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Patrick Vancura, University Medical Center of the Johannes Gutenberg University
Shaima Abdelhadi, University Medical Center of the Johannes Gutenberg University
Erika Csicsely, University Medical Center of the Johannes Gutenberg University
Kenkichi Baba, Morehouse School of Medicine
Gianluca Tosini, Emory University
Paul Iuvone, Emory University
Rainer Spessert, University Medical Center of the Johannes Gutenberg University

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**RESEARCH ARTICLE**

**Gnaz couples the circadian and dopaminergic system to G protein-mediated signaling in mouse photoreceptors**

Patrick Vancura1*, Shaima Abdelhadi1, Erika Csicsely1, Kenkichi Baba2, Gianluca Tosini2, P. Michael Iuvone3, Rainer Spessert1

1 Institute of Functional and Clinical Anatomy, University Medical Center of the Johannes Gutenberg University, Mainz, Germany, 2 Neuroscience Institute and Department of Pharmacology and Toxicology, Morehouse School of Medicine, Atlanta, Georgia, United States of America, 3 Department of Ophthalmology, Emory University School of Medicine, Atlanta, Georgia, United States of America

* p.vancura@uni-mainz.de

**Abstract**

The mammalian retina harbors a circadian clockwork that regulates vision and promotes healthiness of retinal neurons, mainly through directing the rhythmic release of the neurohormones dopamineD4 acting on dopamine D4 receptors and melatoninD2 acting on MT1 and MT2 receptors. The gene GnazDa unique Gi/o subfamily memberDwas seen in the present study to be expressed in photoreceptors where its protein product Gαz shows a daily rhythm in its subcellular localization. Apart from subcellular localization, Gnaz displays a daily rhythm in expressionDw ith peak values at night in preparations of the whole retina, microdissected photoreceptors and photoreceptor-related pinealocytes. In retina, Gnaz rhythmicity was observed to persist under constant darkness and to be abolished in retina deficient for Clock or dopamine D4 receptors. Furthermore, circadian regulation of Gnaz was disturbed in the db/db mouse, a model of diabetic retinopathy. The data of the present study suggest that Gnaz links the circadian clockworkDvia dopamine acting on D4 receptorsDto G protein-mediated signaling in intact but not diabetic retina.

**Introduction**

The mammalian retina is known to harbor an intrinsic circadian clock system [1, 2] where circadian clocks are localized in various types of retinal neurons including horizontal cells, amacrincells [3, 4] and photoreceptors [5±7]. The molecular clock enables the retina to adjust its physiology to adapt to daily changes in environmental demands. In particular, the retinal clock promotes adjustment of visual processing [8] that manifests in circadian changes in the retinal electrical responses to light, which can be measured using the ERG [9, 10]. Clock-dependent regulation of retinal physiology involves the neurohormones melatonin and dopamine [11, 12]. Both neurohormones play opposing roles in retinal adaptation. While melatonin by acting on MT1 and MT2 receptors promotes adaptation to darkness [13, 14] dopamine supports adaptation to light by acting on D4 receptors [15±18].
Heterotrimeric G proteins mediate the stimulation of G protein coupled receptors (GPCRs) to regulate a broad range of physiological functions in various tissues [19]. Each G protein consists of an α-subunit that binds and hydrolyzes GTP, as well as a β- and a γ-subunit. Sixteen types of α-subunits, five types of β-subunits and thirteen types of γ-subunits are known in humans. The sixteen α-subunits are encoded by a gene superfamily that can be subdivided into four different classes: Gna1, Gnas, Gnaq and Gna12/13. The Gna1 class encompasses the four families Gna1, Gnaz, Gnao and Gnat. Based on their sequence similarities the Gnaz family consisting of exclusively the gene Gnaz and the Gnat family involving the genes Gnat1 and Gnat2 are considered α-transducins. However, Gnaz has no function in vision and for this reason is referred to as non-visual α-transducin while Gnat1 and Gnat2 are involved in phototransduction and therefore are referred to as visual α-transducins.

The transcription of α-transducin display a 24-h rhythm in the rodent retina [20±22]. However, it is an open question whether the α-transducin investigated refers to the non-visual α-transducin Gnaz and/or the visual α-transducins Gnat1 and/or Gnat2. The aim of the present study was to investigate (1) as to what extent expression of Gnaz, Gnat1 and/or Gnat2 is under daily regulation in retina, photoreceptors and photoreceptor-related pinealocytes, (2) depends on a circadian clock, (3) is regulated by the neurohormones melatonin and dopamine and (4) is disturbed in diabetic retinopathy.

Material and methods

Animals

Adult (age of 10±12 weeks) male and female mice (see below) not carrying rd mutations and, when indicated, rats (Sprague Dawley) were used in this study. With the exception of the mouse model for diabetic retinopathy (C57BL/6j db/db), the mice used were melatonin-proficient (C3H/f/+Clock+/+, C3H/f/+Clock/-/-, C3H/f/+MT1+/+, C3H/f/+MT1-/-, C3H/f/+Drd4+/+ and C3H/f/+Drd4-/-). Genotyping was performed by PCR analysis of genomic DNA. C3H/f/+Clock+/+, C3H/f/+Clock/-/-, C3H/f/+MT1+/+, C3H/f/+MT1-/-, C3H/f/+Drd4+/+ and C3H/f/+Drd4-/- were generated by backcrossing Clock mice (strain name: B6.129S4-Clocktm1.1Rep/J) obtained from Jackson Laboratory (Bar Harbor, ME, USA) against C3H/f/+ mice for ten generations. Diabetic (db/db) and non-diabetic (db/+) mice (strain name: BKS.Cq-Dock7m+/+Leprdb/J) were purchased from Jackson Laboratory. 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 to non-diabetic mice (blood glucose level: 138 ± 4 mg/dl; bodyweight: 25 ± 1 g).

Animals were kept under light/dark 12:12 (LD) cycles for 3 weeks under standard laboratory conditions (illumination with 200 lux at cage level during the day and dim (< 5 lux) red light during the night, 20 ± 1°C, water and food ad libitum) and sacrificed at 3-h intervals over a period of 24 hours by decapitation following anesthesia with carbon dioxide. In order to determine 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).

Laser microdissection and pressure catapulting (LMPC)

To prepare the retinae for LMPC, the HOPE technique (DCS, Hamburg, Germany) was applied for fixation [23]. Photoreceptors were isolated from stained sections in a contact- and
contamination-free manner by using the LMPC technique as described previously [6]. The purity grades of the preparations obtained were verified by using a specific gene marker of photoreceptors, namely Nrl as a marker for rods [24], and of inner retinal neurons, namely Th as a marker for amacrine cells [25]. In comparison to 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 polymerase chain reaction (qPCR)

Using the RNeasy Micro Kit (Qiagen, Hilden, Germany) RNA was extracted from the tissue samples as described previously [26]. Subsequently first stranded cDNA was synthesized 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 samples in RNase-free water (1:4) quantitative PCR was performed. Quantitative PCR was carried out in a total volume of 20 µl containing 10 µl Absolute™ QPCR SYBR® Green Fluorescein Mix (Abgene), 0.2 µl of each primer (10 µM), 4.6 µl RNase-free water, and 5 µl sample. Primer sequences are listed in Table 1. PCR amplification and quantification were performed 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). To further confirm the specificity of the primer sets used, particular of those for the genes Gnaz, Gnat1 and Gnat2—sequencing of the generated amplicons was performed. According to the obtained sequences, the designed primer sets were verified as highly selective to their respective targets. The amount of mRNA in the samples was calculated from the measured threshold cycles (Ct) 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.

Western blot analysis

For Western blot analysis, samples were loaded on 4±12% NuPAGE Novex Bis-Tris gels (Invitrogen, Carlsbad, CA, USA), separated and then blotted onto PVDF membrane (Westran S, Whatman Inc., Sanford, ME, USA). For immunodetection, membranes were blocked in 5% skim milk and incubated with rabbit polyclonal anti-Gαz antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, USA, sc-388) overnight at 4°C. Using an ECL detection system (GE Healthcare Amersham, Freiburg, Germany), the horseradish-peroxidase-conjugated secondary antibody (goat anti-rabbit-HRP 1:10.000; Sigma-Aldrich, St. Louis, MO, USA; A0545) was visualized. Monoclonal anti-β-actin HRP-coupled primary antibody (1:40.000; Sigma-Aldrich, St. Louis, MO, USA; A3854) was used to control for equal protein loading. Densitometry measurement was performed using Image Lab 4.1 (Bio-Rad Laboratories, Hercules, CA, USA).

Fluorescence microscopy

Eyes were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan) and frozen in melting 2-methyl-butane (VWR, Radnor, PA, USA). Cryosections (10 µm) were treated with 0.1% Tween 20 in PBS, washed with PBS and then blocked with antibody diluent containing background reducing components (Dako, Capitania, CA, USA) for 30 minutes at room temperature. Subsequently sections were incubated with primary antibodies (anti-Gαz polyclonal antibody, 1:100, Santa Cruz Biotechnology, Santa Cruz, USA, sc-388; anti-Centrin3 polyclonal antibody, 1:100, kindly provided by Prof.
Table 1. Primer sequences used for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primer Sequence 5' to 3'</th>
<th>Length of PCR Product [bp]</th>
</tr>
</thead>
</table>
| m18S | NR_003278.3      | Forward: CAACACGGGAAACCTCAC  
Reverse: TCTCTCCACACACTTAAAGAC  | 110 |
| mDrd4 | NM_007878.2     | Forward: GTGGAACGCTTTCTTCGG  
Reverse: GTGGAAGGCACTGTTGAC  | 116 |
| mGapdh | BC082592.1    | Forward: CATCCCAAGACCTGAAAC  
Reverse: TCAAGATGCTGCTTCCAC  | 144 |
| mGna11 | NM_010301.3    | Forward: GCAATGACAGAGCCTTAGAGG  
Reverse: ACAGGAGAGGAGCCTAGTG  | 106 |
| mGna12 | NM_010302.2    | Forward: TGGAGGCGCTTTCTTCGG  
Reverse: TGGAGGCACTGTTGAC  | 149 |
| mGna13 | NM_010303.3    | Forward: CCCTTGTAACCACCACTTC  
Reverse: GAGCCTGCCTGCTTGC  | 102 |
| mGna14 | NM_008137.4    | Forward: TCTGACAGAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 135 |
| mGna15 | NM_010304.3    | Forward: TGGAGGCGCTTTCTTCGG  
Reverse: TGGAGGCACTGTTGAC  | 143 |
| mGna1 | NM_010305.1    | Forward: CCCTTGTAACCACCACTTC  
Reverse: GAGCCTGCCTGCTTGC  | 101 |
| mGna2 | NM_008138.4    | Forward: TCTGACAGAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 125 |
| mGna3 | NM_010306.3    | Forward: AGCAGTGTCAGGGAATAGGG  
Reverse: CTCGAGGATGCTGCTTGC  | 138 |
| mGna4 | NM_010307.3    | Forward: TGCTTCAAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 114 |
| mGnao1 | NM_010308.3   | Forward: GACGCTGTCAGGGAATAGGG  
Reverse: TCTGAGGATGCTGCTTGC  | 116 |
| mGnaq | NM_008139.5    | Forward: GCCACAGAGGATTGTTAAC  
Reverse: TTAAAGGCAAGGAGTTGAC  | 120 |
| mNas | NM_010309.4    | Forward: CAAGCTGTCAGGGAATAGGG  
Reverse: CTCGAGGATGCTGCTTGC  | 146 |
| mNat1 | NM_008140.2    | Forward: TCTGACAGAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 110 |
| mNat2 | NM_008141.3    | Forward: GCAAGCTGTCAGGGAATAGGG  
Reverse: TCTGAGGATGCTGCTTGC  | 129 |
| mNaz | NM_010311.3    | Forward: GCTGTCAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 123 |
| mNb1 | NM_008142.4    | Forward: CTGTCAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 112 |
| mNb2 | NM_010312.4    | Forward: GTCCATGAGGGCACAGAG  
Reverse: GTGCAAGCGAGGAGCAAG  | 121 |
| mNb3 | NM_013530.1    | Forward: CTGTCAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 142 |
| mNb4 | NM_013531.4    | Forward: TACTCTGTCAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 142 |
| mNb5 | NM_010313.2    | Forward: GCTGTCAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 112 |
| mNgt1 | NM_010314.2    | Forward: AGTTCATGAGGGCACAGAG  
Reverse: GTGCAAGCGAGGAGCAAG  | 103 |
| mNgt2 | NM_023121.2    | Forward: AGTTTTCCTCAGGGAATAGGG  
Reverse: AAGGAGAGGAGCCTAGTG  | 146 |
| mNrl | NM_008736.3    | Forward: AGTTCATGAGGGCACAGAG  
Reverse: GTGCAAGCGAGGAGCAAG  | 149 |
| mTh | NM_009377.1    | Forward: CAGCCCTACAAAGATCAAC  
Reverse: GTACGGGTCAAACTTACAG  | 129 |

(Continued)
Wolfrum, Institute of Zoology, Johannes Gutenberg University, Mainz, Germany) in antibody diluent overnight at 4°C. Following removal of the primary antibody, slides were washed with PBS and incubated with Alexa Fluor488 or Alexa Fluor568 conjugated donkey anti-mouse or donkey anti-rabbit secondary antibodies (Molecular Probes, Leiden, The Netherlands) for 1 hour in antibody diluent at room temperature. Cell nuclei were counterstained with DAPI (Thermo Fisher Scientific, Waltham, USA). Negative immunohistochemistry controls were performed in parallel by omission of primary antibodies. After they were washed, sections were cover slipped with fluorescent mounting medium (Dako, Capinteria, CA, USA). Stained retinal sections were examined by Axiophot microscope (Zeiss, Jena, Germany) and images were obtained with a digital imaging system (JVC, Yokohama, Japan).

### Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM) of four (qPCR and Western blot) independent experiments. 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-h 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 [27, 28]. The model can be expressed according to the equation: $f(t) = A + B \cos \left(2\pi \left(t + C\right) / T\right)$ with the $f(t)$ indicating relative expression levels of target genes, $t$ specifying the time of sampling (h), $A$ 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. Protein levels were calculated relative to actin immunoreactivity of each dataset throughout 24 hours to plot temporal expression. One-way ANOVA (one way analysis of variance) was used to evaluate variations among the groups in the 24-h profile. Significance of daily regulation was defined by showing a $p < 0.05$.

### Results

**Gnaz mRNA is under daily regulation in retina and pineal gland**

To gain insight into the daily regulation of G protein-dependent signal transduction in the mouse retina, transcript levels of different $\alpha$ subunits were determined as a function of time-of-day. Among the $\alpha$ subunits tested, exclusively the non-visual $\alpha$-transducin gene *Gnaz* displayed a daily rhythm (Fig 1, blue lines; for statistical analysis, see Table 2). Neither of the visual $\alpha$-transducins, *Gnat1* and *Gnat2* (Fig 1, blue lines; for statistical analysis, see Table 2), nor any of the other $\alpha$ subunits tested (*Gna11, Gna12, Gna13, Gnal4, Gnal5, Gna1, Gna12,* Table 1. (Continued)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primer Sequence 5’ to 3’</th>
<th>Length of PCR Product [bp]</th>
</tr>
</thead>
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<tr>
<td>r18S</td>
<td>NR_046237.1</td>
<td>Forward: GTTGGTGGAGCGATTTGTC</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TCAATCTCGGTTGGCGAAC</td>
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<tr>
<td>rDrd4</td>
<td>NM_012944.1</td>
<td>Forward: TGGGCTATTCACACAGGTG</td>
<td>112</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: CACAGCGGTTCTCTCAG</td>
<td></td>
</tr>
<tr>
<td>rGapdh</td>
<td>NM_017008.4</td>
<td>Forward: TGACTCTACCCCGCCCAAGG</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TCGAAGATGGTGTGTGGTTT</td>
<td></td>
</tr>
<tr>
<td>rGnaz</td>
<td>NM_013189.2</td>
<td>Forward: CCGAGTACAAAGGTCCAGAAC</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TCGGTTGGCAGAGTAAG</td>
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https://doi.org/10.1371/journal.pone.0187411.t001
Gnai3, Gnal, Gnao1, Gnaq, Gnas) displayed a 24-h rhythm (data not shown). Similarly, rhythmic expression of Gβ subunits (Gnb1, Gnb2, Gnb3, Gnb4, Gnb5), and Gγ subunits (Gngt1, Gngt2) was not observed (data not shown).

Gnaz rhythmicity displayed peak expression in darkness and was similar in retina of mouse and rat (Fig 1, blue versus red lines; for statistical analysis, see Table 2). It also occurred in rat pineal gland, an organ that is phylogenetically related to the retina and is controlled by the body’s master clock in the suprachiasmatic nucleus [29] (Fig 1, black lines; for statistical analysis, see Table 2). This suggests that daily regulation of Gnaz is phylogenetically conserved and may in retina and pineal gland be promoted by different clocks.

### Daily regulation of Goζ protein amount

To investigate whether the observed variations in Gnaz mRNA result in corresponding variations in protein amount, Gαζ immunoreactivity was compared at different ZTs in Western blot analysis by using an antibody that recognizes a band of ~ 39 kDa (Fig 2), a molecular mass in the range of the predicted size from the Gnaz gene (355 amino acids). The intensity of Gαζ immunostaining tended to display a daily change (p = 0.062 in one-way ANOVA) with peak values around ZT21 (Fig 2). The temporal gap between the peaks in Gnaz mRNA (ZT16.9) and Gαζ protein (ZT21) may reflect the time necessary to translate the transcript into protein.
Thus, this observation suggests that daily regulation of Gnaz mRNA amount causes a corresponding rhythm of Gαz protein.

**Daily regulation of Gαz protein localization**

Localization of Gαz protein was investigated in fluorescence microscopy (Fig 3) by conducting double labeling analysis for Gαz and centrin3, a marker of the connecting cilium and the inner
segment of photoreceptors [30]. G\(\alpha_z\) immunoreactivity mainly occurred in photoreceptors where its subcellular localization was seen to vary between day and night. This follows from the observation that G\(\alpha_z\) staining mainly arose in the connecting cilium/inner segment at ZT6 and was most dense in the outer segment at ZT18 (Fig 3). This supports a concept in which subcellular localization of G\(\alpha_z\) protein might exhibit a lighting condition-dependent transport from the connecting cilium/the inner segment to the outer segment.

Daily regulation of Gnaz mRNA amount in photoreceptor cells

The expression of Gnaz in photoreceptors raises the question whether rhythmicity of Gnaz mRNA arises from this cell type. To address this question, daily profiling of Gnaz mRNA was performed in photoreceptors enriched by using the LMPC technique. Gnaz transcript amount was seen to display a daily rhythm (Fig 4, red lines; for statistical analysis, see Table 2) with a 24-h profile resembling that obtained from preparations of the whole retina (Fig 4, blue lines;
for statistical analysis, see Table 2). Therefore, daily changes in retinal Gnaz mRNA amount may partly or fully derive from photoreceptors.

**Gnaz expression depends on a circadian regulator**

24-h regulation of a gene may be promoted by a true circadian clock or light/dark-transitions. To test circadian regulation of Gnaz, 24-h profiling of transcript amount was conducted in mice adapted to DD (Fig 4, black lines; for statistical analysis, see Table 2). Consistent with clock-dependent regulation of Gnaz expression, the daily rhythm of Gnaz transcript persisted...
Under DD. Furthermore, Gnaz was not rhythmically expressed in Clock deficient mice (Fig 5, first row, blue versus red lines; for statistical analysis, see Table 2). This supports the concept that Gnaz rhythmicity is driven by a retinal clock that requires Clock for its functionality [31] and not by the master clock in the suprachiasmatic nucleus (SCN), which does not require Clock for its functionality [32±34].

Circadian regulation of Gnaz requires dopamine D4 receptors

In order to evaluate the contribution of melatonin and dopamine to daily regulation of Gnaz, 24-h profiling of the gene was performed in mice deficient for MT1 (Fig 5, second row, blue versus red lines; for statistical analysis, see Table 2) or Drd4 (Fig 5, third row, blue versus red lines; for statistical analysis, see Table 2). The daily rhythm of Gnaz was seen to persist in MT1 deficient mice but peak expression appeared to be slightly phase-advanced. This observation suggests that melatonin signaling via MT1 receptors does not drive rhythmicity of Gnaz but might influence its phasing. More importantly, daily regulation of Gnaz was absent in Drd4 deficient mice. This suggests that dopamine and D4 receptors play a role in driving circadian changes in Gnaz expression. In mouse retina dopamine release and D4 receptor stimulation occurs in a circadian manner [12]. Therefore, this finding supports the concept that Gnaz expression depends on a clock-driven dopamine signal.

Expression of Gnaz is arrhythmic in diabetic retina

To test the assumption that diabetic retinopathy impairs circadian control of Gnaz, the db/db mouse, a model of Type II diabetes [35] was used. The non-diabetic phenotype (db/+ ) was seen to display a daily rhythm in Gnaz mRNA but with a lower amplitude than that observed in previous experiments (Fig 6, blue lines; for statistical analysis, see Table 2). This may be due to a different genetic background of the db/db mice (C57BL/6Jb) and the other mouse strains, which were on a C3H background. Different to the non-diabetic phenotype (db/+ ), Gnaz expression was arrhythmic in diabetic (db/db) mice (Fig 6, blue versus red lines; for statistical analysis, see Table 2). Therefore, circadian regulation of Gnaz appears to be disturbed in diabetic retinopathy.

Fig 6. Daily profiling of Gnaz mRNA in diabetic retina. Transcript levels of Gnaz and Drd4 were recorded in non-diabetic (db/+ ) mice (blue lines) versus diabetic (db/db) mice (red lines) in preparations of the whole retina under LD 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-h period. Statistical analysis of transcriptional profiling is provided in Table 2. Note that Gnaz expression is arrhythmic in diabetic mice. 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 retinæ)).

https://doi.org/10.1371/journal.pone.0187411.g006
Daily profiling of the clock-dependent gene Drd4 confirms the validity of the experimental system

To test the validity of the experimental system and the obtained results, the clock-driven gene Drd4 was monitored in the same transcriptomes as those utilized for Gnaz mRNA determination. Consistent with the validity of the results obtained for Gnaz, Drd4 expression was observed to be rhythmic in retina of mouse and rat and pineal gland (Fig 1, for statistical analysis, see Table 2). As expected for a gene under circadian regulation, Drd4 rhythmicity persisted under DD (Fig 4, black lines; for statistical analysis, see Table 2) and was damped in mice deficient for Clock (Fig 5, first row, blue versus red lines; for statistical analysis, see Table 2). Beyond what was previously known, Drd4 rhythmicity was seen in the present study to persist in mice deficient for Mt1 (Fig 5, second row, blue versus red lines; for statistical analysis, see Table 2) and, according to statistical analysis, not in diabetic (db/db) mice (Table 2; Fig 6, blue versus red lines).

Discussion

The gene Gnaz encodes Gαz, a unique Gi/o subfamily member, whose tissue distribution is quite restricted to primarily neuronal and endocrine tissue [36], including retina [37±39]. The findings of the present study extend previous knowledge on Gnaz/Gαz distribution [37, 40±42] by showing that it is highly expressed in photoreceptors and pinealocytes. Cell types originating phylogenetically and ontogenetically from a common ancestral cell type [29].

Gnaz mRNA—but not Gnat1 or Gnat2 mRNA—displays a daily rhythm in retina. This finding and large sequence similarities of Gnaz with Gnat1 and Gnat2 (44% and 43% respectively), suggest that the earlier reported daily change in α-transducin mRNA [20±22] relies on Gnaz but not on the visual types of α-transducin. This assumption is furthermore supported by the fact that in transcriptomes of the murine retina, Gnaz displays a higher day/night change than Gnat2 and Gnat1 does not undergo daily regulation at all [8]. Additionally the oligodeoxynucleotide probes used previously to detect α-transducin [20] are highly complementary with all types of α-transducin (Gnaz: 82%, Gnat1: 93%, Gnat2: 79%).

In the context of the functional significance of the rhythmic expression of the Gnaz gene, it is noteworthy that daily rhythmicity is also evident at the level of Gαz protein. The daily profile in Gnaz mRNA resembles that in Gαz protein. This suggests that daily regulation of Gnaz mRNA evokes a corresponding rhythm in Gαz protein. Since Gαz protein expression predominates in photoreceptors (this study), but may also be abundant in the inner retina [37], Gnaz expression might be under daily/circadian regulation not only in photoreceptors but also in inner retinal neurons. This assumption is consistent with the observation that the acrophase of Gnaz expression tends to differ in microdissected photoreceptors (ZT19.9) and preparations of the whole retina (ZT16.9). Furthermore, Gnaz/Gαz is under daily regulation not only in respect to its expression but also in its subcellular localization within photoreceptors. This follows from the finding that Gαz immunostaining was most dense in the connecting cilium/inner segment at ZT6 but in the outer segment at ZT18. Since Gαz de-novo formation should occur in the inner segment, the temporary localization of Gαz protein in the outer segment suggests that newly synthesized Gαz is transported from the inner to the outer segment of photoreceptors.

Regulation of Gnaz expression was observed to be driven by a circadian clock. This is evident from the present observation that Gnaz rhythmicity persists under constant darkness and therefore does not require light/dark transitions. Circadian control of Gnaz appears to be driven by the retinal circadian clock system and not by the master clock in the SCN. This follows from the finding that Gnaz rhythmicity was not observed in Clock-deficient mice, in which circadian rhythms persist in the SCN due to the CLOCK homologue NPAS2 [32±34].
Rhythmicity of Gnaz was also evident in the pineal gland, a neuroendocrine transducer of the circadian system [43]. The pineal gland in general and its gene expression in particular are mainly under the control of the master clock in the SCN [44]. This suggests that Gnaz expression is circadian in both, retina and pineal gland, but in the retina depends on retinal clocks and in the pineal gland on the master clock in the SCN. The coincidence of Gnaz rhythmicity in mammalian photoreceptors and pinealocytes suggests that circadian regulation of Gnaz is evolutionary conserved. In both tissues, rhythmicity of Gnaz may depend on the clock-driven release of neurotransmitters. Thus circadian regulation of Gnaz may be mediated by dopamine in the retina (see below) and by noradrenaline in the pineal gland; the intra-pineal release of the latter neurotransmitter is known to be under the control of the SCN [45].

In murine retina, 24-h regulation of Gnaz appears to be mediated by dopamine signaling via D₄ receptors. This follows from the present observation that the lack of functional D₄ receptors prevents daily periodicity of Gnaz. Since photoreceptors combine daily rhythmicity of Gnaz with the occurrence of D₄ receptors [15], dopaminergic regulation of Gαz may mainly occur in photoreceptors. D₄ receptor-mediated regulation of photoreceptor function appears to depend on the clock-driven [46, 47] release of dopamine from amacrine cells in the inner retina [4, 48]. Therefore, circadian Gαz expression in photoreceptors may be driven by the clock within amacrine cells. Like Gnaz, the genes Cpt-1α, Acadm and Nr4a1 have been identified to be under circadian and dopaminergic control in photoreceptors [49, 50]. Therefore, clock-dependent dopamine release from amacrine cells may be of general importance for driving rhythmicity of photoreceptor gene expression.

In contrast to D₄ receptors, MT₁ receptors do not play a role in driving rhythmicity of Gnaz. This follows from the present finding that notwithstanding the loss of functional MT₁ receptors 24-h changes in Gnaz transcript are retained. However, the removal of functional MT₁ receptors might result in advanced phasing of Gnaz (this study) and other genes shown to be rhythmic in photoreceptors including Cpt-1α and Acadm [50]. Therefore, melatonin acting on photoreceptor MT₁ receptors [51, 52] might influence the phasing of gene expression rhythmicity in photoreceptors. Considering that melatonin is released by photoreceptors and may feedback on photoreceptor MT₁ receptors, autocrine signaling might direct the phasing of gene rhythmicity in photoreceptors. Alternatively, melatonin might alter the phase of the clock in dopaminergic amacrine cells.

Daily rhythmicity of Gnaz is abolished in early diabetic retinopathy in the db/db mouse. Therefore, it might also be disturbed in diabetic retinopathy of humans; one of the most common causes of blindness in Europe and USA [53]. Since diabetic retinopathy affects dopamine content [54] and rhythmicity of Drd4 expression (this study), the disturbed circadian regulation of Gnaz may reflect dysfunction of the retinal dopaminergic system under diabetic conditions. This assumption is consistent with the observation that circadian regulation of other genes under dopaminergic control is disturbed in diabetic retinopathy [50].

Circadian regulation of Gαz suggests a role of Gαz in linking the circadian clock to G protein-dependent signal transduction. Taken into account that Gαz is known to regulate adenyl cyclase activity [55, 56], it may contribute to circadian regulation of adenyl cyclase activity and cAMP levels in retina [16, 57]. Since Gαz is generally known to repress adenyl cyclase activity, this assumption is consistent with the finding that the level of retinal Gαz with a peak at late night and a nadir during day (this study) is inversely correlated with the level of cAMP showing a nadir at late night and a peak at dusk [16].

Gαz has been conclusively linked to various types of GPCRs [58, 59]. Therefore, Gαz might link the circadian clock to GPCR-dependent signal transduction. Interestingly, in tissues other than retina Gαz has been shown to be coupled to D₂-like receptors [60, 61]Da subclass of dopamine receptors that includes the dopamine D₄ receptor typeDand thus to a receptor
family important for vision [17, 18, 62] and healthy retinal function [54]. However, data from immunoprecipitation studies (not shown) do not support direct coupling of Gαz to dopamine D4 receptors in retina.

Interestingly, Gαz has been seen in the SCN to be coupled to Gpr176, an orphan GPRC that sets the pace of circadian behavior [63]. This suggests a role of Gαz in setting the phase of the master clock in the SCN. This assumption combined with rhythmicity of Gnaz in retina (this study) and pineal gland (this study) indicates that Gαz is abundant and might play a clock-related role in all three components of the circadian system [43]. Accordingly, the α-transducin family appears to comprise clock- (Gnaz) and vision-related (Gnat1, Gnat2) G proteins.

In conclusion, the data of the present study suggest a concept in which Gαz links the circadian clock and the dopaminergic system to GPCR signaling. Future investigations using Gαz null mice [61, 64, 65] are warranted to reveal the exact function of Gαz in photoreceptors and herewith its specific functional role in photoreceptor adaptation. The present data may also provide a suitable basis for future investigations dealing with the clock-related role of Gαz in the circadian system.

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Author Contributions

Conceptualization: Rainer Spessert.

Formal analysis: Patrick Vancura.

Funding acquisition: Gianluca Tosini, P. Michael Iuvone.

Investigation: Patrick Vancura, Shaima Abdelhadi, Erika Csicsely.

Methodology: Kenkichi Baba, Gianluca Tosini, P. Michael Iuvone, Rainer Spessert.

Project administration: Rainer Spessert.

Resources: Kenkichi Baba, Gianluca Tosini, P. Michael Iuvone.

Supervision: Rainer Spessert.

Writing ± original draft: Patrick Vancura, Rainer Spessert.

Writing ± review & editing: Patrick Vancura, Gianluca Tosini, P. Michael Iuvone, Rainer Spessert.

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