Rhythmic Regulation of Photoreceptor and RPE Genes Important for Vision and Genetically Associated With Severe Retinal Diseases

Patrick Vancura,1 Erika Csicsely,1 Annalisa Leiser,1 P. Michael Iuvone,2 and Rainer Spessert1
1Institute of Functional and Clinical Anatomy, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany
2Department of Ophthalmology, Emory University School of Medicine, Atlanta, Georgia, United States

Correspondence: Rainer Spessert, Department of Functional and Clinical Anatomy, University Medical Center of the Johannes Gutenberg University Mainz, Saarstrasse 19-21, Mainz 55099, Germany; spessert@uni-mainz.de.
Submitted: April 13, 2018
Accepted: June 11, 2018
Citation: Vancura P, Csicsely E, Leiser A, Iuvone PM, Spessert R. Rhythmic regulation of photoreceptor and RPE genes important for vision and genetically associated with severe retinal diseases. Invest Ophthalmol Vis Sci. 2018;59:3789–3799. https://doi.org/10.1167/iovs.18-24558

PURPOSE. The aim of the present study was to identify candidate genes for mediating daily adjustment of vision.

METHODS. Genes important for vision and genetically associated with severe retinal diseases were tested for 24-hour rhythms in transcript levels in neuronal retina, microdissected photoreceptors, photoreceptor-related pinealocytes, and retinal pigment epithelium-choroid (RPE-choroid) complex by using quantitative PCR.

RESULTS. Photoreceptors of wildtype mice display circadian clock-dependent regulation of visual arrestins (Arr1, Arr4) and the visual cycle gene Rdh12, whereas cells of the RPE-choroid exhibit light-dependent regulation of the visual cycle key genes Lrat, Rpe65, and Rdb5. Clock-driven rhythmicity of Arr1, Arr4, and Rdb12 was observed also in rat pinealocytes, to persist in a mouse model of diabetic retinopathy (db/db) and, in the case of Arr1, to be abolished in retinase of mice deficient for dopamine D4 receptors. Therefore, the expression rhythms appear to be evolutionary conserved, to be unaffected in diabetic retinopathy, and, for Arr1, to require dopamine signaling via dopamine D4 receptors.

CONCLUSIONS. The data of the present study suggest that daily adjustment of retinal function combines clock-dependent regulation of genes responsible for phototransduction termination (Arr1, Arr4) and detoxification (Rdb12) in photoreceptors with light-dependent regulation of genes responsible for retinoid recycling (Lrat, Rpe65, and Rdb5) in RPE. Furthermore, they indicate circadian and light-dependent regulation of genes genetically associated with severe retinal diseases.

Keywords: circadian regulation, visual cycle, retina, visual arrestin

The mammalian retina has the ability to adjust its function to the marked daily changes in the environmental lighting conditions.1,2 This involves the adjustment of photoreception and visual processing3 that manifests in circadian changes in the retinal electrical responses to light, which can be measured using the ERG.4 Daily regulation of the retina might also be important to comply with daily changes in the occurrence of toxic light (elevated during the day) and oxidative stress (elevated at night). Accordingly, circadian output has been seen to affect photoreceptor viability and to promote ganglion cell survival during aging conditions.5

Daily adaptation of retina and photoreceptors is partly driven by the retina’s own circadian clock system,5,6 where circadian clocks are localized in various types of retinal neurons, including photoreceptors.7–9 The circadian regulation of visual function involves the neurohormones melatonin acting on MT1 and MT2 receptors,10,11 and dopamine acting on D4 receptors12–15 as biochemical transducers of night and day, respectively.

Vision in all vertebrates is initiated by the absorption of photons by the photoreceptive pigments rhodopsin (in rods) and opsins (in cones) located in the photoreceptor outer segments.16 In general, photons induce the conversion of the photopigment’s covalently bound 11-cis retinal to all-trans-retinal, inducing a structural switch that activates the photopigment. The reactions responsible for inactivation of photoexcited photopigment include phosphorylation of the photopigment by rhodopsin kinase followed by the binding of arrestin1 (Arr1) in rods and probably arrestin4 (Arr4, also referred to as cone arrestin) in cones.17–21

The maintenance of vision requires the regeneration of 11-cis retinal.22,23 To meet this requirement, all-trans-retinal is reisomerized to 11-cis retinal, a process that is performed by a multistep enzyme pathway called the visual (retinoid) cycle. The first catalytic step of the visual cycle, namely the reduction of all-trans-retinol to all-trans-retinol takes place in photoreceptors and is performed by five isoforms of the enzyme retinoid dehydrogenase/reductase (RDH)24 that are Rdb8 (prRdb), Rdb11, Rdb13, Rdb14, and Dbrs3 (retSdr1). Detoxification of retinaldehydes that exceed the reductive capacity of the outer segment compartment of the visual cycle is conducted by another isoform of Rdb, Rdb12, and a retinal-specific ABC transporter (Abca4).25,26

The remaining steps of the visual cycle are performed in cells of the adjacent retinal pigment epithelium (RPE) that are (1) the esterification of all-trans-retinol to all-trans-retinyl esters...
by Lrat, (2) the hydrolyzation and isomerization of all-trans-retinyl esters to 11-cis retinol by Rpe65, and (3) the oxidation of 11-cis retinol to 11-cis retinal by the Rdb isofoms Rdb5, Rdb10, and Rdb11. Additional key players of the visual cycle are Rlbp1 (also referred to as Crabr1) and Rbp4 (also referred to as Crb1), which catalyze the transport of 11-cis retinal and retinol within the RPE compartment.26 Due to the compartmentalization of the visual cycle into different cell types, retinoid intermediates have to translocate across the interphotoreceptor matrix, a process mediated by the interphotoreceptor retinoid-binding protein (encoded by the gene Rbp3), which is synthesized and secreted by photoreceptors.

Daily adjustment of vision involves 24-hour changes in the expression of Arr1 and Kmen2, a channel essential for vision.27–30 The data included in the present study suggest that it also involves rhythmic regulation of cone Arr1 and key genes of the visual cycle in photoreceptors and RPE.

**MATERIALS AND METHODS**

**Animals**

Adult male and female mice (see below) and rats (Sprague Dawley) with intact photoreceptors not carrying rd mutations were used in this study. With the exception of the mouse model for diabetic retinopathy (C57BL/6j db/db, C57BL/6j b/db/db), the mice used were melatonin-proficient (C3H/f d/db, C3H/f d/db/db) and nondiabetic (C3H/f d/db, C3H/f d/db/db). Where indicated, mice deficient for dopamine D₁ receptors (Drd1−/-) were used. Mice were genotyped by PCR analysis of genomic DNA. Diabetic (db/db) and nondiabetic (d/db) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). They were checked for body-weight and blood glucose level by tail vein sampling using Accu-Check Aviva reagent strips (Roche Diagnostics, Mannheim, Germany) at the age of 10 weeks. Diabetic mice displayed enhanced values of blood glucose (397 mg/dl) and bodyweight (46 ± 3 g) as compared with nondiabetic mice (blood glucose level, 138 ± 4 mg/dl; bodyweight, 25 ± 1 g). Animals were kept under light:dark (LD) 12:12 cycles for 3 weeks under standard laboratory conditions (illumination with 200 lux white light at cage level during the day and dim [<5 lux] red light during the night, 20°C ± 1°C, water and food ad libitum) and sacrificed at the age of 13 to 15 weeks at 3-hour intervals over a period of 24 hours by decapitation following anesthesia with carbon dioxide. In order to determine the putative clock-dependent regulation of genes, mice previously adapted to LD were housed in constant darkness (DD) for one cycle and sacrificed during the next cycle in DD.

Animal experimentation was carried out in accordance with the National Institutes of Health Guide on the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic Vision Research and approved by the Institutional Animal Care and Use Committees of Morehouse School of Medicine, Emory University, and the European Communities Council Directive (86/609/EEC).

**Retina and RPE-Choroid Sampling**

Eyes of mice were enucleated and, after removal of the optic nerve and connective tissue, were incised around the dentate border of the retina (ora serrata). After discarding the lens and vitreous, the neural retinas and adjacent RPE-choroidal complexes were carefully separated and immediately frozen or, in the case of retinas, processed for laser microdissection and pressure catapulting (LMPC). The purity of the preparations obtained were verified by using specific gene markers of the RPE, namely Lrat and Rdb5, and the retina, namely Arr1 and Rdb12. In comparison to retina, enrichment of Lrat and Rdb5 was more than 150-fold in the RPE-choroid complex. In comparison to that of the RPE-choroid complex, enrichment of Arr1 and Rdb12 was more than 100-fold in retina. All dissections during the dark phase were done under dim red light. In order to prepare the neural retinas for LMPC, the HEPES-glutamic acid buffer-mediated organic solvent protection effect (HOPE) technique (DCS, Hamburg, Germany) was applied for fixation. In this procedure, fresh retinas were fixed in HOPE I (DCS) at 0°C to 4°C for 48 hours. Subsequently, dehydration of the retinas was performed with a mixture of HOPE II solution (DCS) and acetone for 2 hours at 0°C to 4°C, followed by dehydration in pure acetone at 0°C to 4°C (repeated twice). Tissues were then embedded with low-melting point paraffin (Tm, 52°C–54°C) and sectioned (10 μm) on membrane-mounted slides (DNase/RNase free PALM MembraneSlides; P.A.L.M. Microlaser Technologies GmbH, Bernried, Germany). Subsequently sections were deparaffinized with isopropanol (2 × 10 minutes each at 60°C), stained using cresyl violet (1% w/v cresyl violet acetate in 100% ethanol), briefly washed in 70% and 100% ethanol, and then air-dried.

**Isolation of Photoreceptor Cells**

To isolate photoreceptors (rod and cones) from the stained sections in a contact and contamination-free manner, LMPC was performed with a PALM MicroBeam system (Zeiss Imaging, Munich, Germany) running PALM RoboSoftware (P.A.L.M. Microlaser Technologies GmbH) as described previously.8 In brief, these cells were selected, cut and catapulted into the caps of 0.5-ml microfuge tubes with an adhesive filling (PALM AdhesiveCaps; P.A.L.M. Microlaser Technologies GmbH) by using a pulsed UV-A nitrogen laser under the 10% objective. To reach total average sample sizes of 4,000,000 pm² per tube, smaller areas of the sections were pooled. The purity of the preparations obtained were verified by using specific gene markers of photoreceptors, namely neural retina leucine zipper (Nrl) as a marker for rods,81 as well as of inner retinal neurons, namely tyrosine hydroxylase (Th) as a marker for amacrine cells.82 In comparison with whole retina preparations, in photoreceptors collected by LMPC, the ratio of Nrl to Th was increased 84-fold.

**RNA Extraction, Reverse Transcription (RT), and Quantitative PCR (qPCR)**

Using the RNeasy Micro kit (Qiagen, Hilden, Germany), RNA was extracted from the tissue samples as described.53 The amount of extracted RNA was determined by measuring the optical density at 260 and 280 nm. Subsequently, single-stranded cDNA was synthesized by using the Verso cDNA Kit (Abgene, Hamburg, Germany) following the manufacturer’s instructions. Briefly, 4 μl RNA solution was reverse transcribed using anchored oligo-dT primers in a final volume of 20 μl. Following dilution of the obtained cDNA sample in RNase-free water (1:4), qPCR, with aliquots of 5 μl being used, was performed. PCR amplification and quantification were carried out in duplicate using an iCycler (BioRad, Munich, Germany) according to the following protocol: denaturation for 30 seconds at 95°C, followed by 45 cycles of 5 seconds at 95°C and 30 seconds at 60°C. By using agarose gel electrophoresis, the generated amplicons for all genes under examination were shown to possess the predicted sizes (Table 1). The amount of mRNA in the samples was calculated from the measured threshold cycles (Ct) by using an internal standard curve with 10-fold serial dilutions (101–108 copies/μl). Expression levels of each transcript were normalized with respect to the amount of Gapdh mRNA and 18S rRNA present.
| Gene | GenBank Accession Number | Primer Sequence 5’ to 3’ | PCR Product, bp |
|------|--------------------------|--------------------------|-----------------|
| m18S | NR_003278.3              | Forward: CAACACGGGAAACCTCAC  
Reverse: TGGGTCACCAACAAATAGAAC | 110  |
| mAbca4 | NM_007378.1            | Forward: TCGAGAGGGTGAGGTCCAGTG  
Reverse: GATGGGAGGCGAGAAAGTG | 118  |
| mArr1 | NM_009118.2            | Forward: TGGTACGCACTTCAGCTG  
Reverse: ATTAAGGAGGGCATCGGAC | 116  |
| mArr4 | NM_133205.3            | Forward: ACCAATCTGGGAGGTACCTG  
Reverse: GCTCAACACCAACATCAC | 148  |
| mArrb1 | NM_177231.2           | Forward: CTGATACCAAGCCCTTTAGG  
Reverse: GTCCTGAGGAGGCTGAAAC | 103  |
| mArrb2 | NM_001271358.1         | Forward: GGGAAGGGCAAGCTGAAG  
Reverse: CGGAGATGTCCTGCTGAGTAG | 111  |
| mDhp | NM_016974.3            | Forward: GAGGTGCTAATGACCTTTG  
Reverse: GGCCTGTCACCAACAAATAG | 146  |
| mDhrs3 | NM_011303.6            | Forward: CCAGAAGCTGGTGAGGTACCTG  
Reverse: GAGCTGTCCTGCTGCTGAGTAG | 106  |
| mE4bp4 | NM_017373.3           | Forward: CTGGGAACCTGGCTTTTG  
Reverse: CTGGGAACCTGGCTTTTG | 144  |
| mGapdh | BC082592.1            | Forward: CATCCACAGCTGACATAC  
Reverse: TCGCTGAGGAGGCTGAAAC | 144  |
| mLrat | NM_023624.4            | Forward: GATTGGGAGGGCTGAGAGG  
Reverse: GCATCGCTTCAGCTGACAC | 146  |
| mNrl | NM_008736.3            | Forward: GCGGATTTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 92  |
| mPer1 | NM_011065.5            | Forward: GAGGTTCCAGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 120  |
| mPer2 | NM_011066.3            | Forward: GATGGGACATTCAAACAAATAG  
Reverse: GCGAAGCAGGCAGGAGGAAGG | 100  |
| mPer3 | NM_011067.2            | Forward: GCGGCCCTACAGCTGACAAAG  
Reverse: GCCGACATTGCTGCTGCTGCTGAGTAG | 145  |
| mRbp1 | NM_011254.5            | Forward: GAGCTGTCAGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 104  |
| mRbp3 | NM_015745.2            | Forward: GAGCTGTCAGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 125  |
| mRdb5 | NM_134006.4            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 89  |
| mRdb8 | NM_001030290.1         | Forward: GAGGCTGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 137  |
| mRdb10 | NM_133832.3           | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 110  |
| mRdb11 | NM_021557.5            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 132  |
| mRdb12 | NM_030017.4            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 104  |
| mRdb13 | NM_175372.4            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 122  |
| mRdb14 | NM_025697.2            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 127  |
| mRlbp1 | NM_020599.2            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 114  |
| mRlbp3 | NM_009377.1            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 129  |
| mLrs1 | NR_046237.1            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 136  |
| mLrs1 | NM_013025.2            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 137  |
| mArri4 | NM_001190993.1         | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 148  |
| mE4bp4 | NM_053727.2            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 107  |
| mGdpdb | NM_017008.4            | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 89  |
| mRdb12 | NM_001108037.1         | Forward: GAGGCTGCTGCTGCTGCTGAGTAG  
Reverse: GGGAGAAGCTGCTGCTGCTGAGTAG | 139  |
Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM) of four qPCR experiments from four independent tissue samples. Transcript levels were calculated relative to average expression of each dataset throughout 24 hours to plot temporal expression. Cosinor analysis was used to evaluate variations among the groups in the 24-hour profile and to fit sine-wave curves to the circadian data to mathematically estimate the time of peaking gene expression (acrophase) and to assess the amplitude. The model can be expressed according to the following equation: f(t) = A + B cos [2π (t + C)/T], with the f(t) indicating relative expression levels of target genes, t specifying the time of sampling (hours after light-on), A representing the mean value of the cosine curve (mesor; midline estimating statistic of rhythm), B indicating the amplitude of the curve (half of the sinusoid), and C indicating the acrophase (point of time, when the function f[t] is maximum). T gives the time of the period, which was fixed at 24 hours for this experimental setting. Significance of daily regulation was defined by showing a P < 0.05.

RESULTS

Visual Arrestins and Rdh12 are Under Circadian Regulation in Neuronal Retina and Photoreceptors

To investigate whether the neural retina (devoid of the RPE-choroid) and photoreceptors of mice display daily regulation of genes important for visual processing, 24-hour profiling of the mRNA levels of visual arrestins and enzymes of the photoreceptor compartment of the visual cycle was performed. Among the genes tested, Arr1, Arr4, and Rdh12 were seen to display significant daily rhythms in neural retina and photoreceptors of mouse (Fig. 1, blue and red lines; for statistical analysis, see Table 2). Peak expression occurred for Arr1 in retina at Zeitgeber time (ZT) 8.2 and in photoreceptors at ZT7.7, for Arr4 in retina at ZT12.5 and in photoreceptors at ZT11.4, and for Rdh12 in retina at ZT9.4 and in photoreceptors at ZT7.8. No daily periodicity was observed for the visual cycle genes Rdh8, Rdh11, Rdh13, Dbrs3, Abca4, and Rbp3.

The daily rhythm of Arr1, Arr4, and Rdh12 may be driven by a true circadian clock or light/dark transitions. To test circadian regulation, 24-hour profiling of the genes was performed in mice kept for one cycle under constant darkness (Fig. 1, black lines; for statistical analysis, see Table 2). Consistent with the concept that daily rhythmicity of the genes is promoted by a true circadian clock, daily changes of the genes under investigation persisted in the absence of light/dark transitions.

To test the validity of the experimental system used, the clock-controlled gene E4bp4 was recorded in the same transcriptomes as those used for analysis of the other genes. Consistent with the validity of the results obtained, E4bp4 transcript amount was observed to be rhythmic in retina and photoreceptors, under LD 12:12 and DD (Fig. 1; for statistical analysis, see Table 2).
Daily Regulation of Visual Genes

Visual Arrestins and Rdh12 Are Also Rhythmic in Rat Pineal Gland

Mammalian photoreceptors and pinealocytes phylogenetically and ontogenetically descend from a common ancestor cell type.\textsuperscript{36} To investigate whether rhythmicity of the genes is phylogenetically conserved, the 24-hour profiling of the genes was compared in retina and pinealocytes. Because the pineal gland is much larger in rat than in mice, rat tissue was used to obtain sufficient mRNA levels for this purpose. \textit{Arr1}, \textit{Arr4}, and \textit{Rdh12} were rhythmically expressed in rat pinealocytes with similar profiles as those observed in rat retina (Fig. 2, blue and red lines; for statistical analysis, see Table 2).

Consistent with the validity of the results obtained, the clock-controlled gene \textit{E4bp4} was observed to be rhythmic in the same transcriptomes as those used for profiling the other genes (Fig. 2, blue and red lines; for statistical analysis, see Table 2).

Expression of \textit{Arr1} Is Arrhythmic in Dopamine D\textsubscript{4} Receptor–Deficient Mice

In order to evaluate the contribution of dopamine to circadian regulation, 24-hour profiling of the genes was performed in mice deficient for the D\textsubscript{4} receptor (Fig. 3, blue versus red lines; for statistical analysis, see Table 2). Daily regulation of \textit{Arr1} was not observed in retina of \textit{Drd\textsubscript{4}}-deficient mice, but \textit{Arr4} and \textit{Rdh12} were rhythmically expressed. This suggests that circadian regulation of \textit{Arr1}, but not that of \textit{Arr4} and \textit{Rdh12}, requires dopamine signaling via D\textsubscript{4} receptors.

As expected for a clock-controlled gene, \textit{E4bp4} rhythmicity was evident in the same transcriptomes as those used for analysis of the other genes. Interestingly, \textit{E4bp4} rhythmicity mitigates in mice deficient for D\textsubscript{4} receptors (Fig. 3, blue versus red lines; for statistical analysis, see Table 2). Because \textit{E4bp4} transcription is known to be directed by clock gene products,\textsuperscript{37} abolished \textit{E4bp4} rhythmicity may mirror an influence of dopamine signaling on the retinal clock function.

Circadian Regulation of Visual Arrestins and Rdh12 Expression Persists in Diabetic Retinopathy

In diabetic retinopathy, visual function is impaired. To investigate whether disturbed circadian control of \textit{Arr1}, \textit{Arr4}, and \textit{Rdh12} plays a role in this context, the \textit{db/db} mouse, a worldwide adopted model of type II diabetes and diabetic retinopathy,\textsuperscript{38} was used. Irrespective of the melatonin deficiency of the \textit{db/db} mouse (C57BL/6J\textsubscript{b} background), the nondiabetic phenotype (\textit{db/db}) was used to display daily rhythms in \textit{Arr1}, \textit{Arr4}, and \textit{Rdh12} mRNA levels (Fig. 4, blue lines; for statistical analysis, see Table 2). This suggests that circadian regulation does not require melatonin signaling. Moreover, rhythmicity of the genes persisted in diabetic (\textit{db/db}) mice (Fig. 4, blue versus red lines; for statistical analysis, see Table 2). Therefore, circadian regulation of \textit{Arr1}, \textit{Arr4}, and \textit{Rdh12} appears not to be affected in diabetic retinopathy.

Consistent with the validity of the experimental system used, \textit{E4bp4} was observed to be rhythmic in the nondiabetic phenotype (\textit{db/db}). The daily profile in the \textit{E4bp4} transcript was statistically arrhythmic in diabetic (\textit{db/db}) mice, although it resembled that in nondiabetic (\textit{db/db}) mice (Fig. 4, blue versus red lines; for statistical analysis, see Table 2).

Visual Cycle Genes Are Under Daily Regulation in the RPE-Choroid

Because the different steps of the visual retinoid cycle are diversified on photoreceptors and the adjacent RPE, and the
visual cycle gene *Rdh12* was seen to be rhythmic in mouse photoreceptors, possible daily fluctuations in the expression of genes of the RPE part of the visual cycle were investigated in preparations of the RPE-choroid obtained from mice. Among the genes tested, *Lrat, Rpe65*, and *Rdh5* were seen to undergo daily rhythms with peaks at the early daytime (*Lrat*, ZT4.2; *Rpe65*, ZT1.3; *Rdh5*, ZT1.9) (Fig. 5, blue lines; for statistical analysis, see Table 3). No daily periodicity was evident for the visual cycle genes *Rbp1, Rdh10, Rdh11*, and *Rlbp1*.

To check clock-dependent regulation of *Lrat, Rpe65*, and *Rdh5*, 24-hour profiling of the genes was performed in mice kept for one cycle under DD (Fig. 5, black lines; for statistical analysis, see Table 3). Consistent with the concept that daily rhythmicity of *Lrat, Rpe65*, and *Rdh5* expression does not depend on a true circadian clock but requires LD transitions, daily periodicity was evident for the visual cycle genes *Rbp1, Rdb10, Rdb11*, and *Rlhbp1*.

Consistent with the presence of a true circadian clock in RPE-choroid and the validity of the experimental system used, the clock-controlled gene *E4bp4* was seen to display periodicity not only under LD 12:12 but also under DD (Fig. 5, blue versus black lines; for statistical analysis, see Table 3).

**DISCUSSION**

In the present study, the genes *Arr1, Arr4, Lrat, Rdb5, Rpe65*, and *Rdh5* were observed to display daily rhythms in either photoreceptors (*Arr1, Arr4, and Rdb12*) or RPE-choroid (*Lrat, Rpe65*, and *Rdh5*). Since they encode indispensable components of either the phototransduction pathway (*Arr1, Arr4*), or the visual retinoid cycle (*Rdh12, Lrat, Rpe65*, and *Rdh5*), two processes essential to vision, daily regulation appears to contribute to daily adjustment of vision to comply with 24-hour changes in lighting conditions. Remarkably, rhythmic regulation of the photoreceptor genes (*Arr1, Arr4*, and *Rdb12*) was seen to be driven by a circadian clock, whereas that of the RPE genes (*Lrat, Rpe65*, and *Rdh5*) was observed to depend on LD transitions. This suggests that daily

---

**TABLE 3.** Statistical Analysis of Transcriptional Profiling Illustrated in Figure 5

| Gene   | LD 12:12 | DD       |
|--------|----------|----------|
|        | P Value  | Acrophase, h | Amplitude, % | P Value | Acrophase, h | Amplitude, % | See Figure |
| Lrat   | <0.05    | 4.2       | 38.1         | >0.05   | -           | -          | 5          |
| Rdb5   | <0.05    | 1.9       | 42.8         | >0.05   | -           | -          | 5          |
| Rpe65  | <0.05    | 1.3       | 41.7         | >0.05   | -           | -          | 5          |
| E4bp4  | <0.05    | 21.1      | 35.7         | <0.05   | 21.1        | 67.1       | 5          |
adjustment of visual processing combines clock-driven gene regulation in photoreceptors with light-driven gene regulation in RPE. Circadian regulation of photoreceptor genes should derive from clocks located in photoreceptors, inner retinal neurons, and RPE-choroid but not from the master clock within the suprachiasmatic nucleus (SCN).2 Arr1 is abundant in rod and cone photoreceptors.18–21 Due to the limitation of the present study that photoreceptor transcript preparations derive from both rods and cones, Arr1 rhythms could reflect average rod and cone values and may not necessarily be valid for each type of photoreceptor. However, in the rod-dominant mouse retina, the observed 24-hour changes in Arr1 expression may mainly derive from rods.2

**Figure 3.** Daily rhythmicity of Arr1, Arr4, and Rdh12 in retina deficient for dopamine D4 receptors. Transcript levels of the genes were recorded in wildtype mice (blue lines) versus mice deficient for dopamine D4 receptors (red lines) in preparations of the whole retina under LD 12:12 by using qPCR. The mRNA levels are plotted as a function of ZT. The lines represent the periodic sinusoidal functions determined by cosinor analysis (solid and broken line for P < 0.05 and P > 0.05). Data represent a percentage of the average value of the transcript amount during the 24-hour period. Statistical analysis of transcriptional profiling is provided in Table 2. Note that rhythmicity of Arr1 and E4bp4 is not observable in mice deficient for dopamine D4 receptors. The value of ZT0 is plotted twice at both ZT0 and ZT24. The solid bars indicate the dark period. Each value represents mean ± SEM (n = 4; each n represents one animal [two retinas]).
daytime may be necessary to compensate for an Rpe65-dependent increase in retinaldehyde release.

The genes observed to be under daily regulation in the present study are important for maintaining vision and for protecting photoreceptors from cytotoxic byproducts of the visual pathways. Accordingly, mutations of these genes have been genetically linked to various forms of severe retinal diseases. Not only is Arr1 genetically associated to Oguchi disease\(^56,57\) and retinitis pigmentosa, \(^58\) but also are Lrat, Rpe65, and Rdh12 to Leber’s congenital amaurosis. \(^59\) Mutations of Rdh5 are associated with fundus albipunctatus. \(^60\) Moreover, mice deficient for Arr1, Arr4, Rpe65, and Rdh12 suffer from dystrophy of rods (Arr1, \(^61\) Rdh5, \(^62\) and Rdh12\(^52\)) and/or cones (Arr1, \(^19\) Arr4, \(^20,21\) Rpe65, \(^63\) and Rdh12\(^52\)). These findings indicate that the abundance of each of the gene products is a prerequisite for retinal health. Thus, correct upregulation of the genes during early (Lrat, Rpe65, Rdh5, and Rdh12) or late (Arr1 and Arr4) daytime might be essential for retinal health.

Rhythmicity of Arr1, Arr4, and Rdh12 persisted in the db/db mouse, a mouse model of diabetic retinopathy. Therefore, daily regulation of these genes might also be unaffected in diabetic retinopathy of humans, one of the most common causes of blindness in Europe and United States.\(^64\) Accordingly, the pathogenesis of diabetic retinopathy appears not to derive from disturbed circadian regulation of visual arrestins or Rdh12.

Circadian regulation of Arr1 appears to be mediated by dopamine signaling via D4 receptors. This follows from the present observation that Arr1 periodicity is disrupted in retinas deficient of functional D4 receptors. D4 receptor-dependent control of photoreceptors\(^12\) appears to derive from the clock-driven release of dopamine from amacrine cells in the inner retina and/or from circadian expression of Drd4, the gene that encodes the dopamine D4 receptor.\(^13\) Therefore, circadian regulation of Arr1 in photoreceptors may be promoted by a molecular clock located within amacrine cells and photoreceptor cells.

Daily regulation of Arr1, Arr4, and Rdh12 was seen in the present study to also occur in rat pineal gland, a neuroendocrine transducer of the circadian system.\(^27,65\) In mammalian pineal gland, rhythmicity of gene expression is driven by the master clock in the SCN.\(^66\) This suggests that regulation of the visual genes is circadian in retina and pineal gland but depends on different clocks, viz. the intraretinal clock system and the master clock in the SCN. Mammalian photoreceptors and pinealocytes phylogenetically and ontogenetically descend from a common ancestor cell type even if pinealocytes have lost direct photoreception and endogenous clock function during evolution.\(^67\) Therefore, the circadian regulation of the visual genes under investigation appears to be evolutionary conserved.

In conclusion, the data of the present study suggest that genes important for phototransduction shutoff and retinoid renewal are not only important for maintaining vision, but also for mediating adjustment of vision to comply with 24-hour changes in lighting conditions. As a consequence, mutations of the respective genes might impair daily adjustment of the retina and this deficiency might contribute to the pathogenesis of the respective gene associated retinal disorders. Moreover,
circadian regulation of Rdh12 may adjust the detoxification capacity of photoreceptors to changing amounts of cytotoxic byproducts of visual pathways. Therefore, Rdh12 is a candidate gene for mediating the positive influence of the retinal clock on photoreceptor survival.68

Acknowledgments

The authors thank Ute Frederiksen and Kristina Schäfer for their excellent technical assistance, Susanne Rometsch and Bettina Wiechers-Schmied for secretarial help, and Russell G. Foster for providing us with C3H/þ/þ (rd++) mice. The data contained in this study are included in the theses of Patrick Vancura and Annalisa Leiser as a partial fulfillment of their doctoral degree and of Erika Csicsely as a partial fulfillment of her bachelor's degree at the Johannes Gutenberg University, Mainz, Germany.

Supported in part by grants from the National Institutes of Health (R01EY004864, R01EY027711, and P50EY006360 to PMI) and by an unrestricted departmental grant from Research to Prevent Blindness to Emory Ophthalmology.

Disclosure: P. Vancura, None; E. Csicsely, None; A. Leiser, None; P.M. Iuvone, None; R. Spessert, None

References

1. Iuvone PM, Tosini G, Pozdeyev N, Haque R, Klein DC, Chaurasia SS. Circadian clocks, clock networks, arylalkylamine N-acetyltransferase, and melatonin in the retina. Prog Retin Eye Res. 2005;24:433–456.

2. McMahon DG, Iuvone PM, Tosini G. Circadian organization of the mammalian retina: from gene regulation to physiology and diseases. Prog Retin Eye Res. 2014;39:58–76.

3. Storch KF, Paz C, Signorovitch J, et al. Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information. Cell. 2007;130:750–741.

4. Cameron MA, Barnard AR, Hut RA, et al. Electroretinography of wild-type and Cry mutant mice reveals circadian tuning of photopic and mesopic retinal responses. J Biol Rhythms. 2008;23:489–501.

5. Tosini G, Davidson AJ, Fukuhara C, Kasamatsu M, Castanon-Cervantes O. Localization of a circadian clock in mammalian photoreceptors. FASEB J. 2007;21:3866–3871.

6. Schneider K, Tippmann S, Spiwoks-Becker I, et al. Unique clockwork in photoreceptor of rat. J Neurochem. 2010;115:585–594.

7. Baba K, Pozdeyev N, Mazzoni F, et al. Melatonin modulates visual function and cell viability in the mouse retina via the MT1 melatonin receptor. Proc Natl Acad Sci U S A. 2009;106:15043–15048.
11. Sengupta A, Baba K, Mazzoni F, et al. Localization of melatonin receptor 1 in mouse retina and its role in the circadian regulation of the electroretinogram and dopamine levels. *PLoS One.* 2011;6:e24485.

12. Pozdnev N, Tosini G, Li L, et al. Dopamine modulates diurnal and circadian rhythms of protein phosphorylation in photoreceptor cells of mouse retina. *Eur J Neurosci.* 2008;27:2091-2700.

13. Jackson CR, Chaurasia SS, Hwang CK, Iuvone PM. Dopamine D(4) receptor activation controls circadian timing of the adenylyl cyclase 1/cyclic AMP signaling system in mouse retina. *Eur J Neurosci.* 2011;34:57-64.

14. Jackson CR, Ruan GX, Aseem F, et al. Retinal dopamine mediates multiple dimensions of light-adapted vision. *J Neurosci.* 2012;32:9359-9368.

15. Hwang CK, Chaurasia SS, Jackson CR, Chan GC, Storm DR, Iuvone PM. Circadian rhythm of contrast sensitivity is regulated by a dopamine-neuronal PAS-domain protein 2-adenyl cyclase 1 signaling pathway in retinal ganglion cells. *J Neurosci.* 2013;33:14989-14997.

16. Ingram NT, Sampath AP, Fain GL. Why are rods more sensitive than cones? *J Physiol.* 2016;594:5415-5426.

17. Chan S, Rubin WW, Mendez A, et al. Functional comparisons of visual arrestins in rod photoreceptors of transgenic mice. *Invest Ophtalmol Vis Sci.* 2007;48:1968-1975.

18. Nikonov SS, Brown BM, Davis JA, et al. Mouse cones require an arrestin for normal inactivation of phototransduction. *Neuron.* 2008;59:462-474.

19. Brown BM, Ramirez T, Rife L, Craft CM. Visual arrestin 1 contributes to cone photoreceptor survival and light adaptation. *Invest Ophtalmol Vis Sci.* 2010;51:2372-2380.

20. Deming JD, Pak JS, Brown BM, et al. Visual cone arrestin 4 contributes to visual function and cone health. *Invest Ophtalmol Vis Sci.* 2015;56:5407-5416.

21. Deming JD, Pak JS, Shin JA, et al. Arrestin 1 and cone arrestin 4 have unique roles in visual function in an all-cone mouse retina. *Invest Ophtalmol Vis Sci.* 2015;56:7618-7628.

22. Kiser PD, Golczak M, Maeda A, Palczewski K. Key enzymes of the retinoid (visual) cycle in vertebrate retina. *Biochim Biophys Acta.* 2012;1821:137-151.

23. Kiser PD, Palczewski K. Retinoids and retinal diseases. *Annu Rev Vis Sci.* 2016;2:197-234.

24. Parker RO, Crouch BK. The interphotoreceptor retinoid binding (IRBP) is essential for normal retinoid processing in vertebrate retina. *Rev Vis Sci.* 2008;4:613–618.

25. Mirshahi M, Thillaye B, Tarraf M, de Kozak Y, Faure JP. Light-induced changes in S-antigen (arrestin) localization in retinal photoreceptors: differences between rods and cones and defective process in RCS rat retinal dystrophy. *Eur J Cell Biol.* 1994;63:61-67.

26. Liu X, Li A, Brown B, Weiss ER, Osawa S, Craft CM. Mouse cone arrestin expression pattern: light induced translocation in cone photoreceptors. *Mol Vis.* 2002;8:462–471.

27. Zhang H, Cuenca N, Ivanova T, et al. Identification and light-dependent translocation of a cone-specific antigen, cone arrestin, recognized by monoclonal antibody 7G6. *Invest Ophtalmol Vis Sci.* 2003;44:2858–2867.

28. Haire SE, Pang J, Boye SL, et al. Light-driven cone arrestin translocation in cones of postnatal guanylate cyclase-1 knockout mouse retina treated with AAV-GC1. *Invest Ophtalmol Vis Sci.* 2006;47:3745–3753.

29. Alivisatos AP, Thompson DA, Koutalos Y. Reduction of all-trans-retinal in vertebrate rod photoreceptors requires the combined action of RDH8 and RDH12. *J Biol Chem.* 2012;287:24662–24670.
51. Maeda A, Golczak M, Maeda T, Palczewski K. Limited roles of Rdh8, Rdh12, and Abca4 in all-trans-retinal clearance in mouse retina. Invest Ophthalmol Vis Sci. 2009;50:5435–5443.

52. Maeda A, Maeda T, Imanishi Y, et al. Retinol dehydrogenase (RDH12) protects photoreceptors from light-induced degeneration in mice. J Biol Chem. 2006;281:37697–37704.

53. Mackay DS, Dev Borman A, Moradi P, et al. RDH12 retinopathy: novel mutations and phenotypic description. Mol Vis. 2011;17:2706–2716.

54. Maiti P, Kong J, Sparrow JR, Allikmets R, Rando RR. Small molecule RPE65 antagonists limit the visual cycle and prevent lipofuscin formation. Biochemistry. 2006;45:852–860.

55. Kiser PD, Zhang J, Badice M, et al. Rational tuning of visual cycle modulator pharmacodynamics. J Pharmacol Exp Ther. 2017;362:131–145.

56. Nakamachi Y, Nakamura M, Fujii S, Yamamoto M, Okubo K. Oguchi disease with sectoral retinitis pigmentosa harboring adenine deletion at position 1147 in the arrestin gene. Am J Ophthalmol. 1998;125:249–251.

57. Hayashi T, Tsuzuranuki S, Kozaki K, Urashima M, Tsuneoka H. Macular dysfunction in Oguchi disease with the frequent mutation 1147delA in the SAG gene. Ophthalmic Res. 2011;46:175–180.

58. Nakazawa M, Wada Y, Tamai M. Arrestin gene mutations in autosomal recessive retinitis pigmentosa. Arch Ophthalmol. 1998;116:498–501.

59. Chacon-Camacho OF, Zenteno JC. Review and update on the molecular basis of Leber congenital amaurosis. World J Clin Cases. 2015;3:112–124.

60. Skorczyk-Werner A, Pawlowski P, Michalczuk M, et al. Fundus albipunctatus: review of the literature and report of a novel RDH5 gene mutation affecting the invariant tyrosine (p.Tyr175Phe). J Appl Genet. 2015;56:317–327.

61. Song X, Seo J, Baumeur E, et al. Rapid degeneration of rod photoreceptors expressing self-association-deficient arrestin1 mutant. Cell Signal. 2013;25:2613–2624.

62. Mettrailler S, Schorderet DF, Cottet S. Early apoptosis of rod photoreceptors in Rpe65(-/-) mice is associated with the upregulated expression of lysosomal-mediated autophagic genes. Exp Eye Res. 2012;96:70–81.

63. Rohrer B, Lohr HR, Humphries P, Redmond TM, Seeliger MW, Crouch RK. Cone opsin mislocalization in Rpe65/- mice: a defect that can be corrected by 11-cis retinal. Invest Ophthalmol Vis Sci. 2005;46:3876–3882.

64. Stitt AW, Lois N, Medina RJ, Adamson P, Curtis TM. Advances in our understanding of diabetic retinopathy. Clin Sci (Lond). 2013;125:1–17.

65. Hastings MH, Maywood ES, Reddy AB. Two decades of circadian time. J Neuroendocrinol. 2008;20:812–819.

66. Bailey MJ, Coon SL, Carter DA, et al. Night/day changes in pineal expression of >600 genes: central role of adrenergic/cAMP signaling. J Biol Chem. 2009;284:7606–7622.

67. Maronde E, Stehle JH. The mammalian pineal gland: known facts, unknown facets. Trends Endocrinol Metab. 2007;18:142–149.

68. Ait-Hmyed O, Felder-Schmittbuhl MP, Garcia-Garrido M, et al. Mice lacking Period 1 and Period 2 circadian clock genes exhibit blue cone photoreceptor defects. Eur J Neurosci. 2013;37:1048–1060.