DIURNAL VARIATION OF MELATONIN RECEPTORS IN *DANIO RERIO* (ZEBRAFISH)

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DIURNAL VARIATION OF MELATONIN RECEPTORS IN

DANIO RERIO (ZEBRAFISH)

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LIST OF ABBREVIATIONS

AANAT	arylalkylamine N-acetyltransferase
bp	base pair
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
˚C	degrees centigrade
cat #	catalog number
cm	centimeter
CREB	cAMP-response element binding protein
C_t	cycle threshold
Cy3 and Cy5	cyanine3 and cyanine5
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
Fig	figure
g	gram
GO	gene ontology
h	hour
HCl	hydrochloric acid
M	molar
mM	millimolar

µM	micromolar
min	minutes
ml	milliliter
µl	microliter
NA	not applicable
NCBI	National Center for Biotechnology Information
ND	not detected
NIH	National Institutes of Health
nm	nanometer
No.	number
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
qPCR	quantitative RT-PCR
RNA	ribonucleic acid
RPE	retinal pigment epithelium
SCN	suprachiasmatic nuclei
SD	standard deviation
SE	standard error of the mean
zfin	the zebrafish model organism database

ABSTRACT

DIURNAL VARIATION OF MELATONIN RECEPTORS IN

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The circadian system provides an integrating mechanism for synchronization of biological processes with the regular 24-hour light and dark changes in the environment. In some teleost species, ocular melatonin levels exhibit a circadian periodicity with elevated levels during the dark as compared to light, thereby regulating the circadian rhythms of several biological functions, such as the diurnal suite of events that help the retina anticipate changes in ambient light. To gain a better understanding of the diurnal variation in gene expression, I analyzed the changes in gene expression in the eye of zebrafish. Dual color oligonucleotide microarrays were used to compare total RNA harvested from eyes of adult zebrafish at midday and midnight. Statistical analyses identified 44 genes which showed significant, 2-fold or more change; 26 genes showed

decreased expression at midnight (D/L $\leq$ 0.5) and 18 genes showed increased expression at midnight (D/L $\geq$ 2). Seven genes were further analyzed using qPCR. The results of qPCR identified AANAT, Mel1a1, Mel1a3, Mel1b1, Mel1b2 and Melc as genes that showed significant change in expression at dawn, dusk, midday and midnight. These results suggest that expression of melatonin receptors is subject to diurnal regulation.

CHAPTER 1

INTRODUCTION

Evolution of life on earth has occurred under rhythmic diurnal changes of environmental conditions. Organisms have evolved endogenous circadian clocks which drive daily rhythms of biochemical, metabolic and behavioral processes (see Pittendrigh, 1993). A circadian system comprises all the different components by which light enters the organism and is transformed into a timed nervous or hormonal signal. In fish, beginning from development of larva, locomotor activity, skin pigmentation, oxygen consumption, thermoregulation, and food intake are some of many functions that show daily rhythms (Ekström & Meissl, 1997).

Circadian/biological clocks are time-keeping systems found in most organisms, allowing organisms to prepare for rhythms in physiology and behavior changes associated with day and night. Delaunay *et al*. (2000) showed that the circadian clock functions during early embryogenesis in zebrafish, and the embryonic circadian clock is driven by an endogenous oscillator. Central circadian clocks in vertebrates are located in the hypothalamic suprachiasmatic nucleus (Reppert & Weaver, 2001), the retina and the pineal gland (Klein *et al.,* 1997). The circadian system of zebrafish is composed of both central and peripheral clocks (Schibler and Sassone-Corsi, 2002) and becomes functional and responsive to light between 20 and 26 h post fertilization (Kazimi & Cahill, 1999). The presence of peripheral circadian oscillators in tissues and organs of adult zebrafish and their responsiveness to light has been shown by organ and tissue culture explant

experiments (Cahill, 1996; Cermakian *et al*., 2000). Fish and other non-mammalian vertebrates, have a well organized network of interconnected circadian units with pineal gland and retina holding main positions in the circadian system (Falcón *et al*., 2007).

Melatonin acts as a neuroendocrine messenger involved in the regulation of circadian biological rhythms (Reiter, 1991). Melatonin is produced mainly in the pineal gland and retina, and its concentration in plasma is higher at night than during the daytime. Melatonin rhythms are generated by changes in the activity of serotonin arylalkylamine N-acetyltransferase (AANAT), a pineal gland- and retina-specific enzyme belonging to the acetyltransferase superfamily which catalyzes the rate limiting step in melatonin biosynthesis (Klein *et al*., 1997). As alluded earlier, it becomes functional and responsive between 20 and 26 h post fertilization (Kazimi & Cahill, 1999).

The increase in production of melatonin hormone during the night time indicate increased AANAT activity and increased transcription of the gene encoding AANAT (Ganguly *et al*., 2002; Falcon *et al*., 2003). Rhythmic AANAT expression, AANAT activity and melatonin production are controlled by an internal circadian clock and signals generated by external light (Klein *et al*., 1997).

Melatonin hormone acts via melatonin receptors which belong to the G protein-coupled receptor superfamily (Iigo *et al*., 1994; Reppert *et al*., 1996). Three subtypes of melatonin receptors have been identified in vertebrates: Mel1a, Mel1b and Mel1c (Reppert *et al*., 1995). In various vertebrate species Mel1a and Mel1b are distributed in the central nervous system and peripheral tissues (Dubocovich, 1995; Reppert *et al*., 1996). In mammals, the SCN and hypophyseal pars tuberalis in brain show high levels of expression of Mel1a (Reppert *et al*., 1994). Mel1b is mainly expressed in the retina and

mediates the actions of melatonin on retinal physiology (Reppert *et al*., 1995). Mel1c has been identified in the brain and retina of non-mammalian vertebrates like zebrafish, golden rabbitfish, *Xenopus laevis*, and chicks (Ebisawa *et al*., 1994; Reppert *et al*., 1995; Wiechmann and Smith, 2001; Park *et al*., 2007b).

The different subtypes of melatonin receptors mediate various physiological functions of melatonin. Levels of expression of melatonin receptor genes have been reported to be higher in brain and retina than in peripheral tissues. Expression of Mel1c gene is solely found in the neuronal tissues (Park *et al.*, 2007). Differential expression of multiple subtypes of melatonin receptors seem likely to provide an important molecular basis of melatonin action. Melatonin activity varies depending on the availability of melatonin binding sites, which shows diurnal variation in several vertebrate species (Yuan *et al*., 1990; Falcón *et al*., 1996; Iigo *et al.*, 1994, 1997, 2003; Gaildrat *et al*., 1998). For example, the melatonin binding sites exhibits diurnal variations in goldfish brain (Iigo *et al*., 2003). Diurnal variations in the levels of melatonin receptor mRNAs have been reported in the brain and retina of chum salmon for Mel1a and Mel1b (Shi *et al*., 2004) and in the brain, retina, and pineal gland of golden rabbitfish for Mel1a, Mel1b, and Mel1c (Park *et al*., 2006). Normal daily variations in the levels of six melatonin receptor subtype mRNAs have also been reported in zebrafish larvae at days 5 and 6 post-fertilization (Shang and Zhdanova, 2007).

One of the functions of melatonin is dark adaptive pigment aggregation in *Xenopus* (Pierce and Besharse, 1985). Pigment aggregation is one of the changes that eyes undergo as part of daily rhythmicity. I hypothesized that gene expression in adult zebrafish eyes would exhibit diurnal variation. Following an initial analysis by

microarray, I focused my exploration on the expression of melatonin receptors. Microarray analysis revealed 44 genes to be differentially regulated between midday and midnight, including the gene encoding AANAT, which was expressed at higher levels at midnight. Follow-up studies using qPCR confirmed AANAT up-regulation and additionally showed that some melatonin receptor subtypes were also differentially regulated, being expressed at higher levels at midnight.

CREB regulates cellular gene expression by binding to a conserved CRE that occurs either as a palindrome (TGACGTCA) or half site (CGTCA and TGACG). The CREB family of activators are stimulated by cAMP and induce cellular gene expression after phosphorylation at a conserved serine (Mayr and Montminy, 2001). Phosphorylation of Ser-133 promotes gene activation by recruiting the coactivator paralogs CBP_p300 (Goodman and Smolik, 2000), leading to induction of cellular genes in response to cAMP (Cardinaux *et al*., 2000). A search of full cAMP response element (TGACGTCA) and half CRE (TGACG and CGTCA) sites was performed to determine whether cAMP might play a role in regulating expression of genes found to be upregulated at midnight in this study.

CHAPTER II

MATERIALS AND METHODS

Fish maintenance

Wild-type ZDR zebrafish (*Danio rerio*) were obtained from Animal Wonders, San Marcos, TX, and Aquatica Tropicals, Plant City, FL. These fish were from a wild-type, lab-bred lineage originating from Scientific Hatcheries (Huntington, CA) and have been maintained for more than 30 generations. Fish were conditioned on a 12 hour light/dark cycle for a minimum of 14 days before use. All protocols were approved by the Texas State IACUC (approval # 0703_0122_07 and # 069744F82-0927_0928_30).

Experimental design

The design of this analysis compared the experimental to the control: midnight samples were used as experimental and midday as control. Dual-color microarray was used to compare the midnight and midday samples. A statistically significant (p-value $\leq$ 0.05) and 2-fold or more change in an experimental sample as compared to a control indicated an up- or down-regulation in gene expression. Quantitative PCR was performed on RNA samples collected at dawn, midday, dusk and midnight to see diurnal variation in melatonin receptors gene expression.

Microarray analysis

RNA extraction for microarray analysis

All samples were collected at Texas State University-San Marcos, TX. Midday samples were taken by sacrificing fish in ambient room light, and midnight samples were collected under infrared illumination using a FIND-R-SCOPE Infrared Viewer (FJW Optical Systems, Inc., Palatine, IL). Samples at each time point were collected in triplicate. Eyes from 10 identically treated fish were pooled for each of three triplicate samples. Following pithing, whole eyes were removed from the fish and immediately placed in 1 ml of TRI-Reagent (Ambion; Austin, TX). The tissue was homogenized with a homogenizer (IKA, Ultra Turrax T8), and total RNA was isolated by phenol/chloroform extraction and isopropanol precipitation (Chomczynski and Sacchi, 1987). RNA quantity was assessed using a Nanodrop spectrophotometer (Thermofisher Scientific, Waltham, MA) to determine the 260/280 ratio, and integrity of the RNA sample was evaluated by glyoxal gel electrophoresis with ethidium bromide staining to detect the integrity of 18S and 28S rRNA bands. Samples were sent by overnight express (FedEx) to Michigan State University's Core Genomics Facility, and an additional quality check using an Agilent BioAnalyzer was performed. High quality RNA was reverse transcribed at Michigan State to yield Cy3 or Cy5 labelled cDNA which was analyzed by microarray analysis.

Microarray analysis

Microarray analysis was performed by Dr. Jeff Landgraf at Michigan State University as previously described (Saul, 2007; Saul *et al*., 2010). A total of 3 OciChips (Ocimum Biosolutions, Indianapolis, IN) were used to analyze the triplicate RNA samples. Total RNA was reverse transcribed, and the product cDNA underwent second

strand synthesis in the presence of RNase H. Following subsequent purification cDNA served as a template for *in vitro* transcription enabling modified 5-(3-aminoallyl)-UTP (aaUTP) to be incorporated into the antisense RNA (aRNA) during amplification. The aaUTP contained a reactive amino group that was subsequently coupled to derivatized dyes (Cy3 and Cy5). The dyes were swapped between midday and midnight sample replicates to control for any dye bias, i.e. in one replicate sample, the midday was labeled with Cy3 while the midnight was labeled with Cy5, and in two replicates the labels were switched. Each aRNA sample was fragmented using Ambion's RNA Fragmentation Reagents, added to the hybridization solution (Ocimum Biosolutions, Indianapolis, IN), heated to 95ºC for 3 min, cooled on ice for 3 min, and spun briefly. Hybridization was performed under a 40 mm x 22 mm LifterSlip (Erie Scientific) by the addition of 240 µl of labeled solution to Zebrafish 14K OciChip. Slides were scanned using an Affymetrix 428 ArrayScanner and analyzed with the GenePix Pro 3.0 software (Axon Instruments, Sunnydale, CA).

The "limma: Linear Models for Microarray Data" library module (version 2.2.0) of the R statistical package (version 2.2.0) was used for array normalization and statistical analysis. All genes with signal intensities of less than 1000 in all times points were removed from the analysis. Unpaired t-test was performed on the triplicate time points to determine whether there were statistically significant differences in gene expression between midday and midnight. Slide intensity data were normalized using the global LOWESS (locally weighted scatter plot smoothing) method with the least squares method used for the linear model fit. All genes with a change (up or down) greater than 2-fold change were considered differentially expressed.

Quantitative RT-PCR

RNA extraction from whole eye for quantitative qPCR

The eyes from 3 identically treated fish were pooled in triplicate for a total of 9 fish used for each time point. Whole eyes were removed from the 3 fish at each time point and immediately placed into 1 ml of TRI-Reagent (Ambion; Austin, TX). The tissue was homogenized using a homogenizer (IKA, Ultra Turrax T8), and total RNA was isolated as described above. RNA was cleaned using RNAeasy spin columns (QIAGEN, Valencia, CA), resulting in a pigment free product.

RNA extraction from retina for quantitative qPCR

The retinas from 3 identically treated fish were pooled in triplicate (total 9 fish). Whole eyes were removed from the 3 fish at each time point and immediately placed in RNA*later*® (Ambion; Austin, TX). The sclera and the lens were removed, and the remaining eye tissues (retina, RPE, and choroid) were immediately placed in 1 ml of TRI-Reagent (Ambion; Austin, TX). The tissue was homogenized (IKA, Ultra Turrax T8), and total RNA was isolated as described above. RNA was cleaned using RNAeasy spin columns, resulting in a pigment free product.

Starting with 1 µg of RNA, reverse transcription was performed using MMLV Reverse Transcriptase (Promega) and random and oligo dT primers (Promega) for 60 min at 37^{o}C. qPCR was carried out on an Eppendorf Realplex^{2} Mastercycler (Hamburg, Germany) using Invitrogen's (Carlsbad, CA) Universal SYBR® GreenER™ Two-Step qRT-PCR Universal Kit, following the cycling program: 5 min at 50^{o}C for cDNA

synthesis, 95°C for 10 min, 50 cycles (95°C for 15 sec, 60°C for 15 sec, 68°C for 15 sec). Amplification was followed by melting curve analysis (see figure 1).

As the qPCR reactions were run, SYBR Green incorporated into double stranded DNA was measured, and measured C_t values are reported by the system C_t values for the genes of interest were compared with C_t values from mRNA encoding the ribosomal protein L-24 (see figure 2) which served as a reference for analysis of results performed using the relative C_t method (Huggett J *et al.*, 2005).

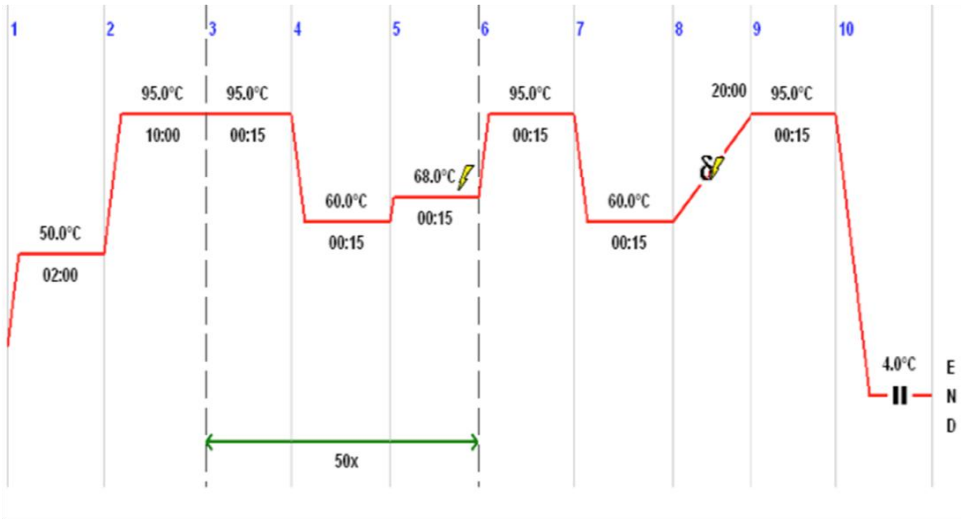

Fig. 1. qPCR was performed according to the temperature template shown above. This figure shows the temperature specifications that were used for qPCR. A total of 50 cycles were run and at the end of each cycle the fluorescence was measured for each sample. Polymerase chain reaction was done in two main steps: holding the reagents at 95°C for 15 sec (denaturation) followed by at 60°C for 15 sec and 68°C for 15 sec (annealing and extension). Step 8 shows the melting curve analysis, where temperature is increasing from 60°C to 95°C with a continuous measuring of fluorescence.

Table 1. Genes and Primer Sequences

Gene	Forward Primer	Reverse Primer
mtnr1aa/zMel1a (NM_131393.1)	CTGGTGATTTTCTCCGTCTACAGA	CCGCCACTGCCAAACTC
mtnr1al/zMel1a-2 (NM_001159909.1)	TTGGTCATTGTGTCAGTCTTCAGAA	GCTATAGCCAAACTCACCACAAAG
mtnr1ab/zMel1a-3 (ZFIN)	TTGGTGATCTTCTCCGTCTACAGA	TCAGCTACGGCCAGACTCA
mtnr1bb/zMel1b (NM_131394.1)	TCGGTGTTCAGGAATCGTAAACTG	GAAGGCCAGACTGACCACAAA
mtnr1ba/zMel1b-2 (NM_131395.1)	TCGGTGCTCCGAAATAGAAAACTC	CGAATGCCAGACTTACTACAAATGC
mtnr1c/zMel1c (NM_001161484.1)	CCGTCTACAGGAACAAGAAACTGA	GGTCAGCCACAGAAAGACTCA
AANAT (NM_131411.1)	ACACCACTGAGACGAGCAGACAAA	ACTGGAAACGCTGATGGGTGTCTT
rpl24/L-24 (NM_173235)	ATGTGAGTCTGCGTTTCTGTCCAAG	GCTTCTTCGACACCTCCTCAGACTG

The melatonin receptor primer sequences are from Shang and Zhdanova (2007); AANAT and L-24 receptor primers were designed by Elizabeth L. Capalbo, Texas State University-San Marcos.

Statistical Analysis

In order to statistically analyze our qPCR results, R-statistical software (version R-2.12.1) was used. The gene expression was normalized using zebrafish L-24 expression level. qPCR data from each biological replicate were analyzed and relative Ct change was calculated using the formula-

$$\text{Relative } C_t = C_t(\text{target}) - C_t(\text{L-24}).$$

Analysis of variance (ANOVA) with post hoc Tukey HSD was used for analysis. A p-value of ≤ 0.05 was taken to indicate significant differences.

Gene Ontology Analysis

The GO categorization is a comprehensive summarization of the genes based on biological process, molecular function, and cellular localization of gene products

(Ashburner *et al.*, 2000). All genes that displayed greater than 2-fold change in expression levels based on microarray analysis were individually analyzed for known functional characteristics and gene ontology, using zfin (http://zfin.org), NCBI (http://www.ncbi.nlm.nih.gov) and ontology information provided by the chip manufacturer. The genes were sub-grouped into the GO categories. Biological processes and molecular functions were annotated to the correlating and predictive genes.

Bioinformatic Analysis.

Genome sequences and annotations were obtained from the zfin site (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg). A whole genome search of full CRE (TGACGTCA) and half CRE (TGACG and CGTCA) sites was performed on six melatonin receptor subtypes, and the transcription factor ATF4 gene was used as positive control. All CRE hits were mapped to the 3'UTR, promoter, exonic, intronic, intergenic and 5'UTR regions according to the locations of RefSeq genes.

CHAPTER III

RESULTS

Microarray Analysis

Total RNA was isolated from whole eyes at midday and midnight on 3 different days. Gene expression in midnight samples was compared to gene expression at midday, and results were expressed as dark *vs*. light ratio (D/L). The Zebrafish 14K OciChip$_{TM}$ Oligo-nucleotide Array (Ocimum Biosolutions) includes 14,067, unique ~50-mer probes representing 8,839 genes. Statistical analyses showed 44 genes differentially expressed by at least a 2-fold difference with D/L ratio ≤ 0.5 or ≥ 2 ($p \leq 0.05$). At midnight, 26 genes showed decreased expression levels (see table 2 and figure 2) while 18 showed increased expression relative to midday (see table 3 and figure 2).

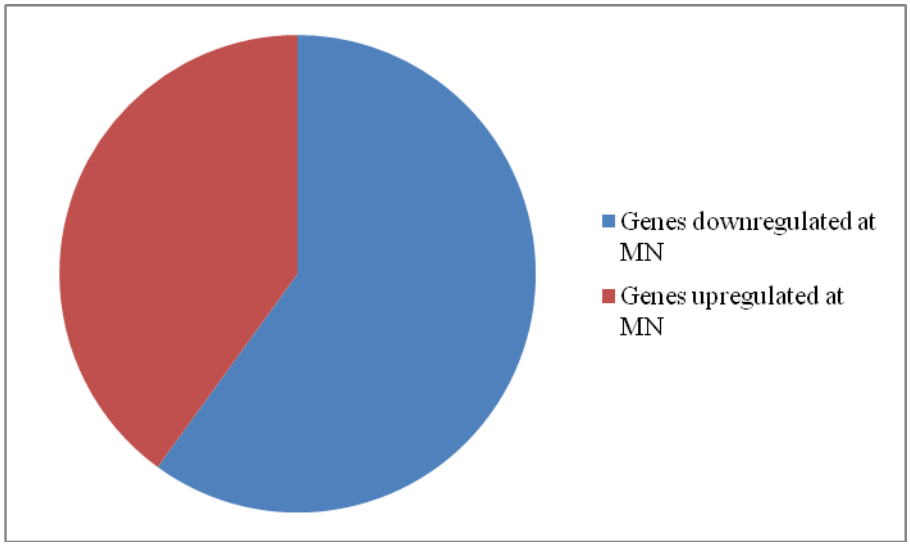

Fig.2. A total 44 genes showed significant change of 2-fold or more. At midnight there were 26 genes that showed decreased expression and 18 genes that showed increased expression as compared to midday.

All genes that showed greater than 2-fold differences were individually analyzed for known functional characteristics and gene ontology. The majority of the genes were classified ontologically in the major categories of biological process and molecular function. Analysis of gene ontology for 44 genes according to biological function category showed 3 genes have biological function in sensory/visual perception, 3 genes in development, and 2 genes in catabolic proteins: 16 genes were from other categories, and 20 were unclassified (see figure 3).

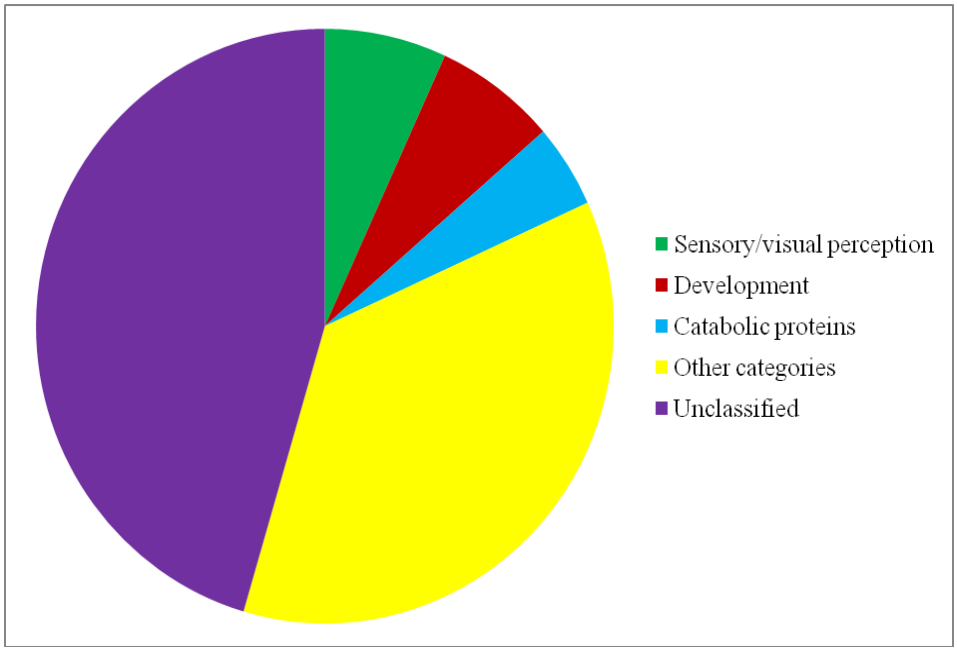

Figure 3. **Analysis of gene ontology for 44 genes according to biological function**.

Classification according to molecular functions showed five genes in calcium ion binding, four of which increased and one of which decreased expression at midnight. Three genes were in the nucleic acid binding category and two in the protein binding category. Twelve were unclassified and twenty-two were the sole occupants of other categories.

The genes up-regulated in the dark included AANAT **(**D/L= 3.062 and p= 9.47 x 10^{-5}). Because AANAT is the rate limiting enzyme in melatonin synthesis, and because of melatonin's well established role in regulating daily rhythms, I decided to examine the expression of melatonin receptors by qPCR.

Table 2. Microarray Results: Genes Showing Decreased Expression (D/L ≤ 0.5):

Accession No.	Name	Ratio (D/L)	Molecular function	Biological function
NM_200785.1	phosphodiesterase 6H	0.13	3',5'-cyclic-nucleotide phosphodiesterase activity	visual perception
BX511213.5	Retinol binding protein 4, like	0.20		
NM_199518.2	phosphoribosyl transferase domain	0.25	hypoxanthine phosphoribosyltransferase activity	nucleoside metabolic process
NM_152955.1	dachshund a	0.30	nucleotide binding	inner ear development
NM_199989.1	glutamic-oxaloacetic transaminase 2b	0.30	L-aspartate:2-oxoglutarate aminotransferase activity	biosynthetic process
NM_212991.2	heterogeneous nuclear ribonucleoprotein K, like	0.31	RNA binding	
NM_001002405.1	arrestin 3, retinal (X-arrestin)	0.34		sensory perception
NM_131039.1	es1 protein	0.39		
NM_131465.2	l-isoaspartyl protein carboxyl methyltransferase	0.40	methyltransferase activity	protein modification process
NM_001025189.1	vitellogenin 5	0.40	lipid transporter activity	lipid transport
NM_173222.1	creatine kinase, brain	0.41	creatine kinase activity	
NM_001040052.1	zgc:136896	0.42	structural constituent of ribosome	translation
XM_001338177.1	similar to KIAA 1447	0.43		
XM_690758.2	similar to U2-associated SR140	0.43		
XM_681309.2	similar to SLIT and NTRK-like factor	0.43		
NM_001008574.1	zgc:92134	0.44	cysteine-type peptidase activity	modification-dependent protein catabolic process
NM_194393.1	guanylate cyclase activator 1C	0.45	calcium ion binding	
NM_001033749.1	S-antigen; retina and pineal gland (arrestin)	0.45		sensory perception
NM_198372.1	family with sequence similarity 107, member B	0.47		
XM_001337224.1	casein kinase 1, alpha 1	0.47	similar to casein kinase I alpha LS	

Table 2 continued

Accession No.	Name	Ratio (D:L)	Molecular function	Biological function
NM_001045073.1	heat shock protein 90-alpha 2	0.47		
NM_200692.1	N-myc downstream regulated gene 1, like	0.47		
NM_001005393.1	brother of CDO	0.48	identical protein binding	axon guidance
NM_213058.1	heat shock protein 5	0.48	ATP binding	response to stress
NM_131225.2	retinal homeobox gene 1	0.49	DNA binding	multicellular organismal development
NM_173270.1	ATPase, H+ transporting, lysosomal, V1 subunit H	0.49	binding	retina development in camera-type eye

Table 3. Microarray Results: Genes Showing Increased Expression (D/L ≥ 2):

Accession No.	Name	Ratio (D:L)	Molecular function	Biological function
NM_131619.1	myosin, light polypeptide 3, skeletal muscle	2.00	calcium ion binding	
NM_001017767.1	zgc:112077	2.00	ligase activity	modification-dependent protein catabolic process
NM_001109741.1	similar to zona pellucida glycoprotein 3.2	2.02		
NM_001077742.1	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1a	2.04	L-ascorbic acid binding	oxidation reduction
NM_212845.1	Rhesus blood group-associated glycoprotein	2.10	ammonium transmembrane transporter activity	ammonium transport
NM_213063.1	WD repeat domain 24	2.14	protein coding	
NM_201024.1	phosphoglycerate mutase 2	2.14	catalytic activity	glycolysis
NM_130927.1	non-metastatic cells 2b.2	2.18	nucleoside diphosphate kinase activity	GTP biosynthetic process
NM_131786.2	cryptochrome 3	2.18	transcription repressor activity	response to light stimulus
NM_001017871.1	myosin, light chain 10, regulatory	2.20	calcium ion binding	
NM_131870.1	guanylate cyclase activator 1A	2.20	calcium ion binding	
NM_131005.2	ependymin	2.43	calcium ion binding	cell-matrix adhesion
NM_001007775.1	protein tyrosine phosphatase type IVA, member 1	2.43	phosphatase activity	dephosphorylation
NM_001007368.1	nuclear receptor subfamily 2, group E, member 3	2.60	DNA binding	regulation of transcription
NM_131108.1	type I cytokeratin	2.69	structural molecule activity	
NM_131411.1	arylalkylamine N-acetyltransferase	3.06	acetyltransferase activity	circadian rhythm
NM_200751.1	retinal pigment epithelium-specific protein 65a	3.10		
NM_001024815.1	BTB (POZ) domain containing 6	3.16	protein binding	

Quantitative PCR Analysis:

AANAT expression shows significant difference between midday and midnight:

Quantitative PCR was conducted on triplicate samples obtained from whole eye to validate the microarray data. AANAT was chosen based on its D/L ratio of 3.06 on the microarray (table 3), and its known role as the rate limiting enzyme in the melatonin biosynthetic pathway (Klein, 1985). Quantitative PCR findings were consistent with the observations made in microarray analysis and indicate that AANAT expression level is significantly up-regulated at midnight (see figure 4), being 2.41-fold higher at midnight relative to midday.

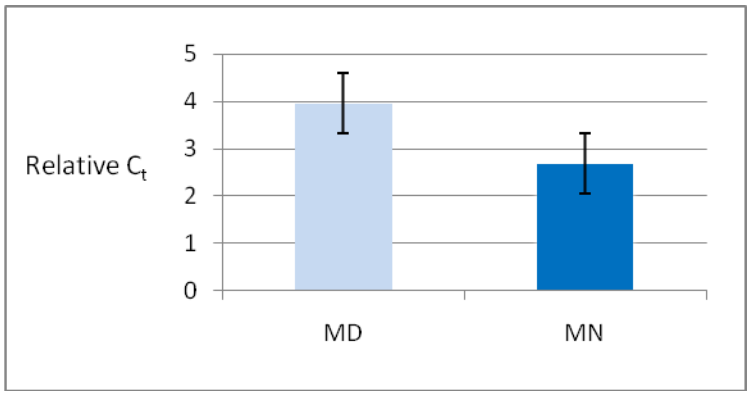

Fig 4. qPCR results indicate that AANAT expression is significantly higher level at midnight (MN) relative to midday (MD). The relative mRNA expression for AANAT gene, was measured using the relative C_t method. Y-axis shows relative C_t values (C_t of gene of interest – C_t of reference gene). The lower relative C_t value means a higher amount of mRNA. L-24 was used as the reference gene so that the level of melatonin receptor gene expression could be compared between midday and midnight.

Diurnal variation of melatonin receptors mRNA:

Quantitative PCR was conducted on RNA samples collected in triplicate obtained from retinal samples at dawn, midday, dusk and midnight to determine diurnal variation of melatonin receptor subtypes. AANAT and all melatonin receptor subtypes except Mel1a2 showed significant diurnal variation ($p < 0.05$). Mel1a1, Mel1a3 and AANAT expression levels reached highest at midnight while Melb1, Melb2 and Melc mRNA were at their peaks at dusk. Quantitative PCR findings differed from the observations made in microarray analysis, where all the genes representing melatonin receptors (2 spots for melatonin a, 2 spots for melatonin b and 1 spot for melatonin c) showed no significant change ($p > 0.05$); however, all the spots were intensity flagged. qPCR results showed 5 out of 6 melatonin receptors (Mel1a1, Mel1a3, Mel1b1, Mel1b2 and Mel1c) mRNAs were expressed at significantly higher levels at midnight as compared to midday. Only Mel1a2 showed no significant change. Combined, these data suggested that using retina-enriched samples enhanced the signal to noise ratio, enabling detection of differences between expression levels at midday and midnight (see figure 5).

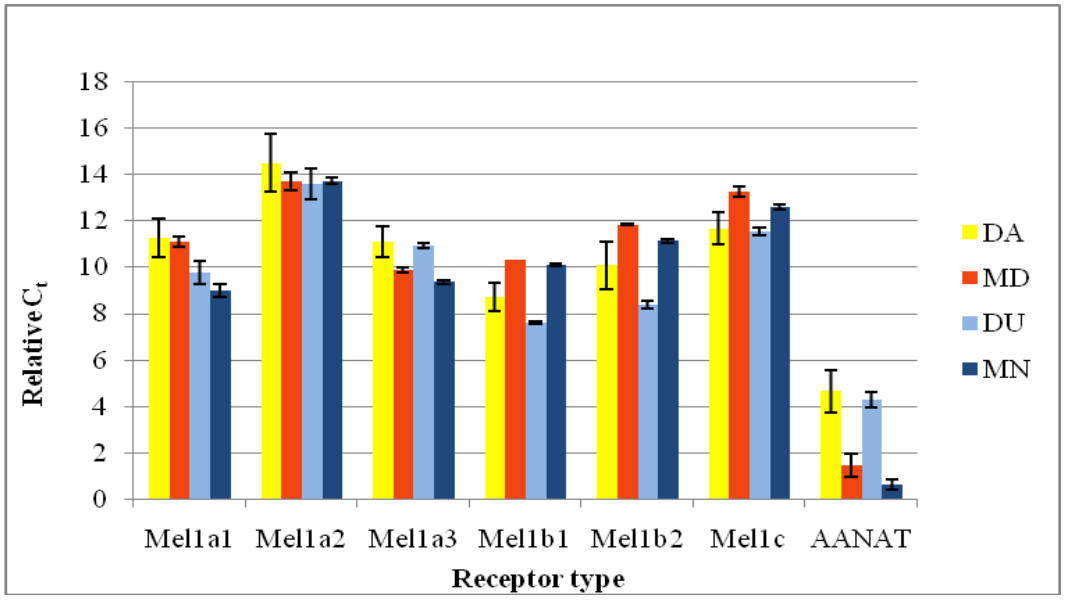

Fig: 5. qPCR results indicate that except for Mel1a2, all melatonin receptors and AANAT shows significant diurnal variation. The relative changes in mRNA expression for known receptor target genes, were measured using the relative C_t method. Y-axis shows relative C_t values (C_t of gene of interest – C_t of reference gene). The lower relative Ct value means higher amount of mRNA. Mel1a1, Mel1a3, Mel1b1, Mel1b2 and Mel1c showed significant difference at four different time points. Mel1a2 showed no significant difference. The relative levels of the mRNA transcript as shown mean relative C_t ± [SEM]; n=3 fish at each time point. L-24 was used as the reference gene so that the level of melatonin receptor gene expression could be compared between dawn, midday, dusk and midnight.

Determination of CRE regions

A whole genome search of full CRE (TGACGTCA) and half CRE (TGACG and CGTCA) sites was performed in all melatonin receptor subtypes using transcription factor ATF4 as positive control. Half CRE's are more abundant than full CRE's and were found in 3'UTR, exon, 5'UTR and also in intron region (see table 4).

Table 4: Location and counts of CRE

Genes	**Full CRE (TGACGTCA)**		**Half CRE (TGACG)**		**Half CRE (CGTCA)**	
	Location	Count	Location	Count	Location	Count
Mel1a1	3'UTR	-	3'UTR	19	3'UTR	-
	Exon	-	Exon	1	Exon	1
	Intron	2	Intron	-	Intron	-
	5'UTR	-	5'UTR	1	5'UTR	18
Mel1a2	3'UTR	-	3'UTR	1	3'UTR	-
	Exon	-	Exon	-	Exon	2
	Intron	-	Intron	9	Intron	4
	5'UTR	1	5'UTR	1	5'UTR	1
Mel1a3	-	-	-	-	-	-
Melb1	3'UTR	-	3'UTR	2	3'UTR	3
	Exon	-	Exon	3	Exon	-
	Intron	1	Intron	11	Intron	10
	5'UTR	-	5'UTR	1	5'UTR	1
Melb2	3'UTR	4	3'UTR	10	3'UTR	5
	Exon	-	Exon	3	Exon	3
	Intron	-	Intron	12	Intron	15
	5'UTR	-	5'UTR	1	5'UTR	3

Table 4 Continued

Genes	**Full CRE (TGACGTCA)**		**Half CRE (TGACG)**		**Half CRE (CGTCA)**	
	Location	Count	Location	Count	Location	Count
Melc	3'UTR	-	3'UTR	-	3'UTR	-
	Exon	-	Exon	-	Exon	-
	Intron	-	Intron	5	Intron	7
	5'UTR	-	5'UTR	2	5'UTR	-
ATF4	3'UTR	-	3'UTR	1	3'UTR	1
	Exon	-	Exon	1	Exon	1
	Intron	1	Intron	1	Intron	2
	5'UTR	-	5'UTR	6	5'UTR	5

Number against the location denotes the count of hits in the genome. 3' UTR- three prime untranslated region, 5'UTR- five prime untranslated region. Gene sequence of Mel1a3 was not found.

CHAPTER IV

DISCUSSION

In this project, over 8000 genes were analyzed for diurnal variation in *Danio rerio* eyes and retina, and six melatonin receptor genes along with the gene for AANAT were further scrutinized for variability in their expression. Melatonin receptors belong to G-protein coupled receptor family with seven trans-membrane domains (Dubocovich, 1988; Reppert *et al*., 1996; Shiu and Pang, 1998). They have highly conserved amino acid sequences which are important in G-protein functioning (Witt-Enderby *et al*., 2003; Gruijthuijsen *et al*., 2004; Kokkola *et al*., 2005). The present study revealed daily rhythmicity in ocular AANAT and retinal melatonin receptors subtypes Mel1a1, Mel1a3, Mel1b1, Mel1b2 and Mel1c mRNA levels in the retina of adult zebrafish.

In my study, the genes representing melatonin receptors (2 spots for melatonin a, 2 spots for melatonin b and 1 for melatonin c) showed no significant change on microarray ($p > 0.05$); however, the spots were intensity flagged, indicating inadequate amounts of mRNA of those species in the original samples to permit analysis. In contrast, qPCR resulted in a significant differential expression. The microarray chips showed high background fluorescence during analysis, as reported by Dr. Jeff Landgraf. However, the discrepancies between qPCR and the microarray analysis can be explained by the sensitivities of the two methods. qPCR is a more sensitive and specific method (Bustin, 2000) and can be considered more reliable. The experimental design of comparing retina-only samples had several benefits over looking cDNA samples derived from whole eye. I

successfully limited extraction primarily to the tissues known to express the receptors thereby eliminating "noise" and dilution of relevant signal. This approach seems to have enhanced my ability to detect changes specific to retina, RPE and choroid. I was also able to show that differences do exist among the melatonin receptor subtypes expression level.

Parallels in circadian rhythms and changes of AANAT level

My microarray and qPCR results show significant up-regulation of AANAT mRNA levels at midnight relative to midday (D/L > 3), a result demonstrated here in adult zebrafish and in line with observations made in other vertebrates. For example, AANAT mRNA levels were found up-regulated in rat retina (Sakamoto & Ishida, 1998) based on northern blot analysis. Using northern blot analysis Klein *et al.* (1997) showed daily rhythms of AANAT expression: the levels are low at midday and higher at midnight in the pineal gland of chicken, rat and sheep. The increased transcript presumably correlates with increase enzyme production since melatonin hormone levels also rise during night (King & Steinlechner, 1985; Klein, 1985).

Role of melatonin in osmoregulation, pigmentation, metabolic and immune functions have also been described (Falcón *et al*., 2010); however, melatonin is better known for the role it plays in the regulation of a variety of daily and annual physiological rhythms (Arendt, 1995; Ekstrom & Meissl, 1997). Early development of melatonin system provides a means by which embryonic activity rhythms can develop according to the environmental light-dark cycle, enabling the young fish to feed and remain active at optimal time of day (Roberts *et al*., 2003). In zebrafish, Kazimi and Cahill (1999) found nocturnal increases in melatonin content were detectable as early as the second night

post- fertilization. Gothilf *et al*. (1999) reported detection of pineal AANAT mRNA expression at 22 h post-fertilization and retinal AANAT mRNA at three days post-fertilization using whole mount *in situ* hybridization of zebrafish embryos. In Senegalese sole (*Solea senegalensis*) daily and seasonal changes in melatonin levels (Vera *et al.,* 2007) have been reported as have changes in melatonin receptor expression based on qPCR analysis (Confente *et al.,* 2010). Therefore a role for both melatonin and melatonin receptors in integrating information about photoperiod, temperature or both to regulate reproductive events according to the daily, lunar and annual cycles has been suggested.

Parallels between circadian rhythms of AANAT and melatonin receptor mRNA expression

The changes in expression of melatonin receptor genes observed support my hypothesis that melatonin receptor expression would parallel AANAT gene expression between midday and midnight. The general increase in the melatonin receptor mRNA expression levels at midnight suggests that there may be melatonin receptor up-regulation, which coupled with increased levels of melatonin hormone release may initiate signaling pathways leading to the response to melatonin hormone. My observation is consistent with earlier findings made on the retina of goldfish, showing that the levels of Mel1a, the major subtype found in the retina, and Mel1b and Mel1c levels increase during the night (Ikegami *et al,* 2009). In golden rabbitfish, diurnal and circadian variations in expression of Mel1a, Mel1b, and Mel1c genes have been reported in the retina, with one peak at nighttime (Park *et al*., 2006, 2007a, b). Daily variations in the melatonin binding sites have also been demonstrated in the retina of goldfish

Carassius auratus (Iigo *et al*., 1995; Ribelayga *et al.*, 2003), catfish *Silurus asotus* (Iigo *et al*., 1997), and European sea bass *Dicentrarchus labrax* using radioreceptor assay techniques (Bayarri *et al*., 2004). Mel1c immunoreactivity in the *X. laevis* retina has also been reported (Wiechmann & Wirsig-Wiechmann, 2001). Mel1b and Mel1c melatonin receptor expression has been localized in the retinal pigment epithelium (RPE) and neural retina of *X. laevis* using immunocytochemistry and *in situ* hybridization (Wiechmann & Smith, 2001; Wiechmann & Wirsig-Wiechmann, 2001). Mel1b receptor mRNA is expressed in the frog retina (Wiechmann *et al*., 1999; Wiechmann & Smith, 2001), and Mel1c receptors are present on retinal photoreceptors (Wiechmann & Wirsig-Wiechmann, 2001). Mel1a and Mel1c receptor proteins are present in the *X. laevis* retina (Wiechmann, A.F., 2002), and their distribution in the photoreceptors and inner retina is similar to that of human retina (Fujieda *et al*., 1999, 2000; Scher *et al*., 2002). Capalbo (2009) showed diurnal variation of muscarinic and dopaminergic receptors in the eye of zebrafish using qPCR and immunohistochemistry, suggesting that diurnal variation in receptor expression may be a general phenomenon.

Studies on circadian variations of melatonin binding sites and melatonin receptor transcripts in fish brains also revealed different daily patterns, indicating the regulatory mechanism for melatonin binding sites differs among species (Amano *et al*., 2003; Iigo *et al*., 2003). Amano *et al*. (2003) found daily rhythms of affinity and density of melatonin binding sites in the masu salmon brain. Day/night and circadian variations of Mel1c transcripts in the retina and whole brain of the golden rabbitfish have been observed (Park *et al*., 2007a, b). The melatonin binding sites in the goldfish brain reveal diurnal changes by radioreceptor assay (Iigo *et al*., 2003). The effects of melatonin depend both

on the melatonin and melatonin receptor rhythms as suggested previously (Falcón *et al.*, 1996; Gaildrat *et al.*, 1998). Wide distribution of Mel1a and Mel1b subtypes mRNAs throughout the brain may be involved in diverse functions of melatonin such as circadian and circannual control of behavioral and physiological rhythms, regulation of sleep, and neuronal apoptosis (Iigo *et al.*, 1994; Pandi-Perumal *et al.*, 2006). From this I conclude that melatonin action at night is the cumulative effect of both increase melatonin hormone production and increased melatonin receptors expression.

Gene Ontology Analysis

Our microarray covers only a portion (14,067 unique probes representing 8,839 genes) of the zebrafish genome. Despite these limitations, some conclusions were made from the available data. The genes differentially expressed are enriched for genes involved in phototransduction, consistent with the function of the fish eye as a photoreceptor organ and the importance of detecting changing light conditions. Many of the genes that were uncovered as differentially expressed by microarray analysis are not well annotated, being referred to as "transcribed locus" or "hypothetical protein." Future expansion of the database and completion of the annotation of the zebrafish genome will promote the analysis of these genes. Nevertheless, we generated a list of many annotated genes. Individual analysis of some of these genes, and of genes that may be annotated in the future, may offer the opportunity to identify new players in circadian rhythm and diurnal function.

CREs and CREB target genes analysis

A long-standing observation has been that cAMP level rise in the retina and pineal gland at night, dependent on the activation of the adenylate cyclase/ protein kinase A. cAMP, acting as a second messenger, induces gene transcription through activation of cAMP-dependent protein kinase. This is followed by phosphorylation of the transcription factor CREB at serine-133, which stimulates melatonin synthesis (Maronde *et al*., 1999; von Gall *et al.,* 2000).

In this study, I speculated that the melatonin receptor genes might be regulated by CREBs, therefore, I examined them for the presence of CREs. With the exception of Mel1a3, the sequence of which was not available, all the melatonin receptor genes had CREs or half CREs that could function as regulatory elements.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

My result shows the daily expression pattern of melatonin receptors, with maximum levels at night-time, represents a shared characteristic in different fish orders, at least in areas involved in the processing of visual/light information as the retina and optic tectum. Our quantitative PCR study has shown the existence of day–night variations in the expression of particular receptors in the retina, which could be involved in mediating circadian and circannual rhythms.

The study presented here aimed to observe diurnal melatonin receptor expression changes. In this project I undertook to examine changes in gene expression between midday and midnight, and later expanded my project to include observations at dawn and dusk. Considering that the eye is an organ of vision specialized for detecting light, surprisingly few genes changed their expression levels between these two time points. To gain a better understanding of the role of melatonin in vision, it would be interesting to examine the function of melatonin at biochemical level. Further studies are required to clarify the interaction between melatonin and its receptors during daily activities in fish. The striking similarities in melatonin receptor expression in fish and mammalian retinas illustrate the usefulness of the *Danio rerio* retina as a model to study circadian processes of the retina. The differential spatial and temporal patterns of expression of the melatonin receptor subtypes suggests that melatonin may convey differential effects on various target cells in the retina. Further studies will aim at further characterizing the role

melatonin plays in vision and clarifying the interaction between melatonin and its receptors during diurnal activities.

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VITA

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