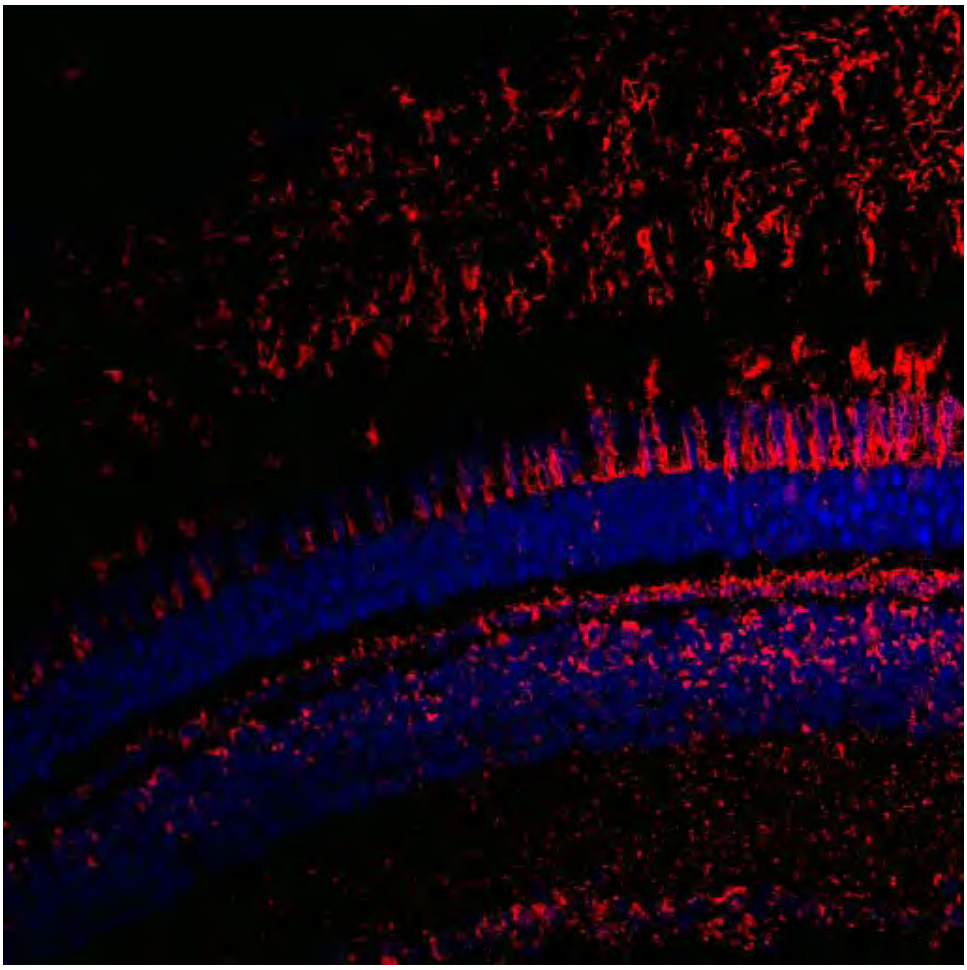

Figure 6. Immunolabeling of M-odd receptors on 90 day old zebrafish eye section. M-odd receptor labeling in the mature adult appears much more spatially restricted than observed at earlier stages.

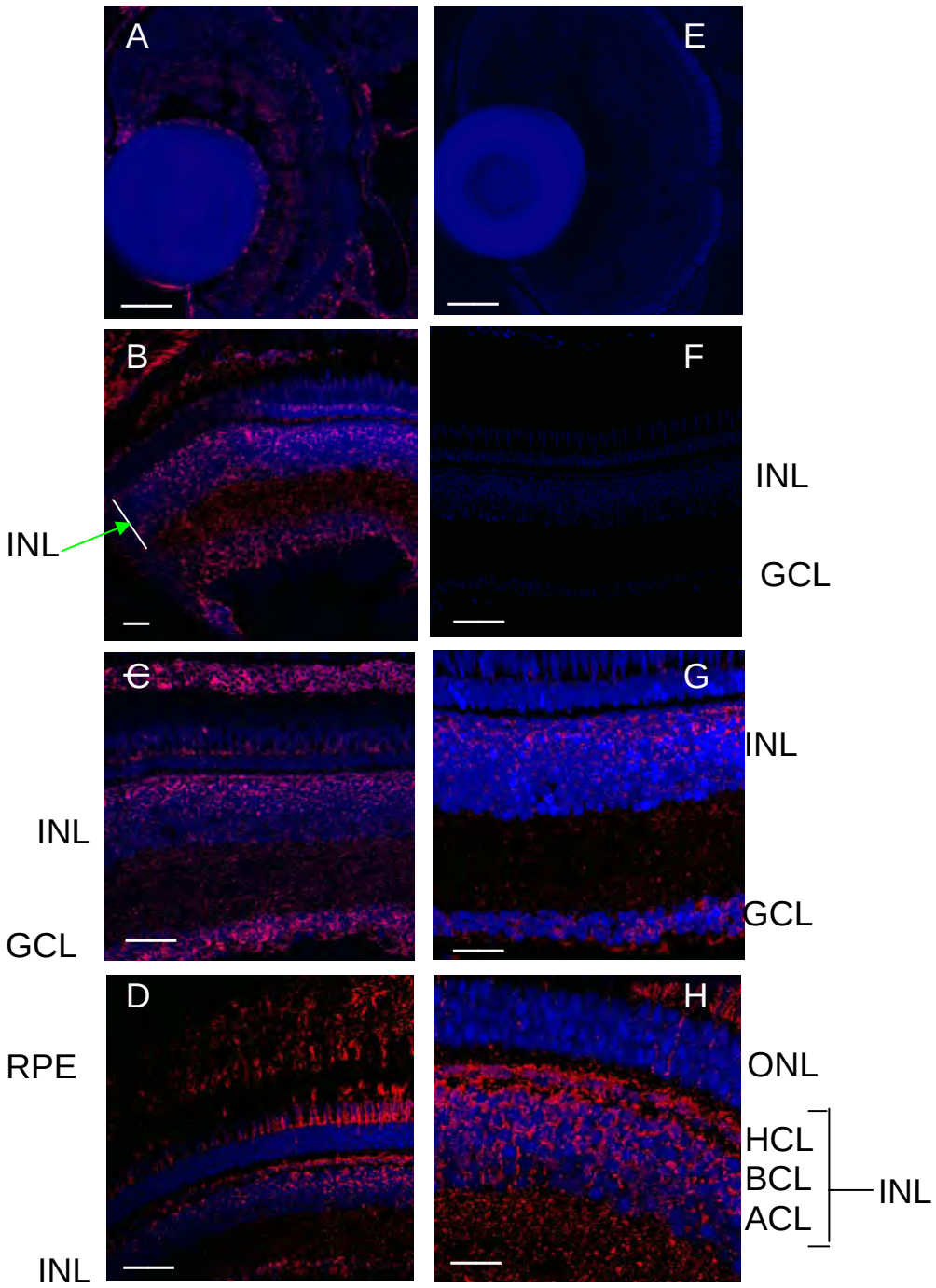

Figure 7. Immunolabeling of M3 including negative control. Immunohistochemistry of M-odd labeling in zebrafish eye sections at four different developmental ages. (A) 7 dpf; (B) 14 dpf; (C) 28 dpf; (D) 90 dpf; (E) Control, 7 dpf; (F) Control, 28 dpf; (G) 28 dpf, higher magnification; and (H) Adult, higher magnification. Abbreviations are as follows: GCL, ganglion cell layer; INL, inner nuclear layer; ACL, amacrine cell layer; BCL, bipolar cell layer; HCL, horizontal cell layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. For controls, no primary antibody was added, but the identical secondary was added along with STYOX, a nucleic acid stain. Scale bars in A-F are 30 µm; G, 18.5 µm; H, 12 µm.

CHAPTER IV

DISCUSSION

The objective of the study reported herein was to uncover all the M-odd receptors expressed by zebrafish and characterize their expression in the developing eye using reverse transcriptase-polymerase chain reaction and immunohistochemistry. I have shown that zebrafish express a minimum of five unique M-odd receptors representing distinct loci in the genome. In addition, I have shown that each has a unique temporal pattern of expression in the developing eye. Finally, at the protein level, the spatial pattern of muscarinic receptor expression changes, becoming more restricted in its distribution as the animal matures.

The original rationale for focusing on the M-odd type of receptors was based on previous evidence supporting the existence of an M-odd type signaling pathway in the RPE of bluegill fish (González *et al*., 2004; Phatarpekar *et al*., 2005). Phatarpekar *et al*. in particular demonstrated that RPE expressed M5 receptors and did not express M2 receptors, but other muscarinic subtypes were not explored at the molecular level. So, although Phatarpekar *et al*.'s data were consistent with an interpretation that acetylcholine induced pigment granule dispersion in RPE by activating M5 receptors, exploration of other receptors was merited. The interest in looking at the developmental expression of receptors was founded on findings by Hodel *et al.* (2006) which showed the zebrafish retinomotor movements were continuing to develop at 28 dpf, raising the possibility that

there might be a gradient of M-odd type receptor expression during development of the eye. When comparing the zebrafish sequence data I have obtained to human homologs, in some cases it seems likely that the putative sequences are what they have been predicted by Ensembl to be. In the case of the zebrafish M3 sequence, there is an average of 63% sequence similarity at the amino acid level when compared to other known M3 receptor sequences. In contrast, when considering the zebrafish M3 sequence, there is only on average 52% similarity with M5 orthologs and 49% similarity with M1 orthologs. This suggests that the putative M3 zebrafish sequence I am examining is likely to be a real M3 sequence. Similar conclusions may be inferred by examining the M5 gene cluster. Both zebrafish M5 and M5v are approximately 60% identical to other M5 orthologs but 54% or less identical to M1 and M3 orthologs. Interestingly, when the zebrafish m5 sequence is compared to the bluegill m5 sequence (NCBI Accession# gbAAW73155.1), there is 77% identity at the amino acid level; however, the zebrafish M5v is only 68% identical to bluegill m5. This suggests that bluegill M5 is more closely related to zebrafish M5 compared to zebrafish M5v. One interesting question to be explored would examine whether bluegill fish also have an M5v gene. A comprehensive statistical phylogenetic analysis should conclude to which muscarinic receptors the putative zebrafish M-odd receptors are most closely related.

Having demonstrated that zebrafish express at least 5 different types of M-odd receptors, I was interested in knowing which types were expressed in the eye and what the developmental time-course for their expression was. Based on my RT-PCR studies, I found that at 7 dpf only M1/M3 and M5v were expressed in the eyes, but by 14 dpf all five M-odd subtypes were expressed. In zebrafish, retinomotor movements are initiated 5

days post-fertilization, but the full complement of retinomotor movements is not observed before adulthood. Even at 28 dpf, juvenile zebrafish lack elaborate retinomotor movements (Hodel *et al*., 2006).

The relationship between muscarinic receptor expression and development of the eyes has been examined in a number of studies. For example, M2 expression in the retina of chick embryos has been shown to be regulated by a muscarinic acetylcholine receptor inducing agent (Belmonte *et al*., 2000), the appearance of which coincides with the development of the Müller glia. An interesting question might be whether there are inducing agents expressed by other retinal cells since Belmonte *et al*. (2000) also showed M3 receptor immunoreactivity in developing chick eyes (E9-E19). Comparatively, these stages in chick are likely younger than 72 hours post-fertilization for zebrafish, suggesting that an examination of muscarinic receptor expression in zebrafish embryos younger than 72 hours post-fertilization would be appropriate.

Muscarinic receptor expression seems to be initiated well after cholinergic neurons begin producing acetylcholine. As described by Arenzana *et al*. (2005) the cholinergic system starts developing in zebrafish as early as 30 hours post-fertilization, based on expression of choline acetyltransferase, the rate limiting enzyme in acetylcholine synthesis. In mammalian retinas, this enzyme is expressed mainly by starburst amacrine cells (Godinho *et al*., 2005). At this stage of development, the target for acetylcholine could be nicotinic receptors. It should be noted that in embryonic chick, there is a developmental switch from nicotinic to muscarinic regulation of the spontaneous bursts of activity in ganglion cells after hatching (Zhao *et al*., 1999; Zhou and Zhao, 1999). Other developmental switches that occur include a change in the role

of acetylcholine and GABA released by starburst amacrine cells (Masland, 2005). Both neurotransmitters are excitatory early in development and later switch to being inhibitory (Zheng *et al.*, 2004). This doubtless reflects a change in the receptor types being expressed.

The RT-PCR studies described herein were not designed to test the cellular sources of muscarinic receptor messenger RNA. Therefore, I performed immunohistochemical studies to determine where in the developing retina muscarinic receptors were expressed. To do this, I used a polyclonal antibody raised against the i2 loop of the human M3 receptor. As shown in Figure 2, this region of the m3 receptor is highly conserved among receptors and species, showing a high percentage of identity at the amino acid level between the M-odd receptor subtypes of zebrafish, human, and other mammals. Comparing the sequences I obtained to the inferred amino acid sequence for the receptors reveals the high percentage of similarity and raises the possibility that this polyclonal antibody could be recognizing any of these receptor types. In contrast, the human M3 i2 loop is only 60% identical to the zebrafish M2 i2 loop, lessening concerns that the antibody fails to discriminate between M-even and M-odd receptors.

The M3 antibody was raised against the second intracellular loop (i2 loop) of the M3 type muscarinic receptor, using a synthetic human M3 sequence. This region is highly conserved across taxa and subtype. ClustalW alignment among several species and other subtypes shows the high degree of similarity, ranging from 95% identity between the M5 i2 loop and the M3 i2 loop to 80% identity when comparing the M1 i2 loop to the M3 i2 loop (Table 8). Knowing only that this antibody is polyclonal and that it recognizes the i2 loop, I cannot be certain that the antibody is specific to the M3-type

receptor. It is possible that the antibody also recognizes M5, M5v, M1 or M1/M3. Although there is ambiguity in the degree of selectivity of the antibody, it does seem likely that the antibody is confined to recognize the M-odd type receptors based on the lesser similarity with the M2 receptor i2 loop of 60%. Taken together with the RT-PCR data which indicates that M3 expression is absent in 7 dpf larval eyes, this M3 antibody is almost certainly recognizing multiple receptor types.

Table 8. Comparison of the i2 loop of zebrafish M-odd type receptors to the i2 loop of human M3.

	% identity to human M3 i2 loop
Zebrafish M1 chromosome 5	80
Zebrafish M1/3 chromosome 14	80
Zebrafish M3 chromosome 17	100
Zebrafish M5 chromosome 17	95
Zebrafish M5v chromosome 20	95
Zebrafish M2	60

Future studies

Several possible future studies and works in progress have been mentioned throughout the discussion. Additional studies might include knocking down expression of each of the subtypes individually and examining the resultant phenotype. Such an approach would provide a relatively simple way to assess the importance of that receptor

in the developing zebrafish. One way to knock down expression would be through the use of morpholino injections, which have been shown to be highly effective in knocking out or knocking down specific transcripts in embryonic and early larval stages of zebrafish (reviewed in Teh *et al*., 2005). Small, interfering RNA (siRNA) assays in isolated cells can also be used to knockdown specific genes (Liu *et al*., 2005). In addition, the creation of transgenic fish has shown to be useful in assessing the function of specific proteins (Teh *et al*., 2005).

The work I report here shows that there are several types of M-odd type receptors that are present in the developing zebrafish eyes from 7 dpf into adulthood. Additionally, the data show that there is strong muscarinic receptor immunoreactivity in specific cell layers of the developing zebrafish retina. Muscarinic acetylcholine receptors have been identified in numerous ocular cell types of mammals (Collison *et al*., 2000), and the data presented here show that this is also true for fish. Previous studies have shown that muscarinic receptors are involved in retinal growth related to myopia (Qu *et al*., 2006) and in ventricular zone stem cell divisions (Pearson *et al*., 2002). Zebrafish provide an interesting experimental model in which to study muscarinic receptor expression partially due to their continually dividing retinal stem cell population (Johns, 1977), and future studies should be done to identify more precisely the cells expressing muscarinic receptors to see whether they include retinal stem cells. One possible aid would be the use of retinal cell specific markers and double immunolabeling experiments that may show colocalization with the muscarinic receptors.

With the observation of new isoforms of two of the muscarinic receptors in zebrafish, and the verification of their expression, further experiments are needed to

determine their necessity and functionality in developing eyes and in other tissues. Pharmacological studies may shed some light as to the presence and function of these new receptor isoforms; however, more intensive molecular characterization through knockouts, transgenics, or mutant analysis studies will likely be necessary.

APPENDIX

SEQUENCE DATA

M1 chromosome 5 DNA sequence

ATGAACACCAATTGTACTTCGCAGACAACAAACCTCACCATGGACCCTCTAGGGGGACA
TCATGAATGGGAGGTGGTTCTGATCGTGCTAGTGACAGGACCTCTTTCCCTCATCACCA
TCCTGGGAAACTCTCTGGTGGTCATATCCATCCGGGTCAACAGTCAGCTCCGGACCATC
AGTAACTACTATCTTCTGAGTCTGGCGGTGGCAGACTTGATCTTGGGTACGGTGTCAAT
GAACTTATATACTGCATACGTAATCTTGGGCCGCTGGACGTTAGGACCTCTGGCTTGTG
ATTTATGGTTGACACTTGACTATGTAGTAAGCAATGCATCGGTAATGAACCTGCTTGTT
ATAAGCTTTGACCGATACTTTTCTGTTACACGACCGCTGACTTATCGAACGAAGCGAAC
TCCAAAAACGGCGGCTGTCTTGATTGCTCTGGCATGGGTGATATCTTTTATATTATGGG
GGCCTGCAATCTTATTTTGGCCTCATGTAATGGGACGACCATCTGGAGCTGAAGCTCAT
GTCTGTTCCATCCCATTTCTCAAGGTACCTCCTATAACATACGGCACGGCGATCGCTGC
TTTCTACCTACCCGTCAGCATCATGATCATTTTATACTGGCGAATATATTGGGAGATTG
AGAACCGAGCGAAAGGGCTTGCTGGTTTTGTTGGGTCTGTGAAAAACACTACAGAAATT
CTGGAAGGGTTGGACAAACAATCAATGCACCAAACCAGCAGTAAGAGCACATTAAGTAT
CTCAAGGGAAGGAATATCCAAAAAGGGACAAGGCAAGTCAGTTGGTTGCTTCACTTGGA
TTTTGAGAAACAAAACAGCAGCTCACGAAAGTTACAAGGAGCCTTACAGCAGCAGTAGC
TGGAACACTGAAGATGTGGATGACAGTGTCTTGTCTTCATCCACAGATGAAGAACATGA
GCCAAACAACAAGTCAAAAGCACCTTCATCTAATCCTTCAGCGATTAAGTTGAAGGATC
TACACTGCAGATCAGACGATACAAAGGGTGTGCAAGAGCAGAACTCTCCTGCTGAGCGT
CTCACCAAACAGTGCCATTTAAAACCCAAAAAGTCTGATCATAGCATTCGCCAACAGCT
CAGAGCTAAACATCGAATTATTTTGATTATTAAAGAAAAGAAAGCAGCCCGGACGCTAA
GTGCCATCTTGTTGGCGTTCATCCTCACATGGACGCCCTACAACATCATGGTTTTGGCC
TCCATTTCATACTGTGTTCCTGAAAAGCTCTGGCAGCTGGGATACTGGCTCTGCTATGT
GAACAGTACAGTGAACCCCATGTGCTACGCCCTCTGCAACGAATCTTTCAGGGTCACAT
TCAAGTCTCTGCTACTCTGTCGAGGTGGAGATAAACGAAAGTGGGACACAAACAGTTGG

Primers used for PCR, cloning and sequencing M1 chromosome5

M1F1 ch5 5'end	ATGAACACCAATTGTACTTCG*
M1R1 ch5 3'end	CCAACTGTTTGTGTCCCAC
M1F3	TTCCATCCCATTTCTCAAGG*
M1R2	CCCACTTCCGTTTATCTCCAC
M1F2	TCGCAGACAACAAACCTCCAC
M1R3	CCAAGTGAAGCAACCAACTG*

* Location of primer is shown underscored in the sequence figure. Sequence data in red represents bidirectionally sequenced DNA. Sequence data in blue shows DNA sequence that has been sequenced in one direction.

M1/M3 chromosome 14 DNA sequence

AGTTGGAATGGATAAGTCTGCGGCCAATAAACCTCACGATGCCACCATGA
ACAACAGCGATCAGTCAGAAAGTCCGAATTCCCTTATGAATCCTGCAACC
ACACAAGCTTCTGCTCTATTACTGAACGCAGGTCAGAGGAGCAGAAAAGC
CCGCACTTCATCWCTGATCAAGGAGAAGAAAGCAGCCCGGACCCTCAGT
GCCATTCTTCTGGCTTTCATTGTAACATGGACACCATATAACATCATGGTT
CTKGTWTCCACGTTCTGCGACAATTGTGTTCCTGAGAGACTTTGGAAACT
GGGATACTGGCTCTGCTATGTTAACAGCACAGTCAATCCGCTCTGCTACG
CGCTCTGTAACAAACACTTCCG

Primers used for PCR, cloning, and sequencing of M1/M3 chromosome 14:

m1f 2rn	GTTCTCGCAGTAATGAAGATG
m1r 2rn	CGGAAGTGTTTGTTACAGAG
m3vch14 f1	TTCATAACTGTTCCCCTGTCC
m3vch14 r1	ATTCGGGGTCCACCTTATTC
m3vch14r2	CTTGTGGATGGTTGTTTGTAGC

Sequence data in red represents bidirectionally sequenced DNA. In the sequence, the letter "K" represents either a guanine or thymine, while "W" represents adenine or thymine.

M3 chromosome 17 DNA sequence

CTCGTCAACCTCACTGCTGTTTTCAAGCTCAATGGGAGTAATGCTGAGGCTGAAGGT
CAATCCTATGATCCTCTGGGTGGACACTCGCTCTGGCAGGTCATTCTCATCGTTGTC
TTTACAGGCCTGCTTTCCCTTATTACTATTATCGGCAACATCTTGGTCATGGTGTCA
TTTAAGGTCAACCGACAGCTCAAAACAGTTAACAATTACTTCCTACTCAGCTTAGCC
GTGGCAGATCTCATCATTGGGGTCATCTCTATGAACCTGTACACTGCCTACATCGTC
ATGGGCCAGTGGGCGATGGGAAACTGGGCATGTGACCTCTGGCTAGCCATAGACTAC
GTAGCCAGCAATGCATCTGTCATGAATCTGCTTGTCATTAGCTTTGACCGATATTTC
TCCATTACGAGGCCTCTGACGTACCGTGCCAAACGCACCACCAAGCGAGCAGGAGTG
ATGATTGGCCTGGCATGGTTTGTGTCACTCATTCTTTGGGCACCTGCCATTTTGTTT
TGGCAGTATTTCGTAGGACAAAGAACTGTTCCACAGGATAAATGCTACATTCAGTTT
CTCTCCGAGCCTATTATTACGTTTTGCACCGCAATGGCGGCGTTCTACCTGCCGGTG
ACGATAATGAGCGTTTTATACTGGCGAATATACAAAGAGACAGAAAACAGATCACGG
GAACTTGCTGGATTGCAAGGTTCAGGTGGACGATTTGGAGGTGTTGAGCGACCACGC
TTCCATCTCCACGCCACTAGGGGGAGCTCTAGGAGCTGCAGCAGCTTTGAACTGGGC
CAACCTGGTCATAAAAAAGCCTCTGTTCACAGTCTGAGTGGGAGATTCCACTGTTGG
GGGTGGAAGTCTGGTGGCAGTGATAAAAGTGGGCCAAATAGGGAGGCAGATCAGAGC
AGCAGTGACAGCTGGAACAACAACGACGCTGGACTTTCAGCTGATCACTCRGGTTCA
TCTGATGAAGACGAGAGCGCACCATCTACAACAAGAGCCATCTTCTCAATTGTTCTC
AGTCTGCCTGGCGTGAGGGCCGCAGTCAACTCCCAGGTCACTTCCTGTGAGGAACTG
GACACAGAGGAAGATCCCTTACGGTCAGCCGAGGAGAAGGACAGCAGGGATGGCAGC
ATCTCACGCTCTGTCACCAACGGGAACAAGCGCTTTGTGGGTGGCATGAGTAAAGTT
CAGTCAATGTCTGCAATACAGCCGACAACAGATACCACCACAGACAGCACTAACACY
ACCATCAAATCTCCCTCAGCACCAATAACTTTCAAAGAGGCAGCTTTAGCAAAACGT
TTTGCAGCTCGCGCAAGGACACAAATCACCAAGCGCAAGCGCATGTCGCTAATAAAG
GAAAAGAAAGCAGCACAGACTCTAAGCGCCATCCTCTTTGCTTTTATCATAACATGG
ACGCCTTATAACATCATGGTAYTGGTGAACACTTTCTGCAATGGCTGCATACCTGAG
AACCTCTGGGCGCTAGGCTATTGGTTATGTTACGTCAACAGTACAGTAAATCCTATG
TGCTACGCACTTTGCAACAAAACTTTTCGTAGCACTTTCAARATGATCCTGCTATGT
CGCTGGGATCAGAAAAAAAGCAAGCCAAGCTTTCCGCAAAGACAGGCTGTGAGGTTT
CACAGGCCCATACCGACAGACTCCACATAG

Primers used for PCR, cloning, and sequencing M3:

CH17M3F1RN	AATGGGAGTAATGCTGAGGCTG
M3F i3loop	CCAACCTGGTCATAAAAAAGCC
CH17M3R1RN	TCCCAGCGACATAGCAGGATC
M3R2	CCTATTTGGCCCACTTTTATC

Sequence data in blue shows DNA sequence that has been sequenced in one direction. The sequence data in black is the remaining unsequenced putative DNA that is predicted by Ensembl. Underlined sequence shows the location of the primers.

M5 Chromosome 17 DNA sequence

ATGGGTGTGGAGAACTTGACCCTCCGCGCCAACATCACGGACGTCCAGCTGGTCACGCA
CAGCCTGTGGGAGGTGATCGCCATCGCCACCGTCTCCGCCATCGTCAGCTTCATCACCA
TCATCGGAAACATCCTGGTCATGCTGTCCTTCAAGGTCAACAGCCAGCTGAAGACGGTC
AACAATTATTATCTGCTGAGTCTGGCCTTCGCTGACCTGATCATCGGCGTTTTCTCCAT
GAATCTGTACACCTCCTACATCCTCATGGGCTACTGGGCGCTCGGGAGTCTGGCCTGCG
ATCTGTGGTTGGCTTTGGATTACGTGGCGAGCAATGCATCAGTTATGAACCTTTTAGTT
ATTAGCTTTGACCGATACTTCTCGATTACGAGACCTCTGACATACCGAGCCAAACGGAC
GCCGAAGAGAGCGGGGATTATGATTGGTTTGGCTTGGCTTGTTTCTTTTATCTTGTGGG
CTCCTCCGATTTTGTGCTGGCAGTATTTTGTTGGTAAAAGAACCGTTCCTGAAAGACAG
TGCCAGATTCAGTTTTTCTCCGAGCCTGTGATCACCTTCGGGACGGCGATAGCCGCGTT
TTATTTTCCTGTATCCGTCATGACTATTCTTTACTGTCGGATATACAAGGAGACCGAAA
GACGCACTAAAGATCTTGCCGAACTTCAAGGCATCAACTCGTCAACAAATTCCAGCGGA
GACGCTCAGCCTCAAAAGATACGGTCTTGTTTCGGCTGCAAACATGTTAGCAATACATC
AAGAACCCAAGCTTTAAGTCACACCAATGCTGCTAAAACTCTGGATGATCAGTTGACCA
ACTTTAACAGCTACGCCTCCTCCGAGGAGGAAGATCGCTCTGGGAACTTCCAGGAGTCT
TGCAGACATCAGGAGAACAAGAGCGAAAGCTACGAGGAAGAGAACTTCTTCCCAACTCC
AGTCAAAGCAAGTCCTACGAAGACCAAGAAGTGTGTTTCGTATAAATTCAAACCGAAAG
ATGTCAGTCCGCTAAAGAACACTAACGGGGACGCAAAACCTGGAGCCTCGTCTTTCTCG
TCGGCCGAATCTGTGAACGCCCCTTCCTCATCGTCATCTTCGAAACCAATCGACGGTAC
GCTGAAATGCCAGATCACGAAGCGTAAACGGATGGTGCTGATCAAGGAGAAGAAGGCGG
CGCAGACTCTGAGCGCCATCCTGCTGGCCTTCATCCTCACCTGGACGCCGTACAACATC
ATGGTGCTGATCTCCACCTTCTGCTCCGACTGCATCCCGCTCTCGCTCTGGCATCTGGG
TTACTGGCTGTGCTATGTCAACAGCACCGTCAACCCCATGTGCTACGCGCTCTGCAACA
AAACCTTCCAGAAGACCTTCCGCATGCTGCTGCTGTGCCAGTGGAGGAAGCAGCGGGCG
GAGGAGAAGCTGTATTGGTGTGGGCAGAACCCGGCCGTGGGCAGCAAACTCACATGA

Primers used for PCR, cloning, and sequencing M5:

Ch17M5F1RN	AACATCACGGACGTCCAGCTG
m5F i3loop	CGAACTTCAAGGCATCAACTCG
Ch17M5R1RN	CCACACCAATACAGCTTCTCCTCC

The sequence data in black is the remaining unsequenced putative DNA that is predicted by Ensembl.

M5variant DNA sequence

ATGCTCTCCTTCAAAGTCAACAGCCAACTGAAGACCGTCAACAATTACTACTTACTC
AGTTTAGCTTTCGCAGACCTGATTATCGGTGTGTTCTCTATGAACCTCTACGCGTGC
TACATTCTCATGGGATACTGGTCTTTAGGGAATACAGCATGTGATCTGTGGTTGGCA
CTTGATTACGTTGCAAGCAATGCATCAGTGATGAACTTGCTGGTCATCAGCTTTGAT
AGATACTTCTCCATTACAAGGCCGCTTACCTACAGGGCTAAACGAACCCCGAAGCGG
GCAGGGATCATGATTGGCATGGCCTGGTTGATATCGTTTATATTGTGGGCTCCACCC
ATTCTGTGCTGGCAGTACTTTGTTGGAGAGAGGAAAGTTCCTCTTGACCAGTGCCAA
ATCCAGTTTTTCTCAGAACCCATAATTACATTTGGGACAGCCATAGCGGCCTTTTAT
GTCCCAGTATCCATCATGACCATACTATATTGCAAAATCTACAAAGAAACAGAAAAA
CGCACTAAGAATTTAGCGGAACTTCAGGGATATCCATCGCTGGACAGCCAGGACGGC
CCTAAAGTACGAAAACCAATCCTTCGATGCTTTAGCTTTAAAAACAAGAGAGACGGG
ACTCAAGCATCATGGTCTTCGTCCAACCAGAGTTACGTAACTCGGACAACATTGCAG
TCTGATGACTTGTGGACAAAATCTGAGCAGGTCACCACCCTGAACAGTTACACCTCA
TCCGAGGACGATGACCGGTCAGTCTCAGAGTCAACGCCGAGAGAATCCTTCAAGAAC
CAGGAACCACCAGTCAGTAAGAACGGACAGCTGGTCTGCTACGAAGATAGCAAATAT
CTAAGTGATGTTGCAAAATGCTCACAAACGAACAATAAAAAGTGCTTGTCATACAAA
TTCAAGCCAATCATTAGCGACATCACTGTTTTGCAAGGTAATAGTGAGAGTGAGAGA
GCAAACCCGGATCAGTCCTCAGACCAACAGGCTCCTTCCTCCACATCCTCTTCTACC
AAACCAGAAGACCCAAACCTTAAGATCCAGATGACCAAACGTAAAAGAATGGTTCTC
ATCAAGGAGAAGAAAGCAGCTCAGACTCTTAGTGCCATCCTTCTAGCCTTTCTTCTT
ACTTGGACACCATACAACATCATGGTGCTCATCTCCACCTTCTGTTCCAACTGCATC
CCAACATCCCTCTGGCAGCTGGGCTACTGGCTCTGCTACGTCAACAGCACCGTCAAC
CCCATGTGCTACGCCTTATGCAACAAGACCTTCCAGAAGACCTTTTGGATGTTAATC
CTCTGCAAGTGGAAGAAGAATAGGGGAGAGGAGAAGCTGTACTGGGGTGGTCAAAAC
CATTAA

Primers used for PCR, cloning, and sequencing M5v:

JSM3F1 ATTTGGGACAGCCATAGCGG
JSM3F2 GACGGGACTCAAGCATCATGG
JSM3R1 AGCACATGGGGTTGACGGTG
JSM3R2 CATGATGTTGTATGGTGTCCAAG
The four primers listed above were designed by Josh Strommen.

m5vrn1f GGGAAATGTATCTGCCATCC
m5vrn2f GGGGAACGTTTTAGTGATGC
m5vrn1r TTAATGGTTTTGACCACCCC

Sequence data in red represents bidirectionally sequenced DNA. Sequence data in blue shows DNA sequence that has been sequenced in one direction. The sequence data in black is the remaining unsequenced putative DNA that is predicted by Ensembl.

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VITA

Richard John Nuckels, Jr. was born in Port Lavaca, Texas on October 7, 1972, the son of Richard John Nuckels, Sr. and Marilyn Rene Nuckels. He received a BS in Biochemistry in May of 2003 from The University of Texas at Austin. In January of 2004, he entered Texas State University-San Marcos to pursue a Masters of Science degree in Biology. He has worked as a research technician/research associate in two zebrafish labs since June of 2002 at UT studying pigment pattern development/evolution and vertebrate eye development. This work resulted in two publications in the journal Development and two others currently in preparation. As a graduate student, he has received a research assistantship and additional support as an instructional assistant for genetics. He will present his research at the 46th American Society for Cell Biology Annual Meeting on December 11, 2006. Upon completion of his graduate degree, he will continue working at UT as a research associate/lab manager studying eye development.

Permanent Address: 335 Kessler Street

New Braunfels, Texas 78130

This thesis was typed by Richard John Nuckels, Jr.