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; spessert@uni-mainz.de.

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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 Rdh5. Clock-driven rhythmicity of Arr1, Arr4, and Rdh12 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 retinae of mice deficient for dopamine D4 receptors. Therefore, the expression rhythms appear to be evolutionarily 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 (Rdh12) in photoreceptors with light-dependent regulation of genes responsible for retinoid recycling (Lrat, Rpe65, and Rdh5) 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
Daily Regulation of Visual Genes

by Lrat, (2) the hydrolysis 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 Rdl isofoms Rdb5, Rdb10, and Rdb11. Additional key players of the visual cycle are Rlpb1 (also referred to as Crlb2) and Rbph1 (also referred to as Crbp), which catalyze the transport of 11-cis retinal and retinol within the RPE compartment. 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 Kcnv2, a channel essential for vision. The data included in the present study suggest that it also involves rhythmic regulation of cone Arr4 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 melanaton-proficient (C3H/f þ Crbp), to as Rlbp1, which is synthesized and secreted by photoreceptors. Daily regulation of visual genes involves 24-hour changes in the expression of Arr1 and Kcnv2, a channel essential for vision.

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**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 MicroImaging, Munich, Germany) running PALM RoboSoftware (P.A.L.M. Microlaser Technologies GmbH) as described previously. 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 µm² 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 nuclear retinal leucine zipper (Nrl) as a marker for rods, as well as of inner retinal neurons, namely tyrosine hydroxylase (TH) as a marker for amacrine cells. 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. 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 (10¹⁰–10⁶ copies/µl). Expression levels of each transcript were normalized with respect to the amount of gapdh mRNA and 18S rRNA present.
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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: \( f(t) = A + B \cos \left[ \frac{2\pi (t + C)}{T} \right] \), with \( f(t) \) 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).
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. 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. Arr1, Arr4, and Rdh12 were rhythmically expressed in rat pinealocytes with similar profiles as those observed in retin (Fig. 2, blue and red lines; for statistical analysis, see Table 2).

Consistent with the validity of the results obtained, the clock-controlled gene 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 Arr1 Is Arrhythmic in Dopamine D4 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 D4 receptor (Fig. 3, blue versus red lines; for statistical analysis, see Table 2). Daily regulation of Arr1 was not observed in retina of Drd4-deficient mice, but Arr4 and Rdh12 were rhythmically expressed. This suggests that circadian regulation of Arr1, but not that of Arr4 and Rdh12, requires dopamine signaling via D4 receptors.

As expected for a clock-controlled gene, E4bp4 rhythmicity was evident in the same transcriptomes as those used for analysis of the other genes. Interestingly, E4bp4 rhythmicity mitigates in mice deficient for D4 receptors (Fig. 3, blue versus red lines; for statistical analysis, see Table 2). Because E4bp4 transcription is known to be directed by clock gene products,abolished E4bp4 rhythmicity may mirror an influence of dopamine signaling on the retinal clock function.

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

In diabetic retinopathy, visual function is impaired. To investigate whether disturbed circadian control of Arr1, Arr4, and Rdh12 plays a role in this context, the db/db mouse, a widely applied model of type II diabetes and diabetic retinopathy, was used. Irrespective of the melatonin deficiency of the db/db mouse (C57BL/6Jb background), the non-diabetic phenotype (db/+) was seen to display daily rhythms in Arr1, Arr4, and 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 (db/db) mice (Fig. 4, blue versus red lines; for statistical analysis, see Table 2). Therefore, circadian regulation of Arr1, Arr4, and Rdh12 appears not to be affected in diabetic retinopathy.

Consistent with the validity of the experimental system used, E4bp4 was observed to be rhythmic in the non-diabetic phenotype (db/+). The daily profile in the E4bp4 transcript was statistically arrhythmic in diabetic (db/db) mice, although it resembled that in nondiabetic (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 of the transcripts vanished under DD.

Consistent with the presence of a true circadian clock in RPE-choroid2,39 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 *Rdh12* were observed to display daily rhythms in either photoreceptors (*Arr1*, *Arr4*, and *Rdh12*) or RPE-choroid (*Lrat*, *Rpe65*, and *Rdh5*). Since they encode indispensable components of either the phototransduction pathway (*Arr1*, *Arr4*),16 or the visual retinoid cycle (*Rdb12*, *Lrat*, *Rpe65*, and *Rdh5*),22,23 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 *Rdh12*) 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**

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<th>Gene</th>
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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). Arr1 is abundant in rod and cone photoreceptors. 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. The phenotype of Arr1/C0 mice suggests the necessity of this protein for phototransduction shutoff and light adaptation of rods. Therefore, circadian regulation of Arr1 may contribute to the reported daily changes in light adaptation.

Different from Arr1, Arr4 expression is restricted to cone photoreceptors and, consequently, circadian regulation of Arr4 should occur in cones. Arr4/C0 mice display visual defects, including decreased contrast sensitivity and visual acuity. This suggests that circadian regulation of the gene contributes to the observed 24-hour changes of these visual parameters. Cone Arr4 is evolutionarily conserved and its function is not redundant with that of Arr1. Accordingly, both types of visual arrestin appear to play complementary roles in the daily adjustment of retinal function.

In rod and cone photoreceptors, protein formation occurs in the cell body and the inner segment. Therefore, increased transcription of the visual arrestins during the day may contribute to the accumulation of arrestin protein in these cell compartments at night. In response to light, both arrestins are translocated to the outer segment, where they influence phototransduction. Hence, circadian regulation of Arr1 and Arr4 may allow rods and cones to prepare an arrestin reservoir at night, ready for translocation to the outer segment in response to light.

Lrat, Rpe65, and Rdh5 encode the key enzymes of the RPE compartment of the visual cycle. Therefore, concurrent upregulation of the genes during the daytime (this study) may result in a daytime peak in the capacity of the RPE to perform chromophore regeneration. This suggests that the 24-hour rhythms of Lrat, Rpe65, and Rdh5 comply with the requirement of the RPE to increase chromophore regeneration during light exposure/daytime.

Rdh12 is localized to the inner segment of both rod and cone photoreceptors. It does not play a significant role in visual cycle function but is necessary to protect photoreceptors from toxic retinaldehydes that exceed the reductive capacity of the outer segment compartment of the photoreceptor cells. Therefore, upregulation of the gene at the earlier time of day may fulfill the demand to protect the photoreceptors from increasing concentrations of retinaldehydes released during light exposure/daytime. Interestingly, the release of retinaldehydes during light exposure/daytime and thus the requirement for protection depends on Rpe65 activity. Therefore, upregulation of Rdh12 during the
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, and retinitis pigmentosa, but also are Lrat, Rpe65, and Rdh12 to Leber's congenital amaurosis. Moreover, mice deficient for Arr1, Rdh12, and Rdh12 suffered from dystrophy of rods, cones, and photoreceptor cells. 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 development is essential for retinal health.

Rhythmicity of Arr1, Arr4, and Rdh12 persists 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. Accordingly, the pathogenesis of diabetic retinopathy appears not to derive from disturbed circadian regulation of visual arrestins or dopamine signaling via D4 receptors. This follows from the upregulation of the genes during early development. These observations indicate that the abundance of each of the gene product is a prerequisite for retinal health.

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 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. Therefore, circadian regulation of Arr1 in photoreceptors may be promoted by a molecular clock located within amacrine cells and photoreceptor cells.
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.

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