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ORIGINAL ARTICLE

Transcriptional analysis of rat photoreceptor cells reveals daily regulation of genes important for visual signaling and light damage susceptibility

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Abstract

Photoreceptor cells face the challenge of adjusting their function and, possibly, their susceptibility to light damage to the marked daily changes in ambient light intensity. To achieve a better understanding of photoreceptor adaptation at the transcriptional level, this study aimed to identify genes which are under daily regulation in photoreceptor cells using microarray analysis and quantitative PCR. Included in the gene set obtained were a number of genes which up until now have not been shown to be expressed in photoreceptor cells, such as *Atf3* (*activating transcription factor 3*) and *Pde8a* (*phosphodiesterase 8A*), and others with a known impact on phototransduction and/or photoreceptor survival, such as *Grk1* (*G protein-coupled receptor kinase 1*) and *Pgc-1α* (*peroxisome proliferator-activated receptor γ, coactivator 1alpha*). According to their daily dynamics, the genes identified could be clustered in two groups: those with peak expression during the second part of the day which are uniformly promoted to cycle by light/dark transitions and those with peak expression during the second part of the night which are predominately driven by a clock. Since *Grk1* and *Pgc-1α* belong in the first group, the present results support a concept in which transcriptional regulation of genes by ambient light contributes to the functional adjustment of photoreceptor cells over the 24-h period.

Keywords: daily adaptation, gene expression, Grk1, Pgc-1α, photoreceptor cell, retina.

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The photoreceptor cell and the mammalian retina as a whole have the ability to functionally adapt to the marked daily changes in ambient illumination which is mirrored in the daily changes of responses to light (for review, see Barlow 2001; Storch *et al.* 2007), which can be measured using the electroretinogram (ERG) (Cameron *et al.* 2008). Functional adjustment of the photoreceptor cell involves transcriptional gene regulation as is evident for the gene *arylalkylamine N-acetyltransferase* (*Aanat*) that codes for the key enzyme in melatonin formation, arylalkylamine N-acetyltransferase (EC 2.3.1.87) (for review, see Iuvone *et al.* 2005). Thus, transcriptional activation of the *Aanat* gene during darkness results in an increase of melatonin formation (Tosini and Menaker 1996; Tosini and Menaker 1998), which appears to be important for the functional adjustment of the

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Abbreviations used: CT, circadian time; DD, constant darkness; LD, light/dark; LMPC, laser microdissection and pressure catapulting; ZT, Zeitgeber time.

photoreceptor cell and the retina as a whole to darkness (Baba *et al.* 2009; Sengupta *et al.* 2011). Transcriptional regulation of the *Aanat* gene is driven by a circadian clock (Niki *et al.* 1998) which is probably located within the retina (Ruan *et al.* 2006, 2008) or even within the photoreceptor cell itself (Tosini *et al.* 2007; Schneider *et al.* 2010; Sandu *et al.* 2011). The clock influences *Aanat* transcription directly by the action of a CLOCK:BMAL1 protein complex on E-boxes contained in the proximal promoter of the gene (Chen and Baler 2000; Tosini and Fukuhara 2003) and indirectly by gating cAMP-induced *Aanat* transcription via the expressional control of the enzyme adenylyl cyclase I (Fukuhara *et al.* 2004).

The understanding of photoreceptor adaptation at the transcriptional level is confined by an insufficient knowledge concerning the genes whose expression is regulated on a day/night basis in the photoreceptor cell. Although numerous genes have been shown to be under daily regulation in preparations of whole eye (Storch *et al.* 2007) or retina (Brann and Cohen 1987; Wang *et al.* 2001; Fukuhara *et al.* 2004; Humphries and Carter 2004; Kamphuis *et al.* 2005; Storch *et al.* 2007; Rath *et al.* 2009; Bedolla and Torre 2011; Mollema *et al.* 2011), only a few besides the *Aanat* gene have been demonstrated to display daily changes of expression in photoreceptor cells. These encompass several clock genes (Tosini *et al.* 2007; Schneider *et al.* 2010; Sandu *et al.* 2011) as well as the genes *Drd4* (Klitten *et al.* 2008), *Fos* (Yoshida *et al.* 1993), *Pde10a* (Wolloscheck *et al.* 2011), *Kcnv2* (Hölter *et al.* 2012), and *Kcnb* (Hölter *et al.* 2012).

To gain a better understanding of the functional adjustment of the photoreceptor cell to daily changes in light intensities, this study was aimed at identifying further genes which are under daily regulation in photoreceptor cells, and in particular those having an impact on phototransduction and/or resistance to light damage of photoreceptor cells. Rats were used as an experimental model as they possess (in contrast to most strains of mice) both intact photoreceptor cells and are capable of melatonin formation, while at the same time allowing effective transcriptome isolation from photoreceptor cells by microdissection (Schneider *et al.* 2010; Sandu *et al.* 2011; Wolloscheck *et al.* 2011).

Materials and methods

Animals

Animal experimentation was carried out in accordance with the European Communities Council Directive (86/609/EEC) and the ARRIVE guidelines. Adult male Sprague-Dawley rats (received at the age of 8 weeks from Harlan Laboratories, Horst, The Netherlands; body weight: 150–180 g) were kept under standard laboratory conditions (illumination with fluorescent strip lights, 200 lux at cage level during the day and dim red light during the night; 20 ± 1°C; water and food ad libitum) under light/dark 12:12 (LD 12:12) for 3 weeks. When indicated, the rats were then kept for one cycle under dim red light and killed during the next cycle. Animals were killed at the indicated time points by decapitation following anesthesia with carbon dioxide. All dissections during the dark phase were carried out under dim red light. Retinas were rapidly removed and immediately processed as follows. For microarray analysis six animals and for quantitative polymerase chain reaction (qPCR) three animals were pooled in each sample.

Sample preparation

Fixation of the retina was carried out using the HOPE technique (Goldmann *et al.* 2006) and embedding with paraffin, deparaffinization with isopropanol and staining with cresyl violet was conducted as described earlier (Schneider *et al.* 2010).

Laser microdissection and pressure catapulting (LMPC)

To isolate photoreceptor cells from the stained sections in a contact- and contamination-free manner the LMPC technique was applied. LMPC was performed using a PALM MicroBeam system (Zeiss MicroImaging, Munich, Germany) with PALM RoboSoftware (P.A.L.M., Bernried, Germany). Under the 10× objective, photoreceptor 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.) by utilizing a pulsed UV-A nitrogen laser. Smaller areas of the sections were pooled to reach total average sample sizes of 4 million square microns per tube. To verify the purity of the preparations, photoreceptors were subjected to molecular analysis with *rhodopsin* as a marker for photoreceptors and *tyrosine hydroxylase* as a marker for inner retinal neurons. In photoreceptors collected by LMPC, the ratio of *rhodopsin* to *tyrosine hydroxylase* was increased about 60-fold compared to preparations of the whole retina. Alternatively, the whole retina was excised using a scalpel, collected in a 0.5 mL microfuge tube and immediately frozen in liquid nitrogen. Cell lysis for RNA preparation was carried out immediately after sample collection.

RNA extraction

RNA from the laser-microdissected tissue samples was isolated using the RNeasy Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, the collected cells were lysed in a guanidine-thiocyanate-containing buffer (RLT buffer) supplied by the manufacturer. The lysates were diluted with RNase-free water and treated with proteinase K. The samples were then cleared by centrifugation, diluted with ethanol, and applied to an RNeasy MinElute Spin Column to bind RNA to the silica-gel membrane. After the first washing step, an on-column DNase treatment with RNase-free DNase I was carried out as described by the manufacturer. Isolated RNA was eluted in a final volume of 12 μL RNase-free water. The concentration of extracted RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) with measurement of the optical density at 260 and 280 nm.

Microarray

Gene expression profiling was performed using arrays of Rat230_2-type from Affymetrix. Biotinylated antisense cRNA was then prepared according to the Affymetrix standard labeling protocol. Afterward, the hybridization on the chip was performed on a GeneChip Hybridization oven 640, then dyed in the GeneChip Fluidics Station 450 and thereafter scanned with a GeneChip

Scanner 3000. All equipment used was from the Affymetrix-Company (Affymetrix, High Wycombe, UK). The Affymetrix chip definition file (CDF) was used to annotate the arrays. The raw fluorescence intensity values were imported and processed using Robust Microarray Analysis (RMA) and the Expression Console 1.2 software (Affymetrix) and normalized by applying quantile normalization. The GO analysis was performed using DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/). The Rat230_2-array Set was used as the background for the GO analysis. The GO terms after correction for FDR at $p \leq 0.05$ (Benjamini Hochberg) were selected for further analysis and interpretation.

Reverse transcription (RT) and quantitative polymerase chain reaction

cDNA was synthesized using the Verso cDNA Kit (Abgene, Hamburg, Germany), following the manufacturer's instructions. Briefly, 4.5 μL RNA solution was reverse transcribed by using anchored oligo-dT primers supplied with the kit in a final volume of 20 μL. cDNA was then diluted 1 : 3 in RNase-free water, with aliquots of 5 μL being used for PCR. Quantitative PCR was carried out in a total volume of 25 μL containing 12.5 μL ABsolute QPCR SYBR® Green Fluorescein Mix (Abgene), 0.75 μL of each primer (10 μM), 6 μL RNase-free water, and 5 μL sample. Primer sequences are listed in Table 1. PCR amplification and quantification were performed in an i-Cycler (BioRad, Munich, Germany) according to the following protocol: denaturation for 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 20 s at 60°C, and 20 s at 72°C. All amplifications were carried out in duplicate. 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 RNA was calculated from the measured threshold cycles (C_t) using a standard curve. Values were then normalized with respect to the amount of *Gapdh* mRNA present. The *Gapdh* expression does not show daily rhythmicity ($p \geq 0.05$; one-way ANOVA).

Western blot

For immunoprecipitation of Pgc-1α, three tissue samples (each obtained by pooling two retinas from two rats) were homogenized

Table 1 Primer sequences

Gene	Accession number	Primer sequence 5′ to 3′	Length of PCR product [bp]
Aanat	NM_012818	Forward GAAGGGAGACAGCAGTTC	144
		Reverse GTCCTGGTCTTGCCTTTG	
Adra1b	NM_016991.2	Forward TTCTGGCTGGGCTACTTC	100
		Reverse ACTGGCACCCAAGGATAC	
Asns	NM_013079	Forward TGGGCAGAGATACCTATGGTG	110
		Reverse GGTGGAGTGTTTCAAGGAGAC	
Atf3	NM_012912	Forward CTGGGTCACTGGTGTTTGAG	103
		Reverse GAGGACATCCGATGGCAAAG	
Cdkn1b	NM_031762.3	Forward AGGGCCAACAGAACAGAAG	100
		Reverse TTTACGTCTGGCGTCGAAG	
Cerk	NM_001134861	Forward AGAGTGGCAAGTGGTATGTG	135
		Reverse CGGATAAGGATGAGGTCAGAAG	
Ctnnal1	NM_001106649	Forward TTCACCGATTCTGCCTACAC	103
		Reverse GCATCCACACGGAAATGAAC	
Dyrk2	NM_001108100	Forward GACAGGTGGACCCAACAATG	111
		Reverse CCCTTCCCGATGACTTTGAG	
Gapdh	NM_017008	Forward ATGACTCTACCCACGGCAAG	89
		Reverse CTGGAAGATGGTGATGGGTT	
Grk1	NM_031096	Forward AAGACCGACCTCTGTCTG	138
		Reverse CAGGCCACTGATGATCTG	
Pax4	NM_031799	Forward CTCCTTCCTGTGGCTTCTTC	106
		Reverse AGGTTGATGGCGCTTGTC	
Pde6d	NM_001108806	Forward GCAGGAGGAAGCTGTACTTG	147
		Reverse GTCAGTGACGATGGAGGTTTG	
Pde8a	NM_198767.1	Forward ACGCCACTGCGTATTTCC	148
		Reverse AGCTCCTTTCCCGCATTG	
Pgc-1α	NM_031347	Forward GCCGTGTGATTTACGTTGG	110
		Reverse ATCCCGCAGATTTACGGTG	
Pla2g1b	NM_031585	Forward GGCCAAGAAGCTGGAAAG	138
		Reverse CGGTCACAGTTGCAGATG	
Rhobtb1	NM_001107622	Forward CCGCCTGGTGTTTACATC	158
		Reverse ACGCTGGTAGTGGTCTTC	

in 1 mL HEPES-sucrose buffer containing protease inhibitors. Insoluble material was pelleted. For antibody immobilization, protein A-agarose beads (30 μL bead volume; Invitrogen, Carlsbad, CA, USA) were washed four times and incubated with rabbit anti-Pgc-1α monoclonal antibody (1 : 250; Cell Signaling Technology, Danvers, MA, USA; #2178) at 4°C. Cell extracts corresponding to 200 μg protein amounts were applied overnight to the antibody-coupled beads at 4°C. Bound proteins were recovered after extensive washes in phosphate-buffered saline. For Western blot analysis, samples were loaded on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen), separated and blotted onto polyvinylidene difluoride membrane (Westran S, Whatman Inc., Sanford, ME, USA). For immunodetection, membranes were blocked in 5% skimmed milk powder and the anti-Pgc-1α (1 : 500) or the rabbit anti-Grk1 polyclonal antibody (1 : 300; Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-84291) was applied overnight at 4°C. The horseradish-peroxidase-coupled secondary antibodies (goat anti-rabbit-horseradish-peroxidase 1 : 5000; Sigma-Aldrich, St. Louis, MO, USA; A0545) were visualized using an ECL detection system (GE Healthcare Amersham, Freiburg, Germany). To ensure that immunoreactivity was derived from equal protein amounts of homogenates staining with rabbit anti-β-actin polyclonal antibody (1 : 300; Sigma-Aldrich; A2066) was conducted. Densitometric measurement was performed using the 'ImageJ 1.46o' software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All PCR data are given as the mean with standard error of the mean (SEM) of four independent experiments. Based on the suggestion that ZT24 is equal to ZT0, cosinor analysis was used to determine the p-value for a daily dynamic, the acrophase (peak expression) and the amplitude of oscillation (half the difference between the highest and lowest values) using the statistic software "R" (version 2.12.1, freely available at www.r-project.org). All statistical analyses should be regarded as being explorative and p-values are given descriptively with no significance level being fixed.

Results

Genes considered under daily regulation in photoreceptor cells

A gene under daily regulation in photoreceptor cells should show a day/night change in the transcriptome of not only photoreceptor cells but also in preparations of the whole retina (Schneider *et al.* 2010; Sandu *et al.* 2011; Wolloscheck *et al.* 2011; Hölter *et al.* 2012) from which transcriptome isolation is more effective. For these reasons, as a first step toward the identification of genes under daily regulation in photoreceptor cells, the transcriptomes of photoreceptor cells and whole retina preparations were screened by microarray analysis for genes showing analog day/night changes in both. This was done by selecting genes that fulfilled the demand of concurrently displaying a ZT6/ZT18 ratio equal or greater than twofold in microdissected photoreceptor cells and whole retina preparations. The resulting gene set comprises 40 genes (Table 2). Of these, 18 genes exhibited enhanced expression during day (Table 2A) and 22 genes showed increased expression at night (Table 2B). The gene set included genes already previously reported to be under daily regulation in photoreceptor cells, that is, *Aanat* (Niki *et al.* 1998; Schneider *et al.* 2010; Sandu *et al.* 2011), *Drd4* (Klitten *et al.* 2008), *Fos* (Yoshida *et al.* 1993; Sandu *et al.* 2011), *Kcnv2* (Hölter *et al.* 2012), *Pde10a* (Wolloscheck *et al.* 2011), and *Rorβ* (Sandu *et al.* 2011). As expected, all these genes showed elevated expression during the night. Clustering of the genes according to their molecular and biological functions (Table 3) revealed that they are distributed over different functional groups. Notably, some of the genes found to be under daily regulation by microarray analysis are important for the function, development and survival of photoreceptor cells (Table 3).

24-h profiling of gene expression under light/dark 12:12

To test and specify their daily regulation, the genes were subjected to daily profiling by qPCR (Fig. 1; Table 4). This effort focussed on 12 genes not yet reported to be under daily regulation in photoreceptor cells or retina.

Consistent with the suggestion that the strategy conducted in this study – two-point day/night sampling in photoreceptor cells and whole retina preparations – is a productive and reliable approach for the detection of genes that are under daily regulation in photoreceptor cells, 10 of 12 genes tested displayed the expected 24-h rhythms in transcript amount in photoreceptor cells with a *p*-value of less than 0.05 (Fig. 1, left columns; Table 4). The genes (*Cerk*, *Ctnnal1*) that failed to show a statistically relevant cyclicity in photoreceptor cells (Fig. 1b, left column; Table 4B) displayed the expected day/night rhythm at least in preparations of the whole retina (Fig. 1b, middle column; Table 4B).

The recording of gene expression over multiple time points in the qPCR study provided a more complete profiling of the daily pattern of transcript amount. This allows the clustering of the genes with respect to their daily profiles. Those genes showing higher expression during the day generally display an increase in mRNA amount after light onset reaching a peak expression between ZT6 and ZT12 (Fig. 1a, left and middle columns; Table 4A). On the other hand, genes exhibiting higher expression during the night generally showed a daily pattern featuring an increase in expression occurring not earlier than the dark onset and reaching peak expression between ZT17 and ZT24 (Fig. 1b; left and middle columns). Notably, among all genes recorded, the amplitude of cycling in photoreceptor cells was most prominent for *Grk1* and *Pla2g1b* (Table 4).

To align the results obtained with previous studies, qPCR measurement was also performed on the following reference genes: (i) *Aanat*, a gene under circadian regulation with peak expression during night (for review, see Iuvone *et al.* 2005), (ii) *Pax4*, a non-circadian gene with peak expression during daytime (Rath *et al.* 2009), and (iii) *Pde6d*, a gene not showing a day/night difference in microarray analysis and therefore not expected to cycle. Consistent with

Table 2 Genes indicated by microarray analysis to display higher expression in photoreceptor cells during daytime (A) or night (B)

				ZT18/ZT6 ratio	
Probe set ID	Gene symbol	Gene name	Uni gene ID	PRC	Ret
(A)					
1369192_at	*Cdkn1b*[c]	cyclin-dependent kinase inhibitor 1B	Rn.29897	0.23	0.50
1394972_at	*Trove2*	TROVE domain family, member 2	Rn.14659	0.27	0.47
1370089_at	*Pgc-1α*[c]	peroxisome proliferator-activated receptor γ, coactivator 1 alpha	Rn.19172	0.28	0.48
1394970_at	*Cars*	cysteinyl-tRNA synthetase	Rn.14865	0.29	0.48
1387925_at	*Asns*[c]	asparagine synthetase	Rn.11172	0.31	0.29
1374794_at	*Kif15*	kinesin family member 15	Rn.45205	0.31	0.20
1374375_at	*RGD1560925*	similar to 2610034M16Rik protein	Rn.13689	0.34	0.29
1376963_at	*Dyrk2*[c]	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	Rn.23189	0.34	0.47
1374034_at	*Cars*	cysteinyl-tRNA synthetase	Rn.14865	0.34	0.37
1394676_at	*Oxa1l*	oxidase (cytochrome c) assembly 1-like	Rn.10589	0.37	0.47
1370140_a_at	*Pax4*[ac]	paired box 4	Rn.14531	0.38	0.44
1390664_at	*Tmem116*	transmembrane protein 116	Rn.39141	0.40	0.29
1393396_at	*Sft2d3*	SFT2 domain containing 3	Rn.14692	0.40	0.47
1385698_at	*Cars*	cysteinyl-tRNA synthetase	Rn.14865	0.42	0.14
1369268_at	*Atf3*[c]	activating transcription factor 3	Rn.9664	0.43	0.45
1369257_at	*Grk1*[c]	G protein-coupled receptor kinase 1	Rn.10548	0.45	0.47
1379340_at	*Lamc2*	laminin, gamma 2	Rn.9278	0.47	0.47
1397882_at	*Zbtb8a*	zinc finger and BTB domain containing 8a	Rn.82564	0.48	0.44
(B)					
1375987_at	*Cerk*	ceramide kinase	Rn.99537	2.03	2.30
1389632_at	*Rhobtb1*[c]	rho-related BTB domain containing 1	Rn.3782	2.15	2.74
1390065_at	*Pde10a*[b]	phosphodiesterase 10A	Rn.44869	2.21	4.29
1390065_at	*Kcnv2*[b]	potassium channel, subfamily V, member 2	Rn.168638	2.21	2.64
1371824_at	*Ak3l1*	adenylate kinase 3-like 1	Rn.1086	2.23	2.30
1394384_at	*Drd4*[b]	dopamine receptor D4	Rn.10159	2.26	2.30
1387255_at	*Aanat*[bc]	arylalkylamine N-acetyltransferase	Rn.88180	2.20	3.48
1398354_at	*Ctnnal1*	catenin (cadherin associated protein), alpha-like 1	Rn.33021	2.40	2.14
1376057_at	*Pde8a*[c]	phosphodiesterase 8A	Rn.24771	2.41	4.29
1386935_at	*Nr4a1*[a]	nuclear receptor subfamily 4, group A, member 1	Rn.10000	2.50	2.06
1396871_at	*Kcnv2*[b]	potassium channel, subfamily V, member 2	Rn.168638	2.55	2.03
1380305_at	*nod3l*	NOD3-like protein	Rn.51041	2.60	2.64
1398580_at	*Wdr31*	WD repeat domain 31	Rn.38938	2.65	2.46
1372448_at	*Zdhhc5*	zinc finger, DHHC-type containing 5	Rn.4240	2.71	2.30
1371257_at	*Rorβ*[b]	RAR-related orphan receptor B	Rn.210157	2.92	3.73
1387520_at	*Drd4*[b]	dopamine receptor D4	Rn.10159	3.13	2.83
1377869_at	*Ccrn4l*[a]	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	Rn.15040	3.62	2.14
1370257_at	*Pla2g1b*[c]	phospholipase A2, group IB, pancreas	Rn.4283	4.39	2.83
1372170_at	*Acy1*	aminoacylase 1	Rn.3679	4.62	2.30
1368574_at	*Adra1b*[c]	adrenoceptor alpha 1B	Rn.10032	4.65	2.62
1368321_at	*Egr1*[a]	early growth response 1	Rn.9096	11.9	2.14
1375043_at	*Fos*[b]	FBJ osteosarcoma oncogene	Rn.103750	17.5	2.87

The genes listed exhibit a ≥ twofold change between Zeitgeber time (ZT) 6 and 18 in both photoreceptor cells (PRC) and preparations of the whole retina (Ret). The listing of the genes is performed according to the extent of their day/night change in photoreceptor cells. The genes *Cars*, *Drd4,* and *Kcnv2* were analyzed by more than one probe set with similar results.

[a]Genes previously reported to be under daily regulation in whole retina.

[b]Genes previously reported to be under daily regulation in photoreceptor cells.

[c]Genes confirmed by 24-h profiling (see Fig. 1, Table 4) to be under daily regulation in photoreceptor cells.

previous reports and the results obtained from transcriptomic profiling, daily rhythms in photoreceptor cells and preparations of the whole retina were observed for *Aanat* with peak expression at night and for *Pax4* with peak expression during daytime (Fig. 1c, left and middle columns; Table 4C). As expected from transcriptomic

Table 3 Grouping of genes according to their molecular and biological functions

Specialized retinal functions	
Visual perception/visual processing	*Grk1*[c], *Rorβ*[b]
Photoreceptor cell development and differentiation	*Grk1*[c], *Pax4*[ac], *Rorβ*[b]
Photoreceptor degeneration	*Grk1*[c], *Pgc-1α*[c]
Non-specialized retinal functions	
Cell cycle, cell death, and cell proliferation	*Asns*[c], *Atf3*[c], *Cdkn1b*[c], *Grk1*[c], *Nr4a1*[a], *Pgc-1a*[c], *Pax4*[ac]
Development	*Pax4*[ac], *Rorβ*[b]
DNA binding	*Atf3*[c], *Egr1*[a], *Fos*[b], *Nr4a1*[a], *Pax4*[ac], *Pgc-1a*[c], *Zbtb8a*
Potassium ion transport	*Cdkn1b*[c], *Kcnv2*[b]
Protein (de)phosphorylation	*Dyrk2*[c], *Grk1*[c]
Receptor activity	*Adra1b*[c], *Drd4*[b]
Response to cAMP	*Aanat*[bc], *Fos*[b], *Pax4*[ac]
Response to drug/hormone	*Adra1b*[c], *Asns*[c], *Cdkn1b*[c], *Drd4*[b], *Fos*[b], *Grk1*[c], *Pax4*[ac]
RNA binding	*Pgc-1a*[c], *Trove2*
Regulation of transcription	*Atf3*[c], *Cdkn1b*[c], *Egr1*[a], *Fos*[b], *Nr4a1*[a], *Pax4*[ac], *Pde8a*[c], *Pgc-1a*[c], *Rorβ*[b], *Zbtb8a*
Intracellular transport/ transport vesicles	*Kif15, Rhobtb1*[c]

The genes listed refer to those identified by microarray analysis to be rhythmic in photoreceptor cells (see Table 2).
[a]Genes previously reported to be under daily regulation in preparations of the whole retina.
[b]Genes previously reported to be under daily regulation in photoreceptor cells.
[c]Genes confirmed by 24-h profiling to be under daily regulation in photoreceptor cells (see Fig. 1; Table 4).

profiling, *Pde6d* failed to show cyclicity in photoreceptor cells or preparations of the whole retina (Fig. 1c, left and middle columns; Table 4C).

24-h profiling of gene expression under constant darkness

To find out whether the daily regulation of the genes in photoreceptor cells is directly illumination-dependent or promoted by the entrainment of a self-cycling clock, rats were kept in DD for one cycle and mRNA recording was conducted during the next cycle (Fig. 1, right column; Table 4). The genes with peak expression during the day uniformly failed to cycle under DD (Fig. 1a, right column; Table 4A) and thus directly depend on ambient light/dark transitions. Four of the six genes showing nocturnal peak expression persisted to cycle under DD (Fig. 1b, right column; Table 4B), thus indicating their clock dependency.

According to previous findings (Schneider *et al.* 2010) and as might be expected for a gene whose expression is governed by the retinal clock system (for review, see Iuvone *et al.* 2005), the transcript amount of *Aanat* persisted to cycle under DD (Fig. 1c, right column; Table 4C). In contrast, *Pax4* – which reportedly does not show a circadian regulation (Rath *et al.* 2009) and *Pde6d* whose expression is not even rhythmic under LD conditions – failed to cycle under DD (Fig. 1c, right column; Table 4C).

Comparison of mRNA levels between photoreceptor cells and retina

On the basis of the qPCR data obtained, the extent to which the daily regulation of the gene expression is specific to photoreceptor cells was investigated. For this, peak mRNA levels of each gene were compared between microdissected photoreceptor cells and preparations of the whole retina under LD 12:12 (Table 5). With the exception of *Pax4*, all genes confirmed to be under daily regulation in photoreceptor cells by 24-h profiling displayed a higher transcript amount in photoreceptor cells than in preparations of the whole retina. Among these genes *Pde8a* (44.5-fold) and *Grk1* (18-fold) showed the most prominent enrichment in photoreceptor cells. Consistent with the validity of our approach, the photoreceptor-specific gene *Pde6d* (for review, see Ionita and Pittler 2007) was seen to show a 28-fold higher mRNA level in photoreceptor cells than in whole retina.

Daily changes in protein levels of Grk1 and Pgc-1α

Among the genes confirmed by 24-h profiling to cycle in photoreceptor cells, *Grk1* and *Pgc-1α* are of particular importance for the function and pathogenesis of the photoreceptor cells (see Discussion). Therefore, daily regulation of both genes was additionally investigated at the protein levels. For this, the strength of immunoreactivity was compared between ZT0, ZT12 and ZT18 by Western blot analysis (Fig. 2). It revealed that for both proteins the intensity of immunoreactivity increased between ZT0 and ZT18. This observation suggests that the daily rhythms in *Grk1* and *Pgc-1α* transcript levels evoke corresponding variations in the protein levels, with the temporal lag reflecting the time necessary to translate mRNA into protein.

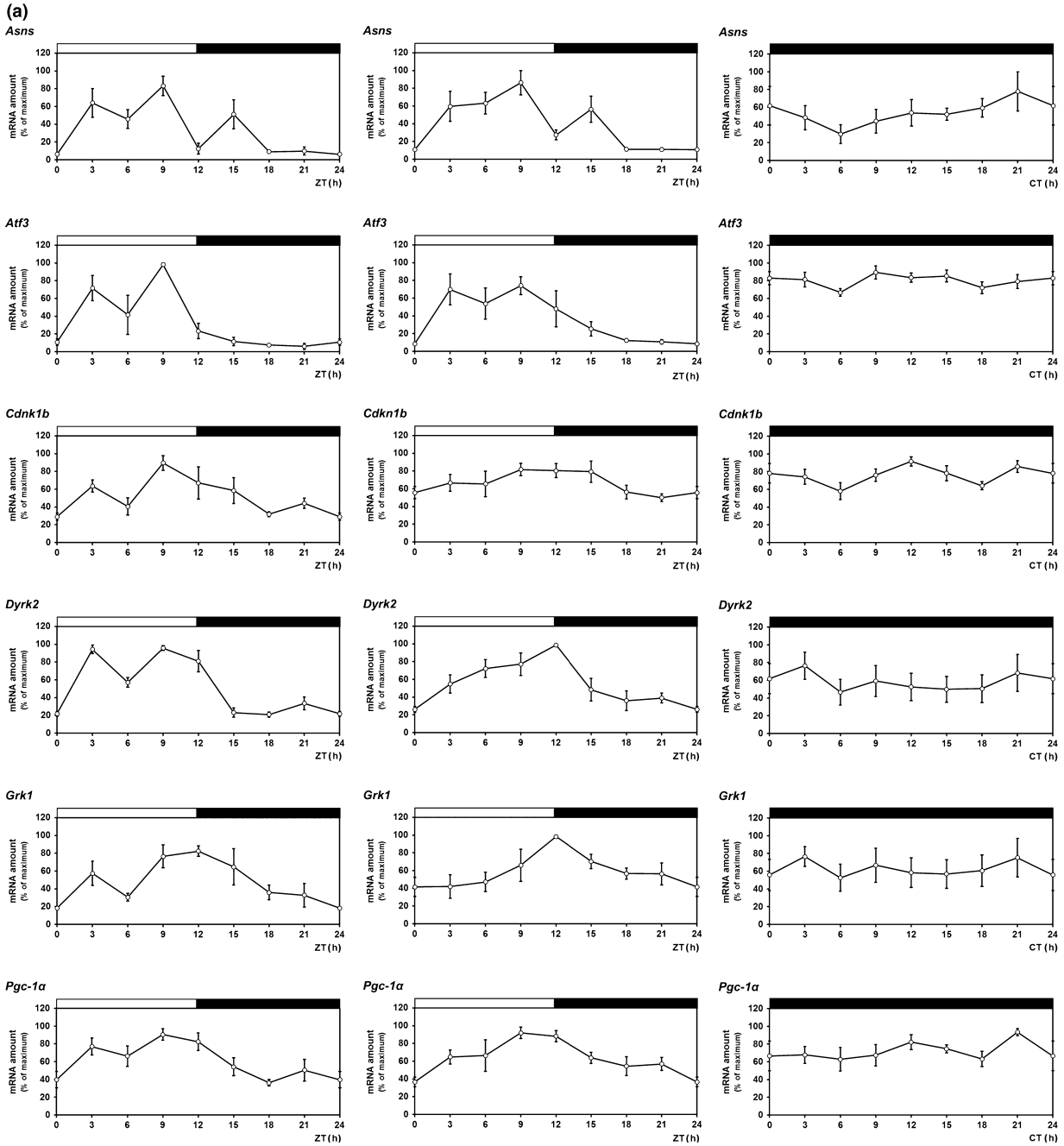

Fig. 1 24-h profiling of (a) genes predicted by microarray analysis to show enhanced expression during daytime, (b) genes predicted by microarray analysis to show enhanced expression at night, (c) reference genes. The mRNA levels are plotted both as a function of Zeitgeber time (ZT) and Circadian time (CT). Transcript levels are recorded in microdissected photoreceptor cells under light/dark (LD) 12:12 (left column), in preparations of whole retina under LD 12:12 (middle column) and in preparations of whole retina under constant darkness (DD) (right column) using qPCR. Statistical analysis of the 24-h profiles illustrated is provided in Table 4. The solid bars indicate the dark period. Data represent a percentage of the maximal value of transcript amount during the 24-h period. The value of ZT0 was plotted twice at both ZT0 and ZT24. Each value represents mean ± SEM (n = 4).

Discussion

As a first step toward the identification of genes whose transcriptional regulation may contribute to photoreceptor adaptation in response to the daily changes in ambient illumination, a gene set indicative for genes under daily regulation in photoreceptor cells was obtained. Since it contains numerous (*Aanat:* Niki *et al.* 1998*; Drd4*: Klitten

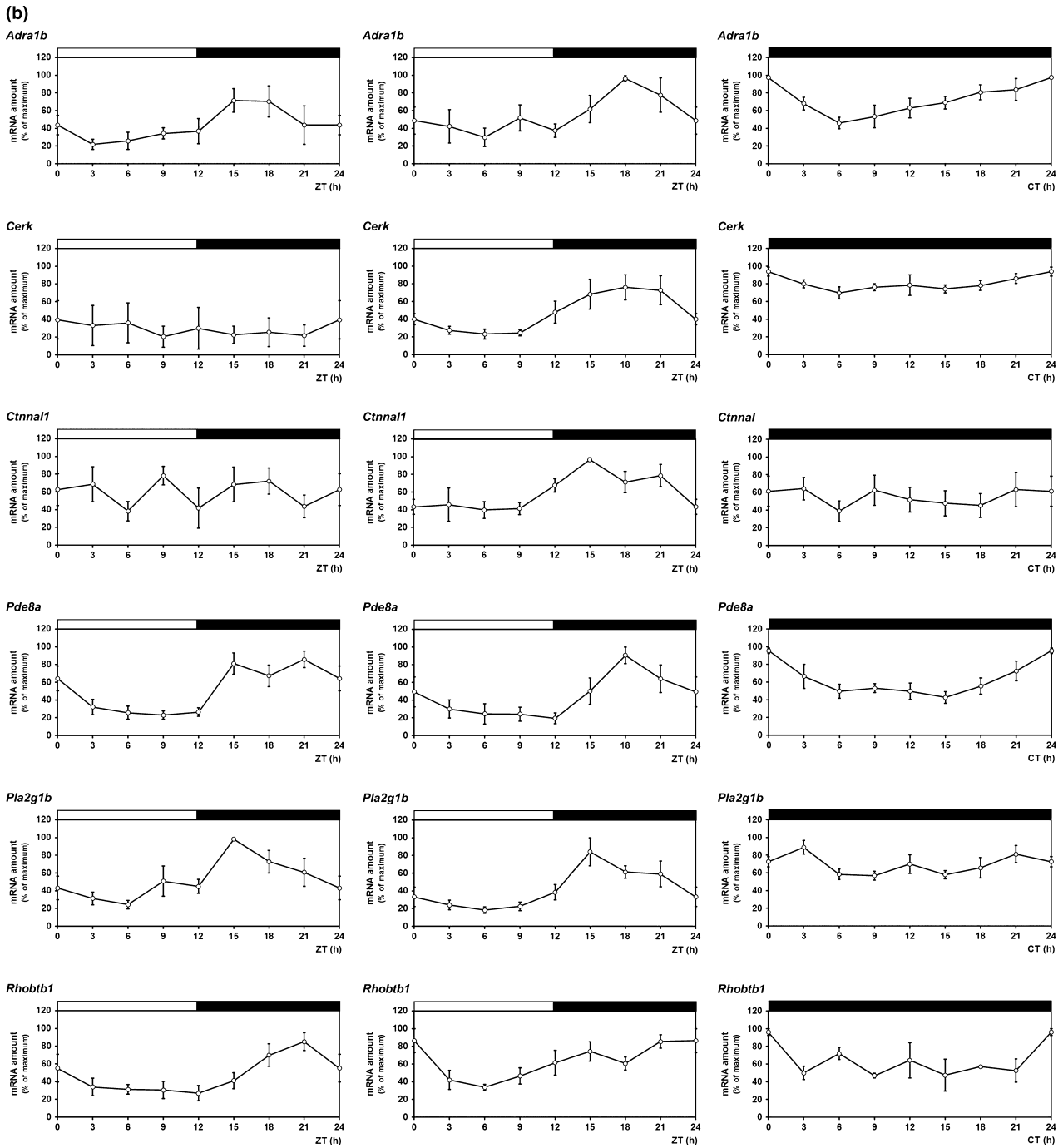

Fig. 1 Continued.

et al. 2008; *Fos*: Yoshida *et al.* 1993; *Kcnv2*: Hölter *et al.* 2012; *Nr1d1*: Sandu *et al.* 2011; *Pde10a*: Wolloscheck *et al.* 2011; *Rorβ*: Sandu *et al.* 2011) (Table 2) but not all of the genes (clock genes: Schneider *et al.* 2010; Sandu *et al.* 2011; *Kcnb*: Hölter *et al.* 2012) previously reported to display cyclicity in photoreceptor cells, this gene set appears to comprise a substantial portion but not the total number of genes under daily regulation in photoreceptor cells. The lack of some relevant genes may be because of the selection criteria applied in this study which do not cover genes whose cycling in photoreceptor cells (i) is smaller than twofold, (ii) follows a 24-h profile which does not result in a difference between ZT6 and ZT18 or (iii) might be negated by a putative cycling in the inner retina in anti-phase with photoreceptor cells.

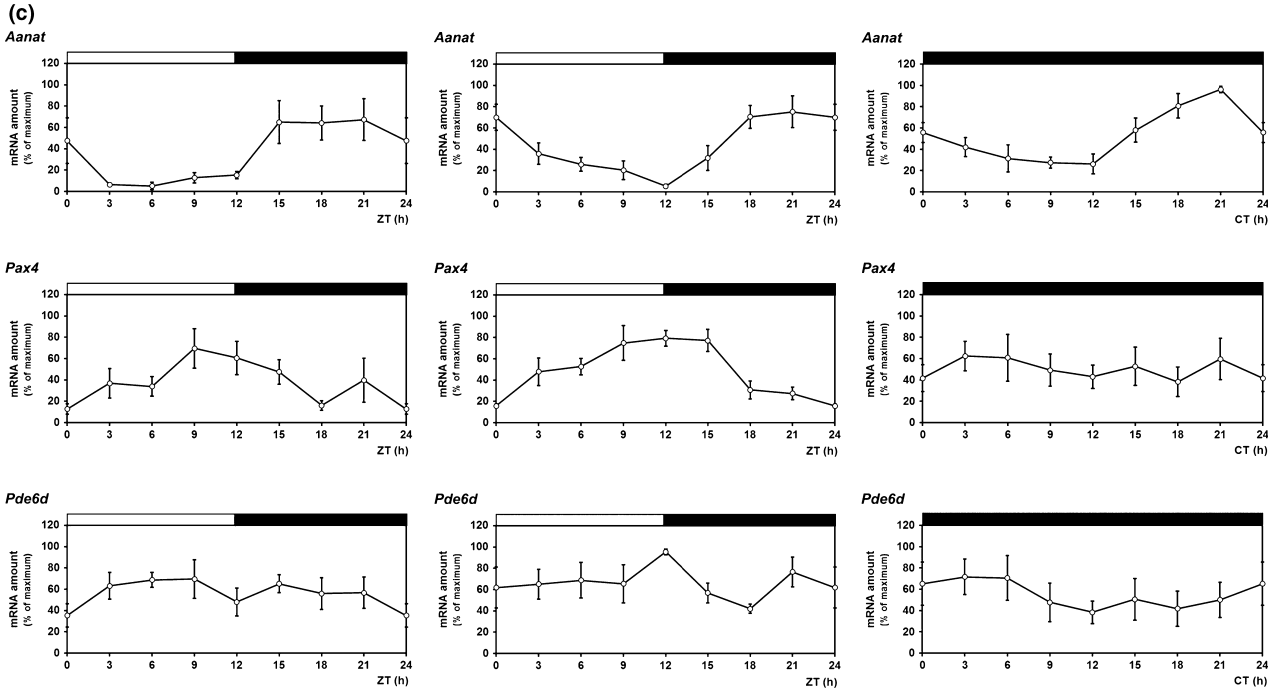

Fig. 1 Continued.

Using this approach, genes could be detected which undergo daily regulation and whose abundance in photoreceptor cells has not yet been confirmed. This provides a basis for the investigation of the precise role of these genes in the function and adaptation of photoreceptor cells and, may even reveal functional capacities of the photoreceptor cell that up

Table 4 Cosinor analysis of the 24-h profiles shown in Fig. 1

Gene	PRC/LD 12:12			Retina/LD 12:12			Retina/DD 12:12		
	p-value	Acrophase	Amplitude	*p*-value	Acrophase	Amplitude	*p*-value	Acrophase	Amplitude
(A)									
Asns	< 0.050	7.46	30.4	< 0.050	8.29	34.3	> 0.050	–	–
Atf3	< 0.050	6.29	30.7	< 0.050	7.48	32.3	> 0.050	–	–
Cdkn1b	< 0.050	10.14	25.4	< 0.050	10.28	17.7	> 0.050	–	–
Dyrk2	< 0.050	7.4	33.6	< 0.050	10.1	35.5	> 0.050	–	–
Grk1	< 0.050	11.1	35.1	< 0.050	12.6	26.1	> 0.050	–	–
Pgc-1α	< 0.050	8.5	24.7	< 0.050	10.5	29.4	> 0.050	–	–
(B)									
Adra1b	< 0.050	19.0	15.3	< 0.050	18.4	38.4	< 0.050	26.0	24.5
Cerk	> 0.050	–	–	< 0.050	19.6	20.9	< 0.050	4.5	12.5
Ctnnal1	> 0.050	–	–	< 0.050	16.4	21.7	> 0.050	–	–
Pde8a	< 0.050	23.5	27.2	< 0.050	21.0	22.2	< 0.050	3.5	32.4
Pla2g1b	< 0.050	17.1	35.1	< 0.050	15.8	20.8	> 0.050	–	–
Rhobtb1	< 0.050	22.4	23.7	< 0.050	22.2	20.2	< 0.050	23.5	0.3
(C)									
Aanat	< 0.050	21.3	28.0	< 0.050	21.4	38.4	< 0.050	21.5	26.0
Pax4	< 0.050	11.1	28.1	< 0.050	11.0	41.3	> 0.050	–	–
Pde6d	> 0.050	–	–	> 0.050	–	–	> 0.050	–	–

(A) Genes with peak expression during daytime (see Fig. 1a), (B) genes with peak expression at night (see Fig. 1b), (C) reference genes (see Fig. 1c). Abbreviations: constant darkness (DD), light/dark conditions (LD), photoreceptor cells (PRC).

Table 5 Comparison of peak mRNA levels between photoreceptor cells (PRC) and preparations of the whole retina (retina) for genes with higher expression during daytime (A), genes with higher expression at night (B) and reference genes (C). The genes listed refer to those subjected to 24-h profiling (see Fig. 1; Table 4)

Gene	PRC	Retina	PRC/retina ratio
(A)			
Asns[c]	119000 ± 2560	9680 ± 1010	12.3
Atf3[c]	15499 ± 4830	12829 ± 4828	1.2
Cdkn1b[c]	771750 ± 6233	89509 ± 14469	8.6
Dyrk2[c]	105042 ± 7340	26409 ± 1773	4.0
Grk1[c]	25760 ± 11452	1435.95 ± 183	18.0
Pgc-1α[c]	5155 ± 969	2485 ± 453	2.1
(B)			
Adra1b[c]	341 ± 151	19 ± 2	17.9
Cerk	1540 ± 422	23102 ± 9770	0.07
Ctnnal1	111696 ± 40932	207767 ± 77647	0.54
Pde8a[c]	3293 ± 1009	74 ± 25	44.5
Pla2g1b[c]	13425 ± 4210	2120 ± 852	6.3
Rhobtb1[c]	162 ± 45	16 ± 6	10.1
(C)			
Aanat[bc]	566 ± 287	150 ± 36	3.8
Pax4[ac]	175826 ± 27926	309335 ± 48120	0.56
Pde6d	654186 ± 180700	23354 ± 4974	28.0

Transcript amount was determined by qPCR and represents the number of transcripts in relation to *Gapdh* x 10^5 (mean ± SEM with n = 4). Note that mRNA levels of the genes are higher in photoreceptor cells than in retina, except for *Cerk*, *Ctnnal1,* and *Pax4*.

[a]Genes previously reported to be under daily regulation in preparations of the whole retina.

[b]Genes previously reported to be under daily regulation in photoreceptor cells.

[c]Genes presently confirmed by 24-h profiling to be under daily regulation in photoreceptor cells (see Fig. 1; Table 4).

until now have not been recognized or have been disregarded. Such a gene is *Atf3* that encodes a member of the ATF/cyclic AMP response element-binding (ATF/CREB) family of transcription factors that – interacting with a number of other transcription factors – bind to cyclic AMP response elements (CREs) in the promoters of various genes (for review, see Hunt *et al.* 2012). As CRE-sequences are also abundant in the *Aanat* promotor (Baler *et al.* 1997; Haque *et al.* 2011) this implies that *Atf3* might contribute to the rhythmicity in *Aanat* transcription and melatonin formation. More generally, *Atf3* is considered an 'adaptive-response' gene whose expression is up-regulated by detrimental and stressful stimuli in many tissues including retina (Takeda *et al.* 2000; Prasad *et al.* 2010; Saul *et al.* 2010; Guo *et al.* 2011) to induce protective and regenerative events (for reviews, see Thompson *et al.* 2009; Hunt *et al.* 2012). The results presented here now suggest that *Atf3* mediates cell adaptation not only in response to pathological signals but also in the context of physiological signals such as ambient lighting conditions.

More importantly, genes with a known impact on photoreceptor physiology and pathology were seen to be under daily regulation, as was the case with *Grk1*, a gene encoding a kinase that phosphorylates the rod visual pigment rhodopsin and in turn thus contributes to the termination of phototransduction (for review, see Arshavsky 2002) and subsequently to the maintenance of rod sensitivity (Cideciyan *et al.* 1998; Sakurai *et al.* 2011). Concurrent daily regulation of *Grk1* activity through differential transcript expression (this study), protein phosphorylation (Horner *et al.* 2005; Osawa *et al.* 2011) and abundance of the *Grk1* inhibitor recoverin (Wiechmann and Sinacola 1997; Zernii *et al.* 2011; Grigoriev *et al.* 2012) is consistent with a key

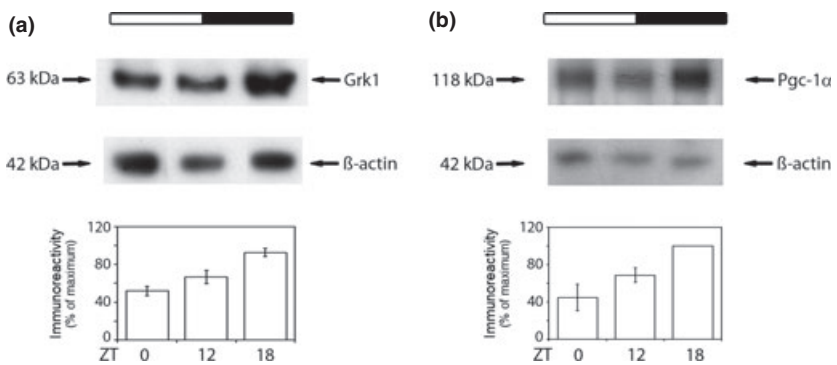

Fig. 2 Western blot analysis of Grk1 (a) and Pgc-1α (b) conducted at different Zeitgeber times (ZTs) during the 24-h cycle. The upper lanes show representative Western blots with Grk1- and Pgc-1α immunostaining at ~63 kDa and ~118 kDa. The lower lanes show the β-actin signal to which the Grk1 and Pgc-1α immunostaining was normalized. The diagrams represent quantifications of immunoreactivity in relation to the corresponding β-actin signal. The solid bar indicates the dark period in LD 12:12. Data were obtained by densitometric measurement and represent percentages of the overall maximal value. Each value is the mean ± SEM ($n = 4$ for Grk1, $n = 3$ for Pgc-1α). Note that for both genes the intensity of immunoreactivity peaks around ZT18.

role of *Grk1* in regulating the daily adjustment of visual processing and sensitivity (for review, see Barlow 2001). Furthermore, *Grk1* deficiency causes light-dependent retinal degeneration (Chen *et al.* 1999; Yetemian *et al.* 2010) and is responsible for the Oguchi form of 'congenital stationary night blindness' (Yamamoto *et al.* 1997; Khani *et al.* 1998; Zhang *et al.* 2005; Hayashi *et al.* 2007; Oishi *et al.* 2007; Azam *et al.* 2009). The daily regulation of *Grk1* expression as reported in this study may therefore also be of pathological interest.

Daily rhythmicity in photoreceptor cells for the first time demonstrates daily changes in *Pgc-1α* gene expression in a tissue of the nervous system. *Pgc-1α* encodes a transcriptional coactivator which coordinates energy metabolism in many tissues and this appears to account for its implication in the pathogenesis of several neurodegenerative disorders including Huntington's and Parkinson's disease (for reviews, see Ross and Thompson 2006; Róna-Vörös and Weydt 2010; Turner and Schapira 2010). *Pgc-1α* was recently found to up-regulate the expression of several genes involved in phototransduction including *Grk1* (see above) (Egger *et al.* 2012). Based on this observation the light-dependent 24-h cyclicity of *Pgc-1α* expression (this study) suggests a role for *Pgc-1α* in mediating the daily adjustment of phototransduction through the transcriptional regulation of relevant target genes. *Pgc-1α* is also reported to play a role in decreasing the light damage susceptibility of the retina and photoreceptor cells (rods), since (i) *Pgc-1α*$^{-/-}$ mice show a pronounced deterioration in retinal morphology and function upon detrimental light treatment, (ii) over-expression of *Pgc-1α* evoked strong anti-apoptotic effects, and (iii) *Pgc-1α* expression is decreased in mouse models of *retinitis pigmentosa* (Egger *et al.* 2012). The protective effect of *Pgc-1α* against light damage (Egger *et al.* 2012), together with the steady increase in *Pgc-1α* expression during the light phase (this study) is consistent with the possibility that the daily regulation of *Pgc-1α* decreases the light damage susceptibility of the retina and photoreceptor cells. Remarkably, Pgc-1α – as a coactivator of Rorα – also regulates the transcription of the clock genes *Bmal1*, *Clock*, *Rev-erbα,* and *Rev-erbβ* and thus influences clock function and physiological rhythmicity (Liu *et al.* 2007). The illumination-dependent regulation of *Pgc-1α* expression (this study) might therefore contribute to the entrainment of the photoreceptor clock/retinal clock system by light (Rohleder *et al.* 2006).

The daily regulation of *Grk1* and *Pgc-1α* observed in this study was found to be directly light-dependent. This suggests that the light-dependent transcriptional regulation of genes which are able to have an effect on visual processing and/or light damage susceptibility contributes to the daily adjustment of photoreceptor function. Of interest is the finding that the daily regulation of *Kcnv2*, a potassium channel essential for visual signal transduction in rods and cones (Wissinger *et al.* 2008, 2011), has recently been observed to be directly clock-dependent (Hölter *et al.* 2012). A hypothetical concept is therefore proposed in which the transcriptional regulation of photoreceptor function is concurrently regulated by ambient illumination (via *Grk1* and *Pgc-1α*) and a circadian clock (via *Kcnv2*).

In conclusion, the present results provide a productive and reliable basis for future research focused on how transcriptional gene regulation has an impact on the adjustment of phototransduction and light damage susceptibility of photoreceptor cells to environmental lighting conditions.

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