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

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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 D_4 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 (*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

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 processing[3] 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.[1]

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 MT_1 and MT_2 receptors,[10,11] and dopamine acting on D_4 receptors[12–15] 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 opsin (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*-retinal to all-*trans*-retinol takes place in photoreceptors and is performed by five isoforms of the enzyme retinoid dehydrogenase/reductase (RDH)[24] that are *Rdh8* (*prRdh*), *Rdh11, Rdh13*, *Rdh14*, and *Dhrs3* (*retSdr1*). Detoxification of retinaldehydes that exceed the reductive capacity of the outer segment compartment of the visual cycle is conducted by another isoform of *Rdh*, *Rdh12*, and a retina-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 *Rdh* isoforms *Rdh5*, *Rdh10*, and *Rdh11*. Additional key players of the visual cycle are *Rlbp1* (also referred to as *Cralbp*) and *Rbp1* (also referred to as *Crbp*), 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 *Kcnv2*, a channel essential for vision.[27–30] 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/6Jb db/+, C57BL/6Jb db/db), the mice used were melatonin-proficient (C3H/f$^{+/+}$, C3H/f$^{+/+}$Drd4$^{+/+}$ and C3H/f$^{+/+}$Drd4$^{-/-}$). Where indicated, mice deficient for dopamine D_4 receptors (*Drd4*$^{-/-}$) were used. Mice were genotyped by PCR analysis of genomic DNA. Diabetic (*db/db*) and nondiabetic (*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 ± 14 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-choroid 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 *Rdh5*, and the retina, namely *Arr1* and *Rdh12*. In comparison to retina, enrichment of *Lrat* and *Rdh5* was more than 150-fold in the RPE-choroid complex. In comparison to that of the RPE-choroid complex, enrichment of *Arr1* and *Rdh12* 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 (T_m, 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 MicroImaging, 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 μm^2 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,[31] as well as of inner retinal neurons, namely tyrosine hydroxylase (*Th*) as a marker for amacrine cells.[32] 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.[33] 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 i-Cycler (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 (C_t) by using an internal standard curve with 10-fold serial dilutions (10^1–10^8 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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Table 1. Primer Sequences Used for qPCR

Gene	GenBank Accession Number	Primer Sequence 5′ to 3′	PCR Product, bp
m18S	NR_003278.3	Forward: CAACACGGGAAACCTCAC Reverse: TCGCTCCACCAACTAAGAAC	110
mAbca4	NM_007378.1	Forward: TCGAGAAGGTTGCCAACTG Reverse: GTGAGAGCTATGGCTGTAGAG	118
mArr1	NM_009118.2	Forward: TCGGTAGCCACTTCACTG Reverse: ATCAAGGAGGGCATCGAC	116
mArr4	NM_133205.3	Forward: ACCAATCTGGCCTCTAGC Reverse: GCTCCACACCAACATCAC	148
mArrb1	NM_177231.2	Forward: CTGATACCAGACCCTTTAGC Reverse: GTGTAGGAAGCCTGAGAAAC	103
mArrb2	NM_001271358.1	Forward: GGGAAGGGAACAGTGAAC Reverse: GATTGGGAGGCAGAAGTG	111
mDbp	NM_016974.3	Forward: GGAGGTGCTAATGACCTTTG Reverse: GGACTTTCCTTGCCTTCTTC	146
mDbrs3	NM_011303.6	Forward: CTTCGCCTTCATGGAGAGC Reverse: CATGCCCTGGAACATCTCG	106
mE4bp4	NM_017373.3	Forward: CGTATTCCACCTCCATCTACC Reverse: AAGACTTGCCCACTACACC	144
mGapdh	BC082592.1	Forward: CATCCCAGAGCTGAAC Reverse: TCAGATGCCTGCTTCAC	144
mLrat	NM_023624.4	Forward: CCATACAGCCTACTGTGGAAC Reverse: AAGACAGCCGAAGCAAGAC	146
mNrl	NM_008736.3	Forward: GTGGAGGAACGGTCCAGATG Reverse: GAACTGGAGGGCTGGGTTAC	149
mPer1	NM_011065.5	Forward: CCAGATTGGTGGAGGTTACTGAGT Reverse: GCGAGAGTCTTCTTGGAGCAGTAG	92
mPer2	NM_011066.3	Forward: AGTGGCAAGATTCAAACCAAAAGT Reverse: TGAAAGCTGTCACCACCATAGAA	120
mPer3	NM_011067.2	Forward: CCGCCCCTACAGTCAGAAAG Reverse: GCCCCACGTGCTTAAATCCT	100
mRbp1	NM_011254.5	Forward: GGACTTCAACGGGTACTG Reverse: GATCATGTGGTCGCCATC	145
mRbp3	NM_015745.2	Forward: ATGGCTACGCTCTTCTTG Reverse: ATGGCTACGCTCTTCTTG	143
mRdh5	NM_134006.4	Forward: TGGAGCCTGGCTTCTTTC Reverse: GTAGTGGGCCTGTATAGCTG	104
mRdh8	NM_001030290.1	Forward: TGGGCTACTTCCGGGACTTG Reverse: GTTGGTCTGTCTGCGGAGTG	137
mRdh10	NM_133832.3	Forward: GTGCTCTGTTGTGTTCTC Reverse: TCAGACTACGCTGATCTC	110
mRdh11	NM_021557.5	Forward: AAGCCGTCACAGGGAACAG Reverse: CGCCCGCATTGTTGATGAG	132
mRdh12	NM_030017.4	Forward: CTCTTCTCACCCTTCTTC Reverse: GATACCCACATCCTCTTG	125
mRdh13	NM_175372.4	Forward: AGACAGTGGACACCAAAG Reverse: GAACAACAGCCAAGTCAG	122
mRdh14	NM_023697.2	Forward: CATTAGAAGGTGCCCAGAC Reverse: GCCACTGACTCATCCATAG	127
mRlbp1	NM_020599.2	Forward: GCTACAGAGGGTCTTTGTTC Reverse: ACTTTGCCGTCGTACTTG	114
mTh	NM_009377.1	Forward: CAGCCCTACCAAGATCAAAC Reverse: GTACGGGTCAAACTTCACAG	129
r18S	NR_046237.1	Forward: GTTGGTGGAGCGATTTGTC Reverse: TCAATCTCGGTGGCTGAAC	136
rArr1	NM_013023.2	Forward: GGCCTCCAGCACCATTATC Reverse: GCACCTCAGTAGCCACTTC	137
rArr4	NM_001190993.1	Forward: ACCAATCTGGCCTCTAGC Reverse: GCTCCACACCAACATCAC	148
rE4bp4	NM_053727.2	Forward: TCGGAACACTGGCATCAC Reverse: TTCTCAGCCTCGGAAACC	107
rGapdh	NM_017008.4	Forward: TGACTCTACCCACGGCAAG Reverse: CTGGAAGATGGTGATGGGTT	89
rRdh12	NM_001108037.1	Forward: GGAATCCAGTGGGAATAG Reverse: AGTAGGCAGGAGTTACAG	139

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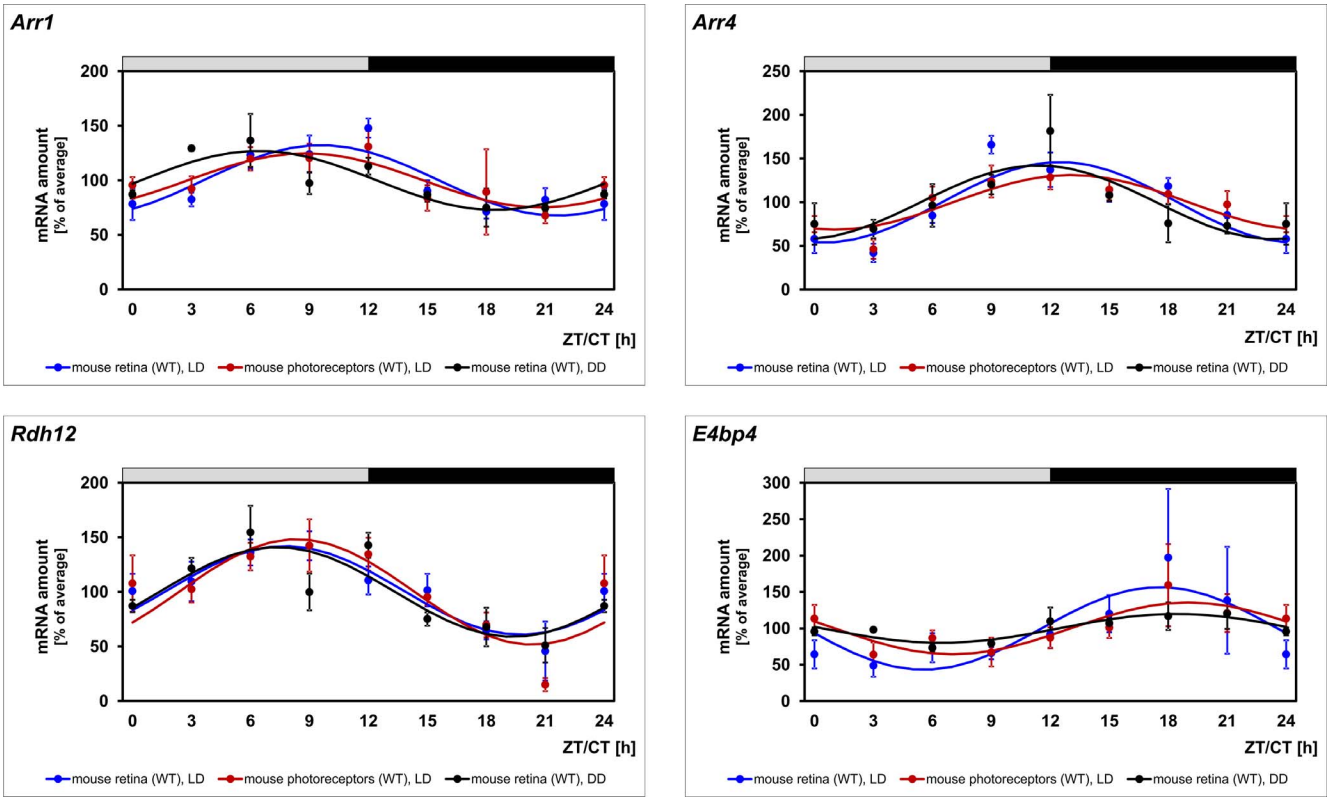

Figure 1. Daily rhythmicity of *Arr1, Arr4*, and *Rdh12* in retina and photoreceptors in LD and under constant darkness. Transcript levels of the genes were monitored in comparison to the clock-controlled gene *E4bp4* under LD 12:12 in mouse retina (*blue lines*) and in isolated mouse photoreceptors (*red lines*), and under constant darkness in mouse retina (*black lines*) by using qPCR. The mRNA levels are plotted as a function of ZT and circadian time (CT). The lines represent the periodic sinusoidal functions determined by cosinor analysis (*solid line* for $P < 0.05$ in cosinor analysis). 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. 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] for whole retina preparations and two animals [four retinas] for photoreceptor preparations).

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.[34,35] The model can be expressed according to the following equation: $f(t) = A + B \cos [2\pi (t + C)/T]$, 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*, *Rdh14*, *Dhrs3*, *Abca4*, and *Rbp3*.

The daily rhythmicity 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 analyzation 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).

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TABLE 2. Statistical Analysis of Transcriptional Profiling Illustrated in Figures 1 to 4

Source of Transcriptomes and Lighting Conditions	*Arr1*			*Arr4*			*Rdh12*			*E4bp4*			See Figure
	P Value	Acrophase, h	Amplitude, %	*P* Value	Acrophase, h	Amplitude, %	*P* Value	Acrophase, h	Amplitude, %	*P* Value	Acrophase, h	Amplitude, %	
Mouse retina (C3H/f$^{+/+}$ (rd^{++})); LD	<0.05	8.2	32.3	<0.05	12.5	46.2	<0.05	9.4	40.6	<0.05	15.8	56.8	1
Mouse photoreceptors (C3H/f$^{+/+}$ (rd^{++})); LD	<0.05	7.7	24.8	<0.05	11.4	31.3	<0.05	7.8	48.4	<0.05	19.7	35.6	1
Mouse retina (C3H/f$^{+/+}$ (rd^{++})); DD	<0.05	7.1	27.1	<0.05	10.8	42.2	<0.05	8.1	41.1	<0.05	18.5	19.8	1
Rat retina (Sprague Dawley); LD	<0.05	8.9	29.9	<0.05	15.0	36.5	<0.05	6.4	25.3	<0.05	18.4	27.5	2
Rat pineal gland (Sprague Dawley); LD	<0.05	12.4	65.9	<0.05	10.6	76.2	<0.05	7.1	36.6	<0.05	21.3	67.6	2
Drd4 WT mouse retina (C3H/f$^{+/+}$ (rd^{++})); LD	<0.05	7.3	42.5	<0.05	9.4	35.5	<0.05	7.2	36.7	<0.05	19.4	17.6	3
Drd4 (KO) mouse retina (C3H/f$^{+/+}$ (rd^{++})); LD	>0.05	-	-	<0.05	8.7	31.3	<0.05	8.2	48.2	>0.05	-	-	3
Nondiabetic (*db/+*) mouse retina (C57BL/6Jb); LD	<0.05	7.7	27.9	<0.05	10.9	23.8	<0.05	8.6	45.4	<0.05	21.6	12.0	4
Diabetic (*db/db*) mouse retina (C57BL/6Jb); LD	<0.05	9.1	35.2	<0.05	12.6	32.7	<0.05	8.8	40.2	>0.05	-	-	4

KO, knock out; WT, wild type.

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.[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. *Arr1*, *Arr4*, and *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 *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 D_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_4 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 D_4 receptors.

As expected for a clock-controlled gene, *E4bp4* rhythmicity was evident in the same transcriptomes as those used for analyzation of the other genes. Interestingly, *E4bp4* rhythmicity mitigates in mice deficient for D_4 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,[37] abolished *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 *Arr1*, *Arr4*, and *Rdh12* plays a role in this context, the *db/db* mice, a worldwide applied model of type II diabetes and diabetic retinopathy,[38] was used. Irrespective of the melatonin deficiency of the *db/db* mouse (C57BL/6Jb background), the nondiabetic 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 nondiabetic 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

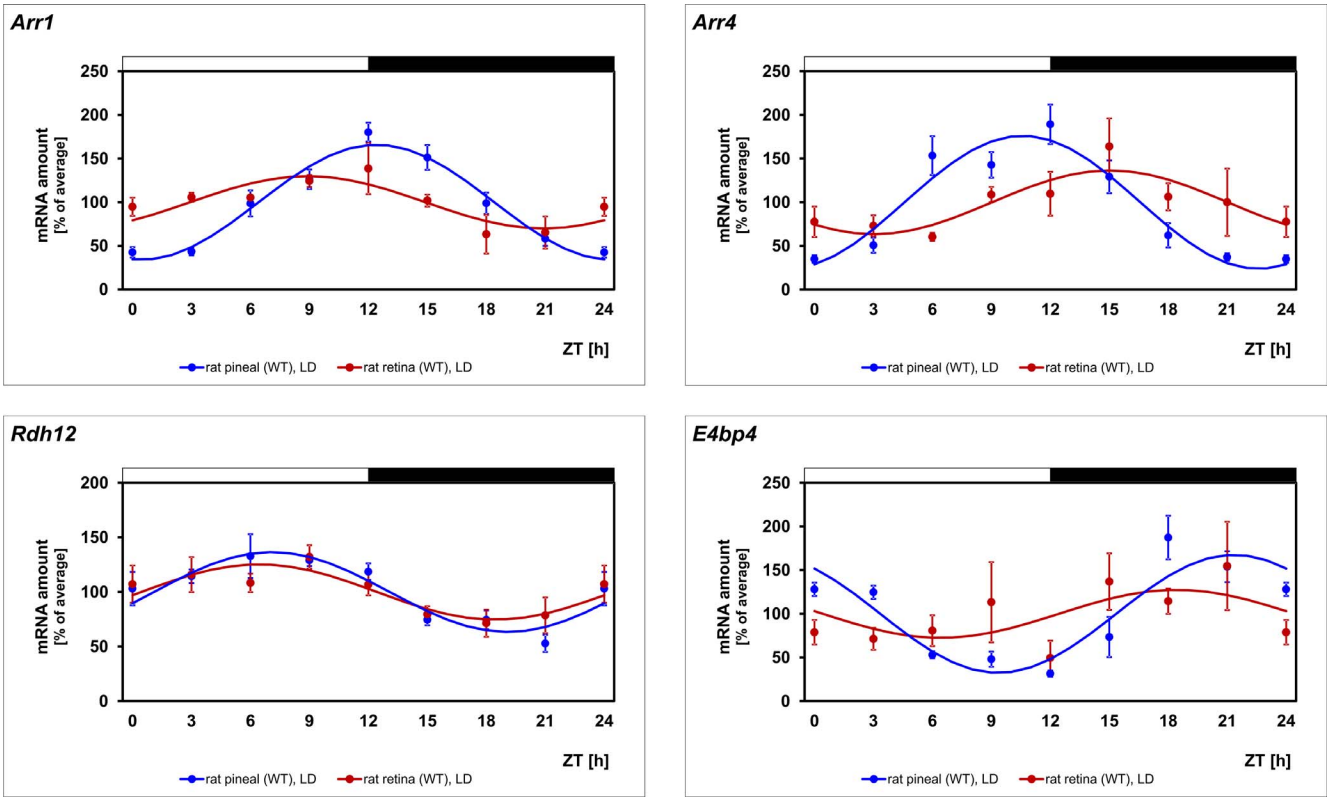

Figure 2. Daily rhythmicity of *Arr1*, *Arr4*, and *Rdh12* in rat pineal gland and rat retina. Transcript levels of the genes were monitored in comparison to the clock-controlled gene *E4bp4* under LD 12:12 in pineal gland (*blue lines*) and retina (*red lines*) of rats by using qPCR. The mRNA levels are plotted as a function of ZT and the lines represent the periodic sinusoidal functions determined by cosinor analysis (*solid line* for $P < 0.05$ in cosinor analysis). 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. 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 two retinas and a pineal gland of one animal).

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-choroid[2,39] 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*, *Rdh5*, *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 (*Rdh12*, *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

	LD 12:12			DD			
Gene	***P* Value**	**Acrophase, h**	**Amplitude, %**	***P* Value**	**Acrophase, h**	**Amplitude, %**	**See Figure**
Lrat	<0.05	4.2	38.1	>0.05	–	–	5
Rdh5	<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

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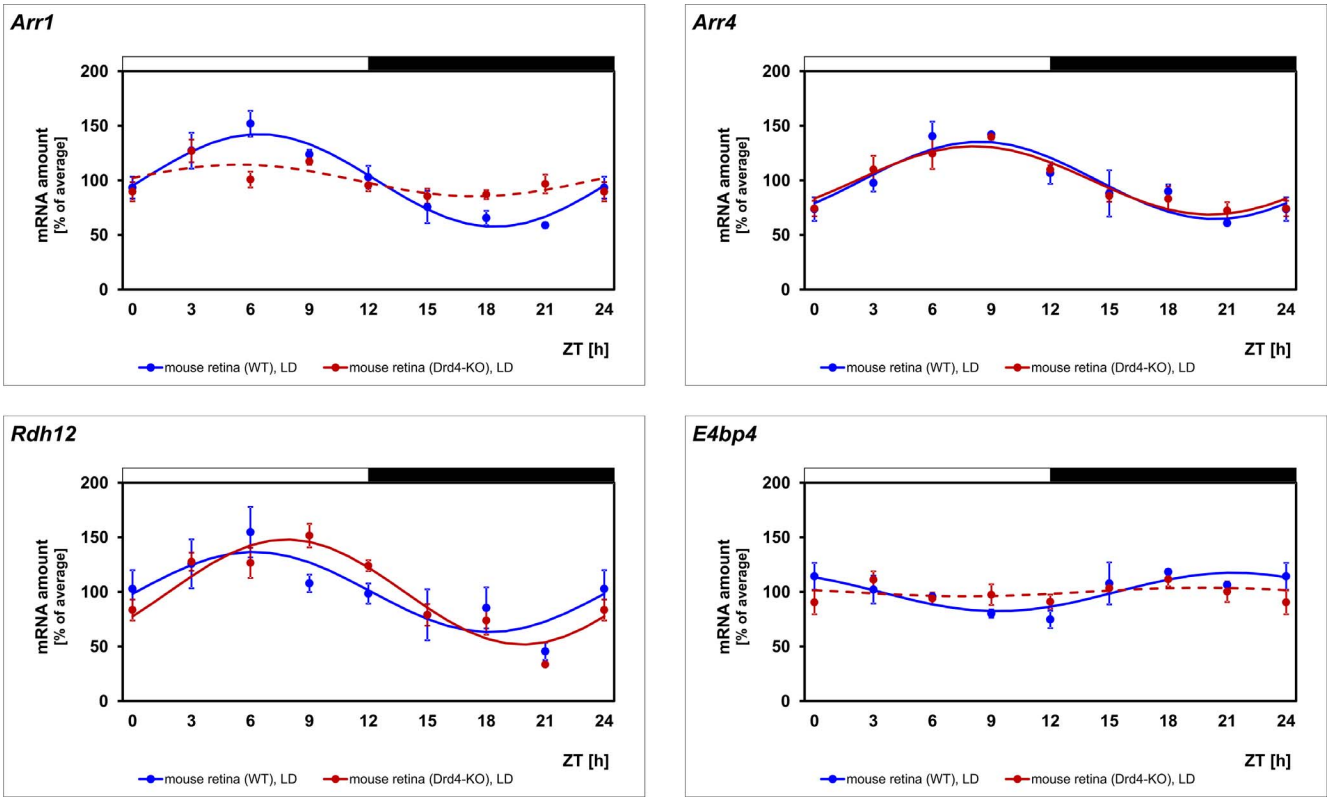

Figure 3. Daily rhythmicity of *Arr1*, *Arr4*, and *Rdh12* in retina deficient for dopamine D_4 receptors. Transcript levels of the genes were recorded in wildtype mice (*blue lines*) versus mice deficient for dopamine D_4 receptors (*red lines*) in preparations of the whole retina under LD 12:12 by 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 D_4 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]).

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[39] 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. The phenotype of $Arr1^{-/-}$ mice suggests the necessity of this protein for phototransduction shutoff and light adaptation of rods.[19,40] Therefore, circadian regulation of *Arr1* may contribute to the reported daily changes in light adaptation.[19] Different from *Arr1*, *Arr4* expression is restricted to cone photoreceptors[20,21] and, consequently, circadian regulation of *Arr4* should occur in cones. $Arr4^{-/-}$ mice display visual defects, including decreased contrast sensitivity and visual acuity.[20,21] This suggests that circadian regulation of the gene contributes to the observed 24-hour changes of these visual parameters.[2,14,15] Cone *Arr4* is evolutionarily conserved and its function is not redundant with that of *Arr1*.[20,21] 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,[41–45] where they influence phototransduction.[46] 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.[22] 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* complies 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.[47] It does not play a significant role in visual cycle function but is necessary to protect photoreceptors from toxic retinaldehydes that exceeds the reductive capacity of the outer segment compartment of the photoreceptor cells.[48–50] 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.[51–53] Interestingly, the release of retinaldehydes during light exposure/daytime and thus the requirement for protection depends on *Rpe65* activity.[54,55] Therefore, upregulation of *Rdh12* during the

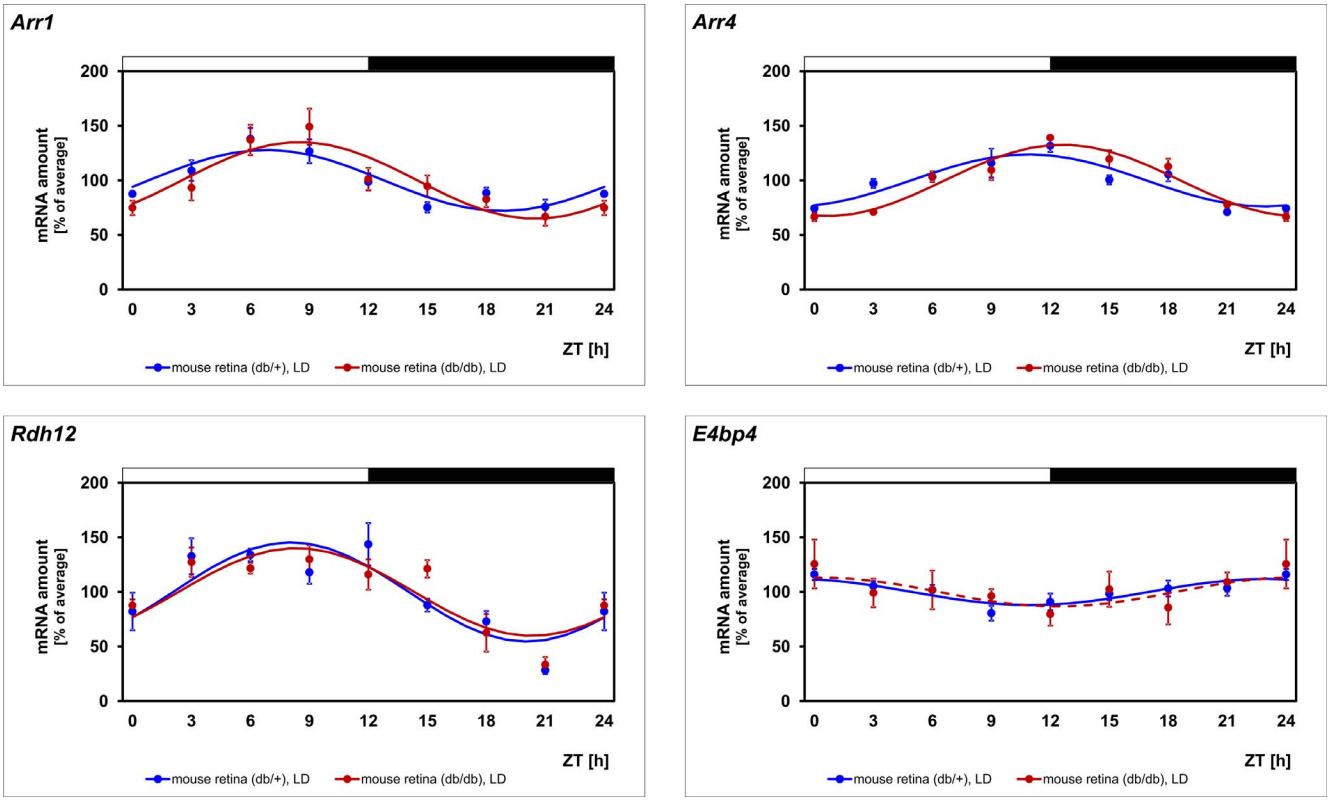

FIGURE 4. Daily rhythmicity of *Arr1*, *Arr4*, and *Rdh12* in diabetic retina. Transcript levels of the genes were monitored in nondiabetic (*db/+*) mice (*blue lines*) versus diabetic (*db/db*) mice (*red lines*) in preparations of the whole retina under LD 12:12 by using qPCR. The mRNA levels are plotted 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. 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] *Rpe65*,[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 D_4 receptors. This follows from the present observation that *Arr1* periodicity is disrupted in retinas deficient of functional D_4 receptors. D_4 receptor-dependent control of photoreceptors[12] appears to derive from the clock-driven release of dopamine from amacrine cells in the inner retina[2] 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,

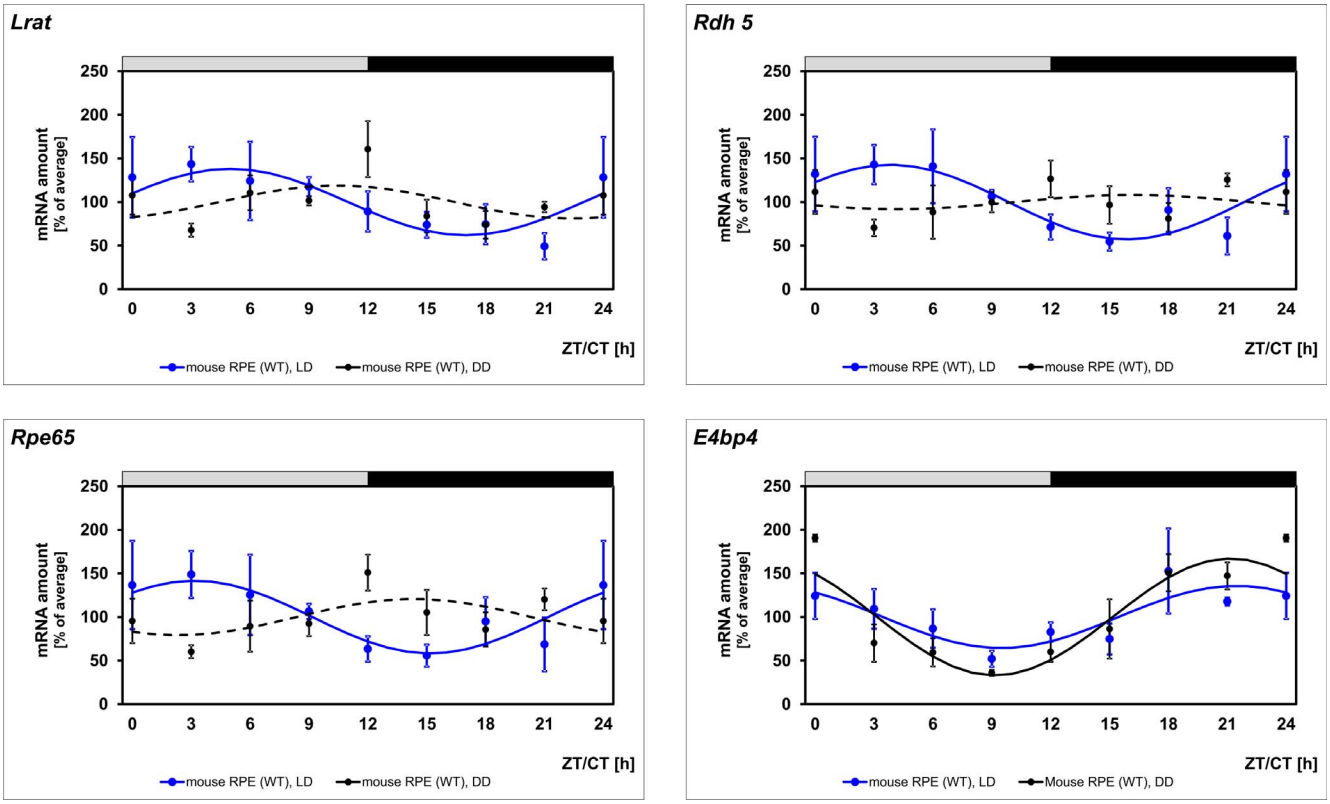

Figure 5. Daily rhythmicity of the visual cycle genes in RPE-choroid. Transcript levels of the visual cycle genes *Lrat*, *Rpe65*, and *Rdh5* were monitored in comparison to the clock-controlled gene *E4bp4* under LD 12:12 (*blue lines*) and constant darkness (*black lines*) in mouse RPE-choroid by using qPCR. The mRNA levels are plotted as a function of ZT and circadian time (CT). 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 3. 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 retinal pigment epithelia]).

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]

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