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Circadian expression of clock genes and clock-controlled genes in the rat retina

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Abstract

The circadian expression patterns of genes encoding for proteins that make up the core of the circadian clock were measured in rat retina using real-time quantitative PCR (qPCR). Transcript levels of several genes previously used for normalization of qPCR assays were determined and the effect of ischemia–reperfusion on the expression of clock genes was studied. Statistically significant circadian changes in transcript levels were found for: Per2, Per3, Cry2, Bmal1, Rora, Rorb, and Rorc with changes ranging between 1.6- and 2.6-fold. No changes were found for Per1, Cry1, Clock, Rev-erb α, and Rev-erb β. Significant differences in transcript levels were observed for several candidate reference genes: HPRT, GAPDH, rhodopsin, and Thy1 and, consequently, the use of these genes for normalization purposes in qPCR or Northern blots may lead to erroneous conclusions. Ischemia–reperfusion leads to a persistent decrease of Per1 and Cry2, which may be related to the selective degeneration of amacrine and ganglion cells. We conclude that while all clock genes are expressed in the retina, only a few show a clear circadian pattern.

Keywords: Retina; Gene expression; Circadian rhythm; Quantitative PCR; Rat; Clock genes; Ischemia

In mammals, the circadian rhythms in behaviour and physiology are organized by a circadian timing system with a central "master" clock located in the suprachiasmatic nucleus (SCN). Entrainment of the master clock with the environment occurs through daily resetting by photic signals, via the retinohypothalamic tract, and in response to non-photic cues [1,2]. The molecular core of the vertebrate circadian clock is composed of clock genes, whose products interact to drive circadian rhythms in electrical activity of SCN cells resulting in changes in physiology [3–5]. In SCN neurons, CLOCK and BMAL1 (also known as aryl hydrocarbon receptor nuclear translocator-like (Arnt1)) form heterodimers that bind to the promoters of Period (Per)1, Per2, cryptochrome (Cry)1, Cry2, and Rev-erb α genes, resulting in transcriptional activation. Thereafter, the accumulating PER proteins form a complex with the CRY proteins resulting in the repression of Clock and Bmal expression. In addition, Bmal1 expression was repressed by Rev-erb α and activated by Rora and Rorc, thus creating an additional regulatory feedback loop [1,6].

Recently, it has been shown that clock genes are not only localized in the SCN but also in peripheral tissues [7–9]. Moreover, microarray studies have indicated that around 8–10% of all genes in liver and heart show a clock-controlled temporal expression [10,11], suggesting that these peripheral oscillators may have a role in the

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daily alterations of physiological processes. In the retina, the synthesis of melatonin shows a rhythmicity with a peak in the night period. Interestingly in SCN lesioned rats and even in cultured mammalian retina cells, this rhythm is not impaired, indicating an independent retinal oscillator [5,12,13]. However, other retinal rhythms are abolished after SCN-lesion, suggesting that different oscillatory mechanisms may co-exist [12]. In line with the view of the presence of a retinal oscillator, expression of clock genes was found in retinal tissue and for some, but not all, circadian changes have been demonstrated by Northern blotting and in situ hybridization techniques [1,12,14–16]. Several studies have presented evidence for a circadian rhythm of Per2 transcript levels in the rat retina with low levels around Zeitgeber Time (ZT) 23-5 and 2- to 3-fold higher levels between ZT14-17 (lights on between ZT 0-12) [12,17–20]. SCN-lesioning abolished this rhythm [19]. The expression levels of Clock showed a minor elevation only at ZT 18 [14]. The expression of Per1 and Bmal1 did not change significantly over a 24 h period in the rat retina [14,17], although Oishi et al. [18] found that Bmal1 levels in the rat eye oscillated 1.6-fold. To our knowledge there are no publications on Per3, Cry1, Cry2, Rev-erb (α,β), and Ror(a,b,c) levels in the rat retina. In the mouse retina, Cry1 and Cry2 are expressed in the ganglion cell layer and inner nuclear layer (INL), but transcript levels did not oscillate [21]. In the retina of other species, circadian patterns were observed for diverse clock genes: mouse (Per1, 2, and 3) [22,23], chicken (Bmal1) [24], and quail (Clock, Per2, and Per3) [25].

Several other genes in the retina are also known to be expressed following a circadian rhythm. A well-studied clock-controlled target gene is arylalkyl-amine *N*-acetyl transferase (AA-NAT), involved in the synthesis of melatonin. The nocturnal increase in melatonin production is associated with a 3- to 5-fold increase in AA-NAT gene expression around ZT18, the rhythm persisting in constant darkness and after SCN-lesioning [12,19,26,27]. The levels of tryptophan hydroxylase 1 and 2 show a parallel pattern [28]. Circadian rhythms have been reported for D-site albumin promoter binding protein (Dbp) [12,19], recoverin [29], parvalbumin (PV) [30], PKC-β [16], rhodopsin [31], and c-fos [32]. Based on these observations, it may be assumed that many genes in the retina show a varying expression level over a 24 h period.

We here present a comprehensive description of the changes in expression levels of the known clock genes over a 24 h period in the rat retina. Furthermore, since circadian changes in gene expression levels may have an effect on the outcome of real-time quantitative PCR assays, either directly or indirectly via changes in transcripts used for normalization, we determined the daily variation in the expression level of several genes used for normalization [33,34]. Finally, in order to examine the possible deleterious effect of ischemia on retinal function through a loss of clock-gene expression we studied whether retinal ischemia–reperfusion may affect the transcript levels of clock genes.

Materials and methods

Animals

Animal handling and experimental procedures were reviewed and approved by the Ethical Committee for Animal Care and use of the Royal Netherlands Academy for Sciences, acting in accordance with the European Community Council directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize suffering and number of animals used for the study presented here. Adult Male Wistar rats (Harlan, the Netherlands), weighing 275–300 g, were housed under controlled lighting conditions 12:12 LD cycle: lights on 7.00 (ZT 0) ~white light 150 lux and lights off 19.00 (ZT12) ~0.5 lux provided by dim red illumination. The animals used in the study presented here were part of a different study which involved either a complete denervation of their liver or a sham treatment. After 2 weeks of recovery from the operation, the animals were killed. Body weight, food (ad lib), and water intake were monitored and were not significantly different between the two groups. Over a period of 24 h, with intervals of 4 h, animals were sacrificed ($n = 5$–7 per group; including both sham and denervated rats in all groups). Animals were sedated with CO_2 and killed by decapitation. Eyes were removed immediately and rinsed in ice-cold phosphate-buffered saline. The retina, free of vitreous, was isolated from the eye and frozen over dry ice. In order to compare the 24 h oscillation of the clock genes in the retina with a known pattern, a tissue sample of the liver was taken from all animals. During tissue isolation and isolation of the retina, the light conditions were kept identical to the housing condition.

Ischemia–reperfusion

The ischemia–reperfusion procedure has been described in detail previously [33]. In short, adult male Wistar rats (Harlan, the Netherlands) weighing 200–300 g were anesthetized. A steel 30-gauge infusion needle connected to a saline reservoir at 1.70 m height was placed in the middle of the anterior chamber of the left eye. The elevated pressure closes all retinal blood vessels until pressure is lowered. Animals were sacrificed at different reperfusion times: 1, 2, 6, and 12 h ($n = 4$ in each group) with an overdose of sodium pentobarbital (60 mg/ml, i.p.). A second series of retinal cDNA samples with longer reperfusion times up to 2 weeks was used to study the long-term effects on Cry2 and Per1 expression [34].

Real-time quantitative PCR

Isolation of total RNA from rat retinas. The processing of tissue for RT qPCR, and controls for the absence of genomic DNA contamination, have been described in detail [33–35]. Total RNA was isolated by the Trizol, following the manufacturer's instructions (Invitrogen). Isolated RNA was dissolved in 16 μl diethylpyrocarbonate (DEPC)-treated water. The concentration of total retinal RNA isolated was around 12 μg/retina (OD_{260} measurement), with no significant differences between the different ZT groups. The RNA samples had sharp ribosomal RNA bands with no sign of degradation (Agilent Technologies, 2100 Bioanalyser).

Reverse transcription. Total RNA, 2 μg in 4 μl water, was DNase I treated (0.5 U DNAseI, Amplification Grade, Invitrogen), reverse transcribed into first strand cDNA with 100 U/μl of Superscript III (Invitrogen) and 50 ng random hexamer primers, during 50 min at

Table 1
Gene nomenclature, GenBank accession code, primer sequences, and predicted size of the amplified product for the different genes studied

Gene	GenBank	Forward primer	Reverse primer	bp
Bmal1/Arnt1	NM_024362	CCGATGACGAACTGAAACACCT	TGCAGTGTCCGAGGAAGATAGC	215
Clock1	NM_021856	TCTCTTCCAAACCAGACGCC	TGCGGCATACTGGATGGAAT	110
Cry1	NM_198750	AAGTCATCGTGCGCATTTCA	TCATCATGGTCGTCGGACAGA	196
Cry2	NM_133405	GGATAAGCACTTGGAACGGAA	ACAAGTCCCACAGGCGGT	155
Per1	XM_340822	TCTGGTTCGGGATCCACGAA	GAAGAGTCGATGCTGCCAAAG	101
Per2	NM_031678	CACCCTGAAAAGAAAGTGCGA	CAACGCCAAGGAGCTCAAGT	148
Per3	NM_023978	ATAGAACGGACGCCAGAGTGT	CGCTCCATGCTGTGAAGTTT	104
Rev-erb α	NM_145775	ACAGCTGACACCACCCAGATC	CATGGGCATAGGTGAAGATTTCT	101
Rev-erb β	NM_147210	CCCAGCAGTAAAAGAGGTGGTAGA	AAGTCCCAGCTTTTAACAGGTTGAC	98
Rora	XM_217192	CCCGATGTCTTCAAATCCTTAGG	TCAGTCAGATGCATAGAACACAAACTC	89
Rorb	XM_219749	CCCGGGATAACAATGTCTGAGA	TGCCAGCTGATGGAGCTCTT	102
Rorc	XM_227436	TCATCAGCTCCATATTCGACTTTTC	GATGAGAACCAAGGCCGTGTAG	92
DbP [1]	NM_012543	CCTTTGAACCTGATCCGGCT	TGCCTTCATGATTGGCTG	116
DbP [2]		CCCGAGGAACAGAAGGATGA	ATCTGGTTCTCCTTGAGTCTTCTTG	101
AA-NAT	NM_012818	GCAGGTTTGTCCCCACACTTC	GAGGCTCCCAAGAACCAGG	79
PKC-β	NM_012713	TTTCAAGGAGCCCCATGCT	ACGGTAAATGATGCCCTTGCT	85
Tph1	XM_341862	GCATAACCAGCGCCATGAA	ACTGGGCCACCTGCTGACT	83
Rcvrn	NM_080901	TTCCGGACTCCGACCCTAA	TCCTTGAAGTCCAGCGTACC	85

Sequences of primers specific for the other transcripts (PV and the reference genes) were published previously [32].

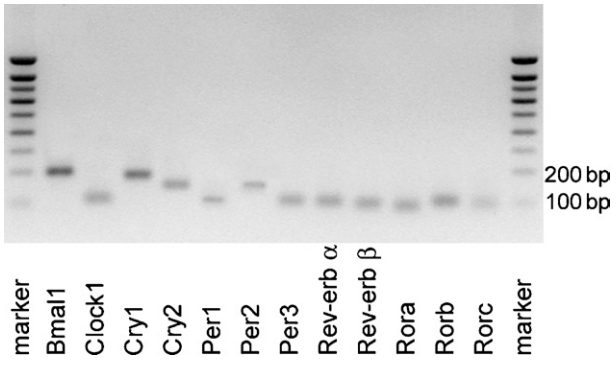

Fig. 1. Agarose gel electrophoresis of qPCR generated amplicons, generated after 40 amplification cycles, for all 12 studied clock genes. The result shows that clock genes are all expressed in the rat retina and that the PCR products are of the expected length (see Table 1). The correct identity of the amplicons was confirmed by sequencing.

50 °C. To the resulting cDNA sample, 15 μl of 10 mM Tris–1 mM EDTA was added, bringing the final volume to a total of 35 μl. From all samples a 1:20 dilution was made and used for qPCR analysis. All samples were stored at −20 °C until analysis.

qPCR primer design. qPCR primer sequences were designed using PrimerExpress V 2.0 software (PE Applied Biosystems, Warrington, UK). The length of the amplicons was kept as close as possible to 100–200 bp and the melting temperature of the primers was set at 59–60 °C [36]. Details of the primers and the GenBank Accession Nos. are given in Table 1. Specificity of the primers was confirmed by BLAST searching. The length of the amplicons was verified by agarose gel electrophoresis (Fig. 1). The qPCR products of all clock genes were partially sequenced and were all confirmed to match the anticipated product.

Real-time quantitative PCR. Methods for the assessment of changes in transcript levels due to ischemia have been described in detail before [33–35]. In short, transcript levels were derived from the accumulation of SYBR green fluorescence in an ABI Prism 5700 Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: 1× SYBR Green PCR buffer, 3 mM $MgCl_2$, 200 μM dATP, dGTP, dCTP, and 400 μM dUTP, 0.5 U Ampli*Taq* Gold, 2 pmol primers, and 2 μl of the 1:20 dilution of the cDNA in a total volume of 20 μl. At the end of the PCR run, the temperature of the sample was slowly raised from 60 to 95 °C while continuously collecting fluorescence data. This allows for the construction of a dissociation curve of the amplified DNA. These curves showed a single amplified product and the absence of primer–dimer formation. Non-template controls were included for each primer pair to check for any significant levels of contaminants. These samples always resulted in at least in a difference of 8 cycles of the C_t values compared to template containing samples. All samples were analysed together on a single 96-well plate.

For all primer pairs, the amplification efficiency (E) was determined on a cDNA dilution series. This allows for a transformation of the observed changes in C_t to the linear domain. The reaction efficiencies had values between 1.8 and 2.0 for all primer combinations. The qPCR C_t values were converted to absolute amounts of cDNA present in the sample (E^{-C_t}) and presented as $C * E^{-C_t}$ with $C = 10^{10}$ [33–35].

Normalization

All cDNA synthesis reactions were performed on 2.0 μg total RNA and it may be expected that the input in the qPCR is not different among the groups and that in fact normalization for the amount of cDNA would not be required. This assumption was tested with a selection of reference genes, used in previous studies on retinal gene expression levels [33,34]. It was found that the transcript levels of some of these genes were significantly different among the ZT groups. The remaining candidate reference genes were subjected to the geNorm-assisted analysis to select the most optimal set of reference genes [37,38].

Statistics

Results are presented as means ± SEM. To demonstrate a statistically significant difference among the different ZT groups, the P value of One-way ANOVA was calculated based on the normalized data (SPSS package V12.0). If the ANOVA indicated such differences, post hoc t tests were performed (Bonferroni) with significance set at 0.05. To exclude any unwanted side effects of the liver denervation on the retinal gene expression levels, a one-way ANOVA was used; no significant effect was disclosed. Moreover, multivariate analysis showed no detectable influence of the liver denervation status while the factor ZT had a significant effect on the gene expression level of diverse transcripts.

Results

Reference genes

Transcript levels of several genes, frequently selected for normalizing PCR or Northern blot assays, showed a differential pattern over a 24 h period (Fig. 2). Genes that showed significant differences among the ZT groups were: hypoxanthine phosphoribosyltransferase (HPRT); $P < 0.0005$, 34% (one-way ANOVA, average coefficient of variation (CV) of the six ZT groups), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; $P < 0.0001$, 31%), rhodopsin ($P < 0.012$, 25%), and thymus cell antigen 1 (Thy1, theta; $P < 0.048$, 31%).

In contrast, β subunit of phosphodiesterase (β-PDE, 26%), 28S (51%), mGluR6 (43%), GluR2 (39%), protein kinase C (PKC-α, 40%), and β-actin (34%) were stably expressed. The geNorm-assisted selection of the most optimal set of reference genes resulted in the selection of β-PDE, GluR2, β-actin, and PKC-α on which the normalization factor was based [37]. Normalization reduced the average CV of all studied genes over the six ZT groups in 30 of the total 35 assays performed in this study, from $37 \pm 9\%$ (mean ± SD) to $30 \pm 8\%$ ($P < 0.0001$; Student's t test for paired samples).

Clock genes

Normalized retinal transcript levels of the studied clock genes over the six ZT groups are presented in Fig. 3. Statistical significant effects of ZT on transcript levels were found for Bmal1, Cry2, Per2, Per3, Rora, Rorb, and Rorc. Levels of Per1 (ANOVA: $P < 0.09$) showed a weak trend.

Per2 changed with a 2.6-fold difference between peak levels at ZT14 and a low at ZT6 ($P < 0.003$). The transcript levels of Per3 increased between ZT2 and ZT10 by 2.1-fold, remaining constant thereafter ($P < 0.019$). Cry2 levels steadily increased between ZT2 and ZT22 with a 1.7-fold difference ($P < 0.009$). Bmal1 transcript levels were constant between ZT6-ZT14 and reached a peak at ZT22 and least levels at ZT 2, representing a

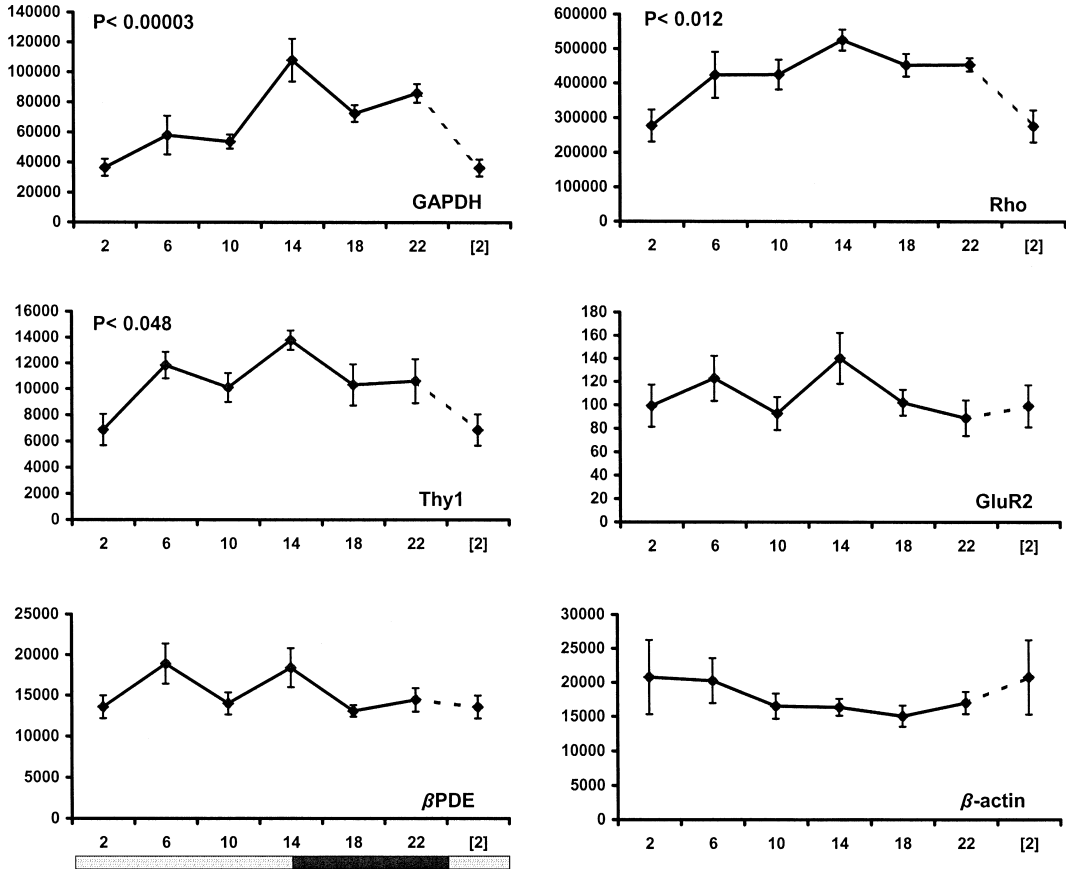

Fig. 2. The 24 h profiles of a selection of candidate reference genes. Transcript levels are presented as mean ± standard error of mean (5–7 animals per ZT) and calculated from the determined C_t values according to $10^{10} * E^{-C_t}$. The presented values are not normalized, statistically significant differences among the ZT groups, indicated by one-way ANOVA, are presented. The horizontal bar at the bottom of the figure represents the lighting conditions 12L:12D. Data from ZT2 are double plotted. Note that from the determined expression levels it may be concluded that, for instance, Cry2 is about 20-fold more abundant than Cry1, but such interpretations between two different transcripts must be made with caution as the amplification efficiency for the first amplification rounds is not known for each PCR [45].

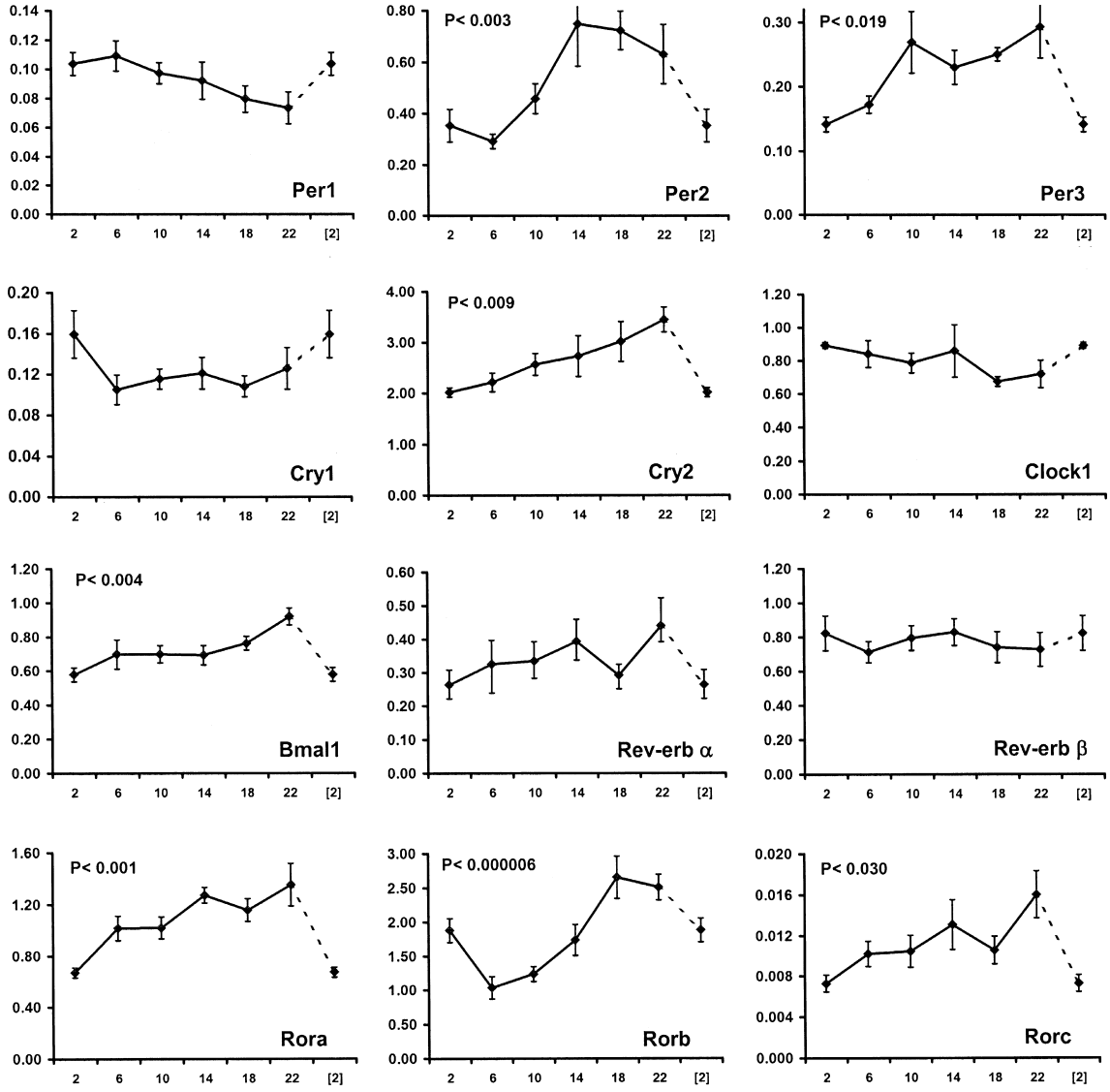

Fig. 3. The circadian patterns of clock genes in the rat retina. The presented values are normalized values, statistically significant differences among the ZT groups, indicated by one-way ANOVA, are given.

1.6-fold difference ($P < 0.004$). The levels of all three Ror isoforms showed circadian patterns. Rora and Rorc levels were both low at ZT2, increasing thereafter with highest levels at ZT22 (Rora: 2.0-fold, $P < 0.001$; Rorc: 2.2-fold, $P < 0.030$). Rorb levels were low at ZT 6 and peaked at ZT18-22 (2.6-fold, $P < 0.0001$). Post hoc statistical analysis tests showed that the differences between groups with the lowest and highest transcript levels were statistically significant. Statistical analysis of the data that were not normalized for differences in cDNA input showed similar results, with patterns for Per2, Cry2, and Rora-c reaching statistical significance.

Transcripts with a circadian expression pattern

Normalized retinal transcript levels of genes with potential circadian expression changes, selected on the basis of a literature survey, are presented in Fig. 4. AA-NAT transcript levels were significantly different among ZT groups ($P < 0.0001$) with a clear peak around ZT18 with levels 3.6-fold higher compared to the levels at ZT6. A gene also involved in the synthesis of melatonin, Tph1, showed a peak expression at ZT2, with stable levels in other ZT groups (1.4-fold; $P < 0.018$). The retinal c-fos transcript levels were 4.2-fold higher during the dark period ($P < 0.008$). The c-jun levels peaked at ZT2 and reduced towards ZT 10 (1.5-fold; $P < 0.02$). Rho transcript levels increased by 1.6-fold between ZT2 and ZT18 ($P < 0.012$). Recoverin (Rcvrn) increased between ZT10 and ZT22 (1.6-fold; $P < 0.0008$). Transcript levels of Dbp assayed with two different primer sets showed no significant effects of ZT. For TH, PV, and PKC-β also no statistically significant differences among ZT groups were found.

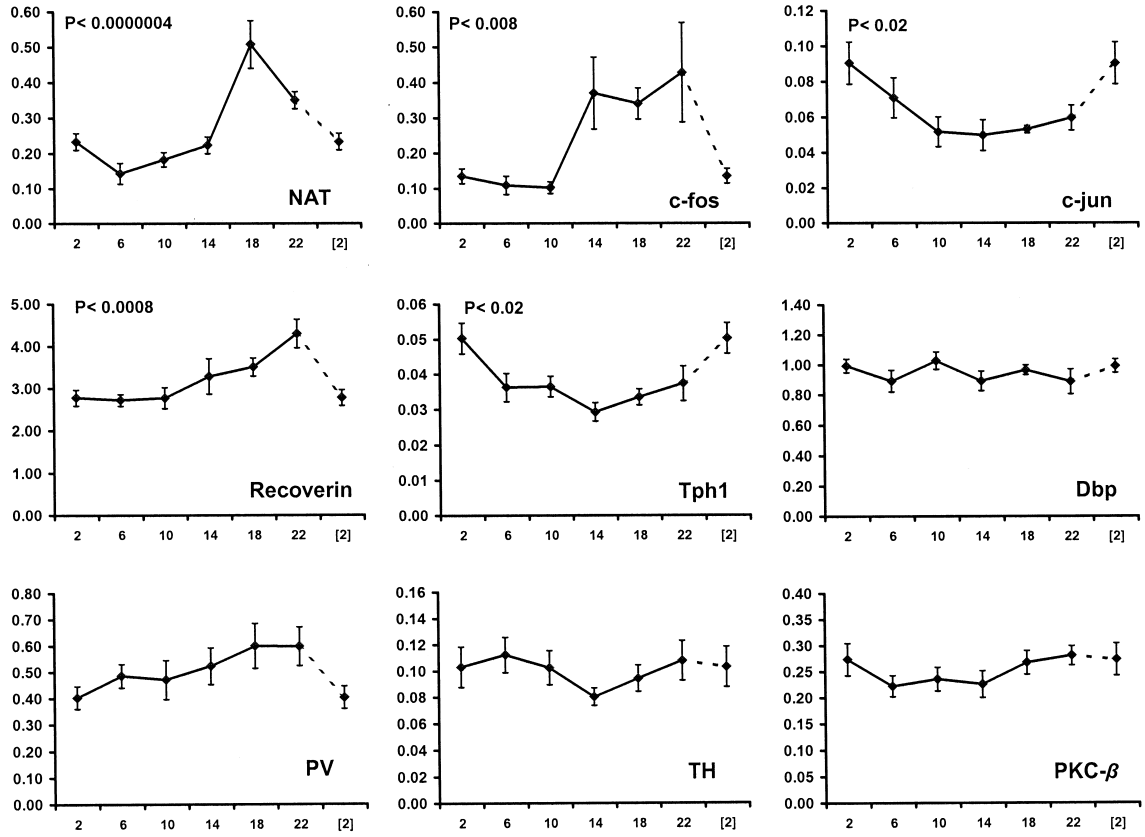

Fig. 4. The circadian patterns of supposed genes with a circadian expression in the rat retina. The presented values are normalized values, statistically significant differences among the ZT groups, indicated by one-way ANOVA, are given.

Ischemia–reperfusion

It was first studied whether clock gene transcript levels from the untreated contralateral eyes showed significant differences in relation to the clock time the rat was sacrificed. No such effects were found and all controls were pooled ($n = 16$). Compared to the control level, ischemia resulted in a transient upregulation in expression level of Rev-erb α with a peak at 6 h ($P < 0.0001$; Bonferroni post hoc; Fig. 5A) and of Per1 with a peak at 1 h ($P < 0.037$; Fig. 5B). Cry2 and Per1 showed at 12 h a trend for decreased transcript levels indicative for a persistent decrease of transcript levels. Because Cry2 and Per1 are localized in the inner retina [39,40] and ischemia leads to neurodegeneration of specific subsets of amacrine cells and of ganglion cells [34,41], the long-term effects of ischemia on these two transcripts were studied. For Cry2, the levels steadily decreased between 2 and 24 h reperfusion to a steady level thereafter for up to 2 weeks (47% decrease; $P < 0.0001$; t test between controls ($n = 26$) and ischemia (48 h–2 weeks; $n = 20$); Fig. 5C). For Per1, the initial upregulation was followed by a gradual decrease corresponding with a loss of 46% between 48 h and 2 weeks ($P < 0.0003$; Fig. 5D).

Discussion

The main results of the study presented here are: (i) Some of the reference genes, used earlier for normalization purposes in qPCR assays like HPRT, GAPDH, and rhodopsin show a distinct circadian variation in their retinal expression level. (ii) Some, but not all, of the known clock genes display a circadian oscillation of their expression level in the rat retina. The oscillation was most evident for Per2, and Rorb. (iii) Retinal ischemia leads to a persistent reduction of Cry1 and Per1.

Implications for normalization in real-time qPCR assays

Real-time qPCR is a powerful and accurate method to detect alterations in the level of transcript in tissues or cell cultures. It is generally accepted that between cDNA samples systematic differences in assessed transcript level are found to be caused by differences in RNA input, efficiency of the reverse transcription, etc. normalization procedures for qPCR assays are commonly applied to correct the outcome of qPCR for such errors. The most frequently used procedure is to express the level of the gene of interest relative to the level of a set of so-called reference genes. The proper way of selecting the optimal

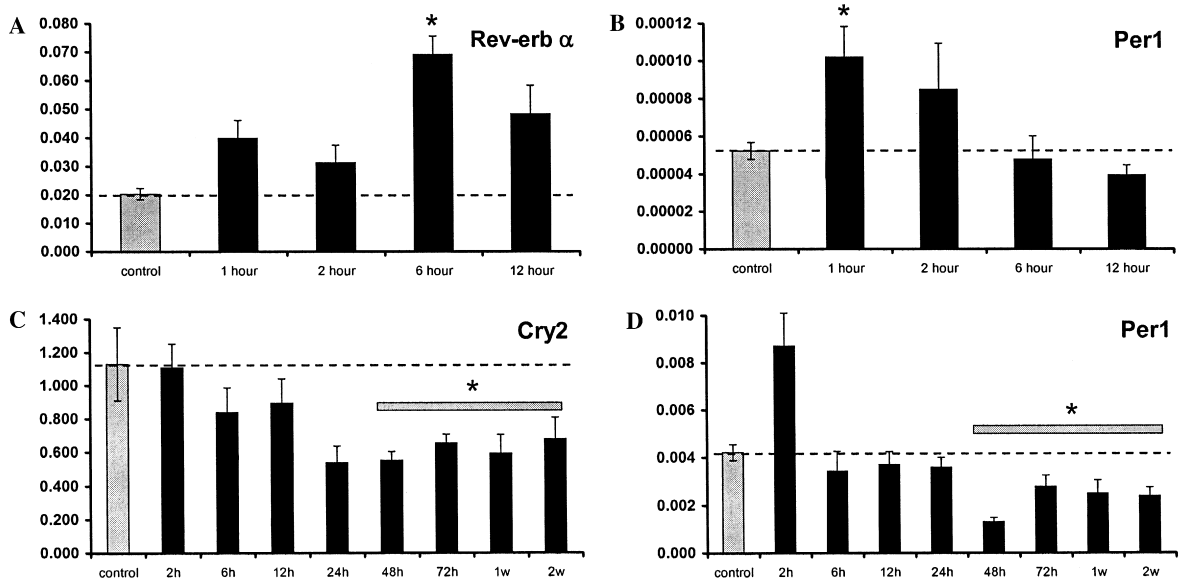

Fig. 5. The effect of 60 min retinal ischemia–reperfusion on the expression level (mean ± SEM) of clock genes resulted in a transient upregulation of (A) Rev-erb α and (B) Per1.The asterisk indicates the reperfusion time at which the Bonferroni post hoc test showed a significant difference. The dashed line indicates the control level. Controls ($n = 16$), all other reperfusion groups ($n = 4$). The long-term effects of 60 min retinal ischemia–reperfusion on the expression level of (C) Cry2 and (D) Per1. At long-term, 24–48 h up to 2 weeks of reperfusion (horizontal grey bar), the transcript levels of Cry2 and Per1 were significantly lower compared to the control level. Controls, $n = 26$; all reperfusion groups $n = 5$–7.

set of reference genes for a particular tissue or experimental setting is an issue of constant deliberation [37,42,43]. However, even after normalization a considerable inter-individual variation in expression level remains [33,34,44]. In this respect, it is of interest to recognize that it is assumed that 8–10% of all gene products display a circadian pattern of expression level, signifying a level of variation that has so far received little attention in the experimental design of qPCR studies [10,11]. Although comparable microarray data are not available for the retina, it may be hypothesized that part of the differences between groups of experimental animals is due to differences in the time of sacrifice with respect to the circadian rhythm. Our finding that HPRT and GAPDH, transcripts often used for normalization in qPCR and Northern blotting assays, exhibited clear circadian patterns in the rat retina with 1.9- and 2.2-fold difference for HPRT and GAPDH, respectively, corroborates this idea. Therefore, in case an experimental design involved groups sacrificed at different ZT points, the use of these genes for normalization purposes may reduce the power of the qPCR assay to detect small differences or even lead to erroneous conclusions. Because no circadian patterns were detected in the levels of β-PDE, 28S, mGluR6, GluR2, PKC-α, and β-actin, these genes seem to be good candidates for compiling a set of reference genes for the rat retina.

Circadian expression levels of clock genes in the retina

After normalization, the transcript levels of Per2, Per3, Cry2, Bmal1, Rora, Rorb, and Rorc showed circadian variations. The daily alteration for Per2 had an amplitude of 2.6-fold. This result is in good agreement with other studies reporting low levels between ZT23 and ZT5 and 2- to 3-fold higher levels around ZT14-17 in the retina [12,17–20] and in other tissues [7,8,45]. The patterns for Per3 were somewhat less pronounced with daily variation of 2.1-fold, while Per1 showed only a weak trend for circadian changes. Namihira et al. [17] were unable to show alterations of Per1 over a 24 h period using Northern blots of rat retina, but in mice Witkovsky et al. [39] showed changes in Per1 immunostaining that is consistent with the low Per1 levels around ZT18-22. Cry2 levels steadily increased between ZT2 and ZT22 with a 1.7-fold difference. Possibly such a low-amplitude daily change cannot be detected by in situ hybridization since in the mouse retina circadian expression of Cry1 and Cry2 was not found [21]. Bmal1 levels showed a significant 1.6-fold alteration with a pattern which is consistent with published data [14,18]. Recently, it was discovered that Bmal1 expression is repressed by Rev-erb α and activated by Rora and Rorc [1,6]. Although we were not able to detect circadian changes in the transcript levels of Rev-erb α or β, all three Ror isoforms displayed clear rhythms with highest levels during the late subjective night. Whether this relates to the upregulation of Bmal1 in the same time window awaits confirmation in further studies and a better understanding of the molecular interactions and cellular localization of peripheral oscillators.

From the qPCR results presented here and the in situ hybridization and Northern blot data presented in other studies, it becomes evident that circadian patterns of

clock genes in samples of total rat retina and mice are either not detectable or relatively minor compared to the changes in other tissues [6,8,23,9]. In fact, transcript levels of liver clock genes in the same animals used in the present study showed a much more robust change over the analysed 24 h period with modulations of 15-fold for Per1 and 7-fold for Cry1 (data not shown, see also [8,16]). Notwithstanding the relative small oscillations in clock gene levels, we were able to demonstrate the well-characterized daily changes in AA-NAT [12,19,26,27], and c-fos [32], evidencing normal circadian patterns in retinal physiology. Circadian patterns were also observed for rhodopsin, recoverin, and Tph1 levels, but the rhythms were somewhat different from those reported [28,29,31]. The alterations at the protein level in PV [30] and PKC-β [16] were not reflected at the transcript level. An unexpected result was that the daily changes in retinal dopamine levels are not associated with detectable changes in TH transcript level. Besides the notion that protein and transcript levels do not necessarily follow parallel changes, it must also be taken into account that the qPCR is performed on samples of the whole retina which limits the interpretation of data obtained from a tissue which, like the retina, is composed of various cell types. When in the different cell types, the daily alterations in the clock genes are out of phase with each other, qPCR will not be able to resolve this in a proper way. This shortcoming may be resolved by using assay techniques with a higher spatial resolution. For instance, using immunocytochemical localization techniques, mouse mPer1 was localized in different subsets of amacrine cells and in ganglion cells. In dopaminergic amacrine cells, the mPer1 expression peaked around ZT 10-14 but whether the levels in other cell types oscillate in parallel remains a topic for further study [39].

Ischemia–reperfusion

Retinal ischemia has a large effect on the gene expression profile in the retina leading to changes in approximately 10% of the genes (unpublished observations). Our recent studies have focused on the changes in AMPA-type glutamate receptors and on cell specific markers of amacrine cell subpopulations using qPCR [33,34]. To investigate whether the clock genes are influenced by ischemia, a characterization of the clock gene response was made. The result shows that in fact the clock genes display rather stable expression levels at least up to 12 h reperfusion with only a transient upregulation for Per1 and Rev-erb α. After 48 h of reperfusion, the ischemic insult leads to a selective partial neurodegeneration in the inner retinal layers, and because Cry2 and Per1 are localized in the inner retina [39,40], the long-term effect of ischemia on these two transcripts was studied. The levels of Cry2 and Per1 were significantly decreased by about 45% between 48 h and 2 weeks of reperfusion. This decrease is in line with the approximate number of ganglion and amacrine cells lost in the inner retina [33,34]. The functional consequences of this loss of Per1 and Cry containing neurons in terms of circadian regulated processes can, on the basis of the current data, only be speculated as the precise role of these clock genes in these cells is not known.

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References

[1] C.B. Green, J.C. Besharse, Retinal circadian clocks and control of retinal physiology, J. Biol. Rhythms 19 (2004) 91–102.
[2] I.H. Kavakli, A. Sancar, Circadian photoreception in humans and mice, Mol. Interv. 2 (2002) 484–492.
[3] H. Okamura, S. Yamaguchi, K. Yagita, Molecular machinery of the circadian clock in mammals, Cell Tissue Res. 309 (2002) 47–56.
[4] R.M. Buijs, A. Kalsbeek, Hypothalamic integration of central and peripheral clocks, Nat. Rev. Neurosci. 2 (2001) 521–526.
[5] G. Tosini, C. Fukuhara, The mammalian retina as a clock, Cell Tissue Res. 309 (2002) 119–126.
[6] T.K. Sato, S. Panda, L.J. Miraglia, T.M. Reyes, R.D. Rudic, P. McNamara, K.A. Naik, G.A. FitzGerald, S.A. Kay, J.B. Hogenesch, A functional genomics strategy reveals Rora as a component of the mammalian circadian clock, Neuron 43 (2004) 527–537.
[7] M.E. Young, P. Razeghi, H. Taegtmeyer, Clock genes in the heart: characterization and attenuation with hypertrophy, Circ. Res. 88 (2001) 1142–1150.
[8] T. Yamamoto, Y. Nakahata, H. Soma, M. Akashi, T. Mamine, T. Takumi, Transcriptional oscillation of canonical clock genes in mouse peripheral tissues, BMC Mol. Biol. 5 (2004) 18.
[9] E. Muhlbauer, S. Wolgast, U. Finckh, D. Peschke, E. Peschke, Indication of circadian oscillations in the rat pancreas, FEBS Lett. 564 (2004) 91–96.
[10] F. Delaunay, V. Laudet, Circadian clock and microarrays: mammalian genome gets rhythm, Trends Genet. 18 (2002) 595–597.
[11] K.F. Storch, O. Lipan, I. Leykin, N. Viswanathan, F.C. Davis, W.H. Wong, C.J. Weitz, Extensive and divergent circadian gene expression in liver and heart, Nature 417 (2002) 78–83.
[12] K. Sakamoto, K. Oishi, M. Shiraishi, S. Hamano, H. Otsuka, Y. Miyake, N. Ishida, Two circadian oscillatory mechanisms in the mammalian retina, NeuroReport 11 (2000) 3995–3997.
[13] G. Tosini, M. Menaker, Circadian rhythms in cultured mammalian retina, Science 272 (1996) 419–421.
[14] M. Namihira, S. Honma, H. Abe, Y. Tanahashi, M. Ikeda, K. Honma, Circadian rhythms and light responsiveness of mammalian clock gene, Clock and BMAL1, transcripts in the rat retina, Neurosci. Lett. 271 (1999) 1–4.
[15] D.J. Kennaway, T.J. Varcoe, V.J. Mau, Rhythmic expression of clock and clock-controlled genes in the rat oviduct, Mol. Hum. Reprod. 9 (2003) 503–507.

[16] R. Gabriel, J. Lesauter, R. Silver, A. Garcia-Espana, P. Witkovsky, Diurnal and circadian variation of protein kinase C immunoreactivity in the rat retina, J. Comp. Neurol. 439 (2001) 140–150.
[17] M. Namihira, S. Honma, H. Abe, S. Masubuchi, M. Ikeda, K. Honmaca, Circadian pattern, light responsiveness and localization of rPer1 and rPer2 gene expression in the rat retina, NeuroReport 12 (2001) 471–475.
[18] K. Oishi, K. Sakamoto, T. Okada, T. Nagase, N. Ishida, Antiphase circadian expression between BMAL1 and period homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats, Biochem. Biophys. Res. Commun. 253 (1998) 199–203.
[19] K. Oishi, K. Sakamoto, N. Ishida, Bimodal circadian expression of serotonin N-acetyltransferase mRNA in the retina of rats under restricted feeding, Neurosci. Lett. 351 (2003) 21–24.
[20] K. Sakamoto, T. Nagase, H. Fukui, K. Horikawa, T. Okada, H. Tanaka, K. Sato, Y. Miyake, O. Ohara, K. Kako, N. Ishida, Multitissue circadian expression of rat period homolog (rPer2) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain, J. Biol. Chem. 273 (1998) 27039–27042.
[21] Y. Miyamoto, A. Sancar, Vitamin B2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals, Proc. Natl. Acad. Sci. USA 95 (1998) 6097–6102.
[22] L.P. Shearman, M.J. Zylka, D.R. Weaver, L.F. Kolakowski Jr., S.M. Reppert, Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei, Neuron 19 (1997) 1261–1269.
[23] M.J. Zylka, L.P. Shearman, D.R. Weaver, S.M. Reppert, Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain, Neuron 20 (1998) 1103–1110.
[24] N.W. Chong, S.S. Chaurasia, R. Haque, D.C. Klein, P.M. Iuvone, Temporal–spatial characterization of chicken clock genes: circadian expression in retina, pineal gland, and peripheral tissues, J. Neurochem. 85 (2003) 851–860.
[25] T. Yoshimura, Y. Suzuki, E. Makino, T. Suzuki, A. Kuroiwa, Y. Matsuda, T. Namikawa, S. Ebihara, Molecular analysis of avian circadian clock genes, Brain Res. Mol. Brain Res. 78 (2000) 207–215.
[26] L. Engel, L. Vollrath, R. Spessert, Arylalkylamine N-acetyltransferase gene expression in retina and pineal gland of rats under various photoperiods, Biochem. Biophys. Res. Commun. 318 (2004) 983–986.
[27] K. Sakamoto, N. Ishida, Circadian expression of serotonin N-acetyltransferase mRNA in the rat retina, Neurosci. Lett. 245 (1998) 113–116.
[28] J. Liang, J.H. Wessel III, P.M. Iuvone, G. Tosini, C. Fukuhara, Diurnal rhythms of tryptophan hydroxylase 1 and 2 mRNA expression in the rat retina, NeuroReport 15 (2004) 1497–1500.
[29] A.F. Wiechmann, M.K. Sinacola, Diurnal expression of recoverin in the rat retina, Brain Res. Mol. Brain Res. 45 (1997) 321–324.
[30] R. Gabriel, J. Lesauter, T. Banvolgyi, G. Petrovics, R. Silver, P. Witkovsky, AII amacrine neurons of the rat retina show diurnal and circadian rhythms of parvalbumin immunoreactivity, Cell Tissue Res. 315 (2004) 181–186.
[31] M. von Schantz, R.J. Lucas, R.G. Foster, Circadian oscillation of photopigment transcript levels in the mouse retina, Brain Res. Mol. Brain Res. 72 (1999) 108–114.
[32] K. Yoshida, K. Kawamura, J. Imaki, Differential expression of c-fos mRNA in rat retinal cells: regulation by light/dark cycle, Neuron 10 (1993) 1049–1054.
[33] F. Dijk, E. Kraal-Muller, W. Kamphuis, Ischemia-induced changes of AMPA-type glutamate receptor subunit expression pattern in the rat retina: a real-time quantitative PCR study, Invest. Ophthalmol. Vis. Sci. 45 (2004) 330–341.
[34] F. Dijk, S. Van Leeuwen, W. Kamphuis, Differential effects of ischemia/reperfusion on amacrine cell subtype-specific transcript levels in the rat retina, Brain Res. 1026 (2004) 194–204.
[35] W. Kamphuis, A. Schneemann, L.M. Van Beek, A.B. Smit, P.F. Hoyng, E. Koya, Prostanoid receptor gene expression profile in human trabecular meshwork: a quantitative real-time PCR approach, Invest. Ophthalmol. Vis. Sci. 42 (2001) 3209–3215.
[36] S.A. Bustin, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, J. Mol. Endocrinol. 25 (2000) 169–193.
[37] J. VandeSompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. DePaepe, F. Speleman, Accurate normalization of rela-time quantitative RT-PCR data by geometric averaging of multiple interval control genes, Genome Biol. 3 (2002) 0034.1–0034.11.
[38] C. Tricarico, P. Pinzani, S. Bianchi, M. Paglierani, V. Distante, M. Pazzagli, S.A. Bustin, C. Orlando, Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies, Anal. Biochem. 309 (2002) 293–300.
[39] P. Witkovsky, E. Veisenberger, J. Lesauter, L. Yan, M. Johnson, D.Q. Zhang, D. McMahon, R. Silver, Cellular location and circadian rhythm of expression of the biological clock gene Period 1 in the mouse retina, J. Neurosci. 23 (2003) 7670–7676.
[40] C.L. Thompson, C.B. Rickman, S.J. Shaw, J.N. Ebright, U. Kelly, A. Sancar, D.W. Rickman, Expression of the blue-light receptor cryptochrome in the human retina, Invest. Ophthalmol. Vis. Sci. 44 (2003) 4515–4521.
[41] F. Dijk, W. Kamphuis, Ischemia-induced Alterations of AMPA-type glutamate receptor subunit. Expression patterns in the rat retina-an immunocytochemical study, Brain Res. 997 (2004) 207–221.
[42] K. Dheda, J.F. Huggett, S.A. Bustin, M.A. Johnson, G. Rook, A. Zumla, Validation of housekeeping genes for normalizing RNA expression in real-time PCR, BioTechniques 37 (2004) 112–119.
[43] W.M. Freeman, S.J. Walker, K.E. Vrana, Quantitative RT-PCR: pitfalls and potential, BioTechniques 26 (1999) 112–115.
[44] J. Alfonso, G.D. Pollevick, A. Castensson, E. Jazin, A.C. Frasch, Analysis of gene expression in the rat hippocampus using real-time PCR reveals high inter-individual variation in mRNA expression levels, J. Neurosci. Res. 67 (2002) 225–234.
[45] H.K.Nogva and K.Rudi, Potential influence of the first PCR cycles in real-time comparative gene quantifications, BioTechniques, 37 (2004) 246-3.