Opisin Photoisomerases in the Chick Retina and Pineal Gland: Characterization, Localization, and Circadian Regulation

Michael J. Bailey1,2 and Vincent M. Cassone1,2

Purpose. The chick retina and pineal gland exhibit circadian oscillations in biochemical and physiological processes in vivo and in vitro, which entrain to 24-hour light–dark cycles. However, the phototransduction mechanisms responsible for entrainment are largely unknown. The present study characterizes two candidate opsin-like genes that may play a role in entrainment of the clocks in these tissues.

Methods. Bioinformatics, cladistics, and in situ hybridization and Northern blot analyses were conducted to characterize, localize, and determine the circadian expression of the candidate opsin-like genes in the retina and pineal gland.

Results. Two candidate photosensors and/or photoisomerases were predominantly distributed within the pineal gland and retina: the retinal pigmented epithelium-derived rhodopsin homologue (peropsin, gRrh) and retinal G-protein-coupled receptor opsin (RGR opsin, gRgr). Northern blot and in situ analyses revealed mRNA expression for both opsins in the pineal gland, retina, and brain tissue. The mRNA for both genes within the pineal gland and retina is regulated on a circadian basis such that they are highest in late subjective day. Digoxigenin in situ analyses showed retinal gRgr message within the inner nuclear layer (INL) and retinal ganglion cell layer (RGL), whereas gRrh message was distributed predominantly in the RGL. In the pineal gland, gRgr message was sparsely distributed among pinealocytes in follicles, but not within the follicles themselves, whereas gRrh was localized in interstitial areas indicative of astrocytic and/or endothelial origin.

Conclusions. The presence of two putative photoisomerases within the pineal gland and in retinal layers associated with biological clock function provides two candidate opsin-like genes that may serve in the visual cycle regulation of the circadian clock. (Invest Ophthalmol Vis Sci. 2004;45:769–775) DOI:10.1167/iovs.03-1125

Several opsin photopigments have been identified and described in cells and tissues beyond the traditionally accepted retinal photoreceptors—the rods and cones—in several vertebrate species.1–3 These tissues include the pineal gland, parapineal structures, and deep tissues in the hypothalamus but also within the inner nuclear layer (INL) and ganglion cell layer (RGL) of the retina itself. Sequence comparisons suggest there are several classes of vertebrate opsins. The photosensory classes include the traditional rod and cone opsins, a separate vertebrate ancient opsin class,4 and a pinopsin class.5 The other classes include exo-rhodopsin6 parapinopsin,7 tmtpinopsin,8 RGR opsin,9 melanopsin,10 peropsin,11 and encephalopsin.12 All these photopigments and their related retinal proteins are characterized 35- to 55-kDa membrane proteins that consist of a single opsin and its retinalaldehyde chromophore. The chromophore is bound to a lysine (K) residue located in the seventh helix of the opsin through a protonated Schiff base linkage.13 In addition, vertebrate opsins contain seven transmembrane helical domains connected by three extracellular and three cytoplasmic loops. These domains are the primary motifs of the G-protein-coupled receptor (GPCR) superfamily.14

The precise functions of the nonvisual opsins are not completely understood. Several of these that have been isolated in pineal tissue5,15,12 have been implicated in photic regulation of the pineal hormone melatonin,16 by virtue of the fact that they are expressed in photoreceptive pinealocytes (PINC).1 Recently, melanopsin expression in the RGL of mammals has been implicated in circadian phototransduction and in nonimage-forming responses, such as pupillary responses to bright light.5,17,18

In addition to photosensory opsins, several opsin-based molecules have been identified as photoisomerases, which catalyze the regeneration of photosensory opsins by converting the all-trans-retinal to produce the chromophore 11-cis retinal in the process known as the “visual cycle.”14 Of these, RGR opsin has been found in retinal pigmented epithelium (RPE) and glial Müller cells9 and is capable of functioning as a photoisomerase that generates 11-cis-retinal in the retinal pigmented epithelium.19 Peropsin (Rrh) was originally identified in human RPE11 and probably functions as a photoisomerase as well.20

Recently, as part of an extensive functional genomics analysis of the chick pineal gland,21 we confirmed the rhythmic regulation of mRNAs involved in melatonin biosynthesis, confirming the microarrays’s validity, and demonstrated circadian variation of mRNAs corresponding to a wide variety of other processes, including orthologues of mammalian “clock” gene, sequences associated with immune function and photoreception. Among the sequences associated with photoreception, genes that encode two opsin-like photopigments, RGR opsin and peropsin, stood out, because these proteins have been primarily associated with the RPE, which is typically not found in the pineal gland.22,23

Because the photopigment(s) and its photoisomerase(s) required for photic entrainment are unknown, we investigated these two classes of opsins within the pineal gland and retina of chicks. In this study, chick orthologues of mammalian RGR opsin and peropsin were isolated and characterized in the pineal gland and retina. The mRNA of both opsins is expressed in cells that have been associated with both melatonin biosynthesis and circadian entrainment. Further, both are regulated on a circadian basis. Whether these function as photosensors

From the 1Center For Biological Clocks Research and the 2Department of Biology, Texas A&M University, College Station, Texas. Supported by Grant P01 NS59546 from the National Institute of Neurological Disorders and Stroke.

Submitted for publication October 10, 2003; revised November 19, 2003; accepted November 21, 2003.

Disclosure: M.J. Bailey, None; V.M. Cassone, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Vincent M. Cassone, Center For Biological Clocks Research, Department of Biology, Texas A&M University, College Station, TX 77843-3258; vmc@mail.bio.tamu.edu.
and/or photoisomerases is discussed in the context of their distribution and sequence analyses.

**METHODS**

**Animals**

White leghorn cockerels were obtained from Hy-Line International (Bryan, TX) and maintained for 3 weeks in a 12-hour light–dark (LD) cycle (lights on Zeitgeber time [ZT], 0 to 12) with food (Startenea; Ralston Purina, Richmond, IN) and water ad libitum. Animals maintained in conditions of constant darkness were killed by using an infrared (IR) viewer.

Animals were treated in accordance with U.S. Public Health Service (PHS) guidelines. The procedures were approved by the Texas A&M University Laboratory Animal Care Committee (AUP 2001-163). The experiments were conducted in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Isolation of gRgr and gRrb**

The cDNAs encoding full-length clones were isolated from a chick pineal cDNA library, as part of our chick pineal expressed sequence tag (EST) sequencing project, previously described. Briefly, the cDNA library was constructed from pineal mRNA collected at ZT-18 with a cDNA synthesis kit (Lambda Zap II; Stratagene, La Jolla, CA). Clones were selected from the library at random for sequencing. Two clones for each gene were initially isolated and then sequenced in sense and antisense orientations to confirm identities. Sequences were deposited into GenBank databases gRrb (accession no. AY339626) and gRgr (accession no. AY339627; available by ftp at zippy.nimh.nih.gov/ or at http://rsh.info.nih.gov/nih-image; developed by Wayne Rashb, National Institutes of Health, Bethesda, MD).

**Bioinformatic Analysis**

Sequence alignments were performed using Clustal W (ver. 1.75) software. Accession numbers used included AH001149: Bos taurus rhodopsin; U15762: Gallus gallus pinopsin; AF036061: G. gallus melatonin; NM_010098: Mus musculus encephalopsin; AB035277: Danio rerio VA opsins; AF028014: Ictalurus punctatus parapinopsin; M62903: G. gallus red cone opsins; AF109372: D. rerio blue opsin; PMR它:1: Petromyzon marinus rhodopsin; AF402774: Tetradon rubripes tmt-opsin; X57143: Takifugu pacificus retinochrome; AF012270: Homo sapiens peropsin; NM_021340: M. musculus RGR opsins; GGU18121: G. gallus Mel1c; AH001062: Drosophila melanogaster rhodopsin; and M76446: human a-1A-adrenergic receptor.

After sequence alignments, cladistic analysis was performed by the neighbor-joining (NJ) method. The NJ method works on a matrix of distances among all pairs of sequence to be analyzed. These distances depend on those used to construct the tree. For example, most tree-building programs output distances as numbers of substitutions per site. For such trees TreeView typically displays a scale of ‘0.1’, meaning 0.1 nucleotide substitutions per site. The actual value depends on the branch lengths in the tree. The chick Mel1c melatonin receptor and the human α-1A-adrenergic receptors, both nonopsin seven-transmembrane-domain receptor proteins, were used as an outgroup for cladistic analysis.

**RNA Analysis**

Total RNA was isolated from tissues using extraction solution (Trizol; Invitrogen, Carlsbad, CA) as described by the manufacturer. Northern blot analyses were performed as previously described. Unless otherwise stated, total RNA (10 μg each lane) was fractionated on a 1.5% agarose-0.66 M formaldehyde gel, and probed for gRgr and gRrb. Probes were labeled with [α-32P] dATP by random priming (DECA Prime II kit; Ambion, Austin, TX). Typically, blots were first hybridized with the gRgr probes and subsequently stripped (twice for 15 minutes each in boiling water) before hybridization with gRrb probe.

The final wash was for 30 minutes at 42°C in 0.1× SSC containing 0.1% SDS. Blots were exposed to x-ray film (BioMax MS; Eastman Kodak, Rochester, NY) for 2 days and their images scanned. Transcript sizes were estimated by comparison with ribosomal RNA bands. Data were normalized for variation in RNA loading and transfer efficiency by comparison to the 18S ribosomal band, which was obtained by digital photography of the blot after transfer. Transcription abundance was determined on computer (Scion Image; Scion, Frederick, MD; available commercially at www.scioncorp.com). Data shown are the average of two blots per gene.

**In Situ Hybridization**

Animals were killed by decapitation, and brains and eyes were removed and rapidly frozen in −60°C isopentane. In situ hybridization (ISH) techniques were performed as previously described. After fixation, deproteinization, and acetylation, slides were hybridized with sense and antisense cRNA encoding the open reading frame (ORF) and 3′ untranslated region (UTR). Probes were transcribed in vitro in the presence of [α-32P] dUTP with T3 and T7 RNA polymerases for sense and antisense probes, respectively. Sections were incubated overnight at 53°C, washed in SSC, and dehydrated in 100% ethanol. Sections were exposed to x-ray film (BioMax MS; Eastman Kodak) for 2 days.

Digoxygenin-labeled probes were synthesized encoding the sense and antisense sequences of both genes, using an RNA transcription kit (DIG; Roche Diagnostics, Indianapolis, IN). After prehybridization, sections were incubated with the RNA probe (200 nmol/mL) in hybridization buffer at 42°C for 16 hours, and subsequently washed three times in 60% formamide/0.2× SSC. To visualize the hybridization, a color reaction was then performed from 8 to 14 hours.

**RESULTS**

**Bioinformatic Analysis of gRrb and gRgr**

Sequence analysis indicates that mRNA encoding each chick opsin protein is very similar to mammalian opsin of the same class. The predicted amino acid sequence from the ORF indicates that gRrb is 77% identical with human peropsin, whereas gRgr is 59% identical with human RGR opsin. Each of the opsin contains seven transmembrane regions indicative of the GPCR superfamily and also contains a lysine in the seventh transmembrane domain, corresponding to 284 K of peropsin and 255 K of RGR opsin (Fig. 1A). This amino acid residue has been shown to be responsible for chromophore binding, leading to the assumption that both of these genes probably encode photosensitive proteins. gRrb and gRgr also contain a cysteine in the first and second extracellular loop necessary for stabilization of the tertiary structure through disulfide bridge formation. The projected protein sequence of gRRH and gRGR contain the DRY sequence motif next to transmembrane domain 3, which is necessary for rhodopsin to activate G-proteins and transducin. In addition, there are several serine-threonine residues present in the C-terminus, which may function as potential phosphorylation sites for kinases.

Cladistic analysis of gRgr and gRrb and several other opsins, using the chick Mel1c melatonin receptor and human α-1A-adrenergic receptor both of which are seven-transmembrane domain G-protein associated receptors, as out-group molecules, clearly indicates that gRgr and gRrb belong within the opsin clade. However, they represent different subgroups.
from either the visual opsins or the many putative extraocular opsins such as pinopsin, parapinopsin, encephalopsin, tmt-opsin, and melanopsin. The sequence of grRh belongs in a separate peropsin class of opsins with human peropsin, whereas gRgr belongs to the RGR opsin class, closer to members of retinochrome 30 and retinochrome-like opsins than to members of other classes of vertebrate opsins (Fig. 1B).

Spatial and Temporal Distribution of grRh and gRgr mRNA

Multiple tissue Northern blot analysis at high stringency revealed that grRh and gRgr mRNA are not widely expressed in the chicken, with high levels of expression visualized only in the brain and retina. The grRh probes hybridized to one transcript at approximately 2.0 kb in the retina and brain tissue excluding the pineal gland. Likewise, gRgr probes revealed two transcripts in the retina and brain, a major one occurring at approximately 2 kb, and minor transcript at approximately 4 kb. No expression was visualized in heart, liver, skeletal muscle, intestine, and kidney (Fig. 2).

ISH of chick brain, with radioactive-labeled probes, indicated very strong expression of both gRgr and grRh in the pinel gland, confirming our initial observation from pineal cDNA libraries, but also lower levels of expression throughout the brain (Fig. 3). This expression was specific, because corresponding sense controls were completely blank (data not shown).

Nonradioactive digoxigenin ISH of grRh and gRgr mRNA revealed expression in both the chick retina and pineal gland. In the pineal gland, expression of gRgr message was primarily observed in parafollicular cells (PFCs) broadly interspersed among PINs (filled arrow) but not within the PINs themselves (Fig. 4A, open arrow). These cells appeared to be astrocytes, although this is very difficult to determine by ISH. Nonetheless, the distribution of grmRNA was different from the distribution of either arylalkylamine-N-acetyl transferase (AANAT) or pinopsin in the chick pineal gland, both of which are localized in follicular PINs, and so it is unlikely that they are expressed by follicular PINs. Sense controls were blank (Fig. 4B). Similarly, grRh mRNA

FIGURE 1. (A) Sequence alignment of RRH and RGR opsin versus B. taurus rhodopsin, performed by using CLUSTAL W analysis. Shaded areas: the seven transmembrane domains; (*) cysteines in the first and second extracellular loop necessary for stabilization of the tertiary structure through disulfide bridge formation; (●) lysine in the seventh transmembrane domain, corresponding to 284 K of peropsin and 255 K of RGR opsin. This amino acid has been shown to be responsible for chromophore binding. (●) DRY sequence motif next to transmembrane domain three, which is necessary for rhodopsin to activate G-proteins and transducin. (B) Phylogenetic analysis of grRh and gRGr-opsins performed with the TreeView program. Proteins used in analysis are as follows: AH001149: B. taurus rhodopsin, U17562: G. gallus pinopsin, AV36601: G. gallus melanopsin; NM_010098: M. musculus encephalopsin; AB095277: D. rerio VA opsin; AF028014: I. punctatus parapinopsin; M62903: G. gallus red cone opsin; AF109372: D. rerio blue opsin; PMB9001: P. marinus rhodopsin; AF402774: T. rubripes tmt-opsin; X57145: T. pacificus retinochrome AF012270: H. sapiens peropsin; NM_021340: M. musculus RGR opsin; GU51821: G. gallus Mel1r; M76446: human α1A-adrenergic receptor.
and brain RNA (10 µg) revealed that one transcript of gRrh was expressed in chicken brain. (A) mRNA expression for gRrb revealed that one transcript of ∼2 kb was present in total retinal and brain RNA (10 µg). (B) Expression of gRgr revealed one major transcript of ∼2 kb and a minor transcript of ∼4 kb. (C) 18S ribosomal RNA. All RNA samples were isolated from the indicated tissues dissected at ZT-12. The blots were repeated with similar results on independently obtained samples.

was expressed in the PFCs (filled arrow) surrounding PINs and in “interstitial” cells (INT, Fig. 4C, open arrow), although the gRrb signal was more widespread than that of gRgr. Sense controls were blank for gRrb as well (Fig. 4D).

In the retina, the relative distributions of gRgr and gRrb differ from their distribution in pineal in that retinal gRgr appears more widespread than gRrb, although the Northern analyses did not indicate differences in level of expression. Retinal gRgr mRNA predominated in the INL (Fig. 4E, open arrow) and the ganglion cell layer (GCL; Fig. 4E, filled arrow), whereas retinal gRrb mRNA expression was observed primarily in the GCL (Fig. 4G, filled arrow). In both cases, expression in the GCL appeared to reside within the retinal ganglion cells themselves, whereas expression in the INL could be of either glial (Müller cells) or neural (e.g., amacrine or bipolar) origin. Little, if any expression was seen in the sense control for each gene (Figs. 4F, 4H). We could not exclude expression in the RPE, because digoxigenin precipitate would be obscured by the pigment, and background hybridization with radioactive probes was very high.

Expression of gRrb and gRgr mRNA was rhythmic in the pineal gland (Figs. 3, 5).ISH of both gRrb (Fig. 3A) and gRgr (Fig. 3B) revealed optical densities that were higher during the day than during the night. Northern blot analysis of pineal tissues confirmed and extended this observation, using RNA prepared from pineal gland tissues collected across 1 day of LD and 1 day of dark-dark (DD; ZT 2–ZT 22, CT 2–CT 22). In the pineal gland, expression of gRrb mRNA oscillated on a 24-hour basis in an LD and DD cycle, so that gRrb mRNA exhibited high levels during the light phase of the day and reduced mRNA abundance in the nighttime (Fig. 5). gRgr mRNA in the pineal gland also oscillated on a 24-hour basis in an LD and DD cycle, so that gRgr exhibited high levels during the light phase of the day similar to those of gRrb (Fig. 5). In addition, Northern blot analysis of retina also showed a rhythm of gRrb and gRgr mRNA, which oscillated on a 24-hour basis in LD and DD, similar to the pattern visualized in the pineal gland. Each gene exhibited high levels during the light phase of the day and reduced mRNA levels at late night (Fig. 6).
produces melatonin, astrocytes, and during daytime and decreased in the night. (E) Rhythm in gRrh mRNA, that persisted in LD and DD in chicken pineal gland. Levels were high during daytime and decreased in the night. The 18S ribosomal RNA was used to normalize blots. Data shown are the average of two blots. (□) Lights-on period; (■) lights-off period with shaded area indicating continuous darkness.

**DISCUSSION**

Nearly all organisms ranging from bacteria to humans possess an endogenous circadian clock that permits the organism to regulate its internal physiology to the surrounding environment temporally. In mammals, the biological clock that controls behavioral, physiological, and biochemical rhythms resides in the suprachiasmatic nucleus (SCN). The localization of a master pacemaker to a single structure, as seen in mammals, varies, depending on the species in question. For example, in birds it comprises a complex system of multiple oscillators coupled together. These oscillators are located in the ocular retina, the pineal gland, and the avian homologue of the mammalian SCN. Previous studies have shown that all three are critical components of the birds’ biological clock, wherein surgical removal of the pineal gland, retina, or SCN disrupts and/or abolishes circadian rhythms in several species of birds. In addition, the avian pineal gland and retina both contain a circadian oscillator and pacemaker to drive circadian rhythms in the biosynthesis of the indoleamine hormone melatonin and photoreceptors to synchronize that rhythm to environmental lighting. This hormone serves a vast array of physiological functions, is under direct regulation of the circadian clock, and is an important circadian marker of the system.

The avian pineal gland is a photosensitive structure but lacks both traditional rod- and cone-type photoreceptor cells and a pigmented epithelium. It is capable, however, of acutely responding to light by suppressing biosynthesis of the hormone melatonin, entrainment of its endogenously generated circadian rhythm of melatonin biosynthesis to an LD cycle in vivo and in vitro and phase shifting that rhythm to pulses of light in vitro. The chick pineal gland contains essentially three cell types, the photoreceptive PIN, which produces melatonin, astrocytes, and “interstitial” cells, which are predominantly B-lymphocytes and endothelial cells. There is no pigmented epithelium and no cells corresponding to Müller cells that could be responsible for visual cycle regeneration of photopigment. The phase-shifting effects of light by the chick pineal gland, but not the acute effects, are remarkably resistant to vitamin A deprivation, suggesting that the two effects of light are mediated by at least two different photopigments.

The understanding of the molecular elements that regulate the generation of circadian rhythm have advanced significantly in recent years. The molecular components that these clocks comprise have been identified in diverse animal species ranging from *D. melanogaster* to several species of mammals. For example, in *Drosophila*, pacemaker cells in the brain, retinas, and other tissues express rhythmic patterns of transcription and translation of “positive elements” comprising the gene products of clock (CLK) and cycle (CYC; homologous to bmal1), which dimerize to activate the transcription of the “negative elements” period (PER) and timeless (TIM), which in turn are translated, dimerize themselves, and feedback to inhibit their own transcription by interfering with the CLK/bmal1 activation. In mammals, this autoregulatory loop is believed to be very similar and to be entrained to LD cycles through the action of both opsin-based photopigments, including melanopsin and, perhaps, the flavin-based blue-light photopigment cryptochrome (CRY). This mechanism by which pineal and retinal photopigments transduce photic information to these molecular clockworks are not fully understood.

Presuming that opsin-based photopigments, whether they are melanopsin, pinopsin, or other pineal specific photopigments, mediate photoentrainment of circadian rhythms and/or regulation of melatonin biosynthesis, the identity of molecules responsible for regeneration of the 11-cis opsin chromophores have not been identified. We believe the presence of two putative photosomeras within pineal tissues and in retinal layers associated with biological clock function provides two candidates for such a function. Both gRgr and gRrh are expressed within the pineal parenchyma, where melatonin biosynthesis is known to occur, and in the INL and RGL of the ocular retina, where circadian photoreception is also present. Both of these putative photosomerases are also expressed throughout the brain. Because it is known that, like most nonmammalian vertebrates, birds possess intracranial photoreceptors that are sufficient for circadian entrainment and photoperiodic time measurement, it is tempting to suggest that these photosomerases may mediate regeneration of extracellular opsin involved in these processes. Conversely, the presence of a DRY motif on the third intracellular loop of each projected protein (Fig. 1A) suggests

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933435/)  
**Figure 5.** Circadian expression of gRrh and gRgr message in the pineal gland. (C) Rhythm in gRrh mRNA under LD conditions that persisted in DD conditions in chicken pineal gland. Levels were high during daytime and decreased in the night. (□) Lights-on period; (■) lights-off period with shaded area indicating continuous darkness.

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933435/)  
**Figure 6.** Circadian expression of gRrh and gRgr message in the pineal gland. All symbols and other designations are as in Figure 5. As in pineal gland, levels were high during the day and decreased at night.
that a second-messenger system may be associated with these two proteins. Whether these motifs function as part of an as yet unidentified second-messenger system involved in chromophore regeneration or a heretofore unknown signaling function for these opsins is at this point unknown. Clearly, further work is needed to delineate the functions of these two molecules in the visual cycles of the circadian clock.

Perhaps, the most interesting feature of the present study is the incomplete overlap of distribution of gRgr and gRrh with the several putative sensory photopigments that have been proposed for phototrafficking of rhodopsin processes in the pineal gland, retina, and brain. For example, although both gRgr and gRrh are present in the pineal gland, they do not directly colocalize with cells known either to produce melatonin or to contain either iodopsin or pinopsin, reputed pineal photosensory photopigments. Both AANAT and the two photopigments are localized exclusively within follicular PINs surrounding the follicular lumens. Conversely, both gRgr and gRrh are localized in the RGCs, which have been shown to express melanosin in mammals, teleost fish, and chicks, suggesting that visual cycle processes may reside in the RGCs themselves. Even so, it is important to point out that colocalization of photosensory photopigments and photoisomersases is not necessary for visual cycle function, because neither gRgr nor gRrh is present in the photoreceptor layers of chicks (Fig. 4) or the several mammalian retinas in which they have been studied. More surprising is the brain expression of both of these mRNA signals, which are generally expressed above sense background. We do not know which cell types express these mRNA species in vivo, but preliminary data in our laboratory strongly suggest that astrocytes express these and other opsin photopigments in vitro. Perhaps, extraocular photosensitivity in avian brain is more pervasive than has been appreciated.

References


**ERRATUM**

Erratum in: In “Assessment of Retinal Structure and Function in Ames Waltzer Mice” by Ball et al. (Invest Ophthalmol Vis Sci. 2003;44:3986–3992, the authors neglected to acknowledge the support received from Research to Prevent Blindness in the publication of their paper.