Die tageszeitliche Regulation der Genexpression in Retina und Fotorezeptorzelle

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Es gibt nur ein einziges Gut für den Menschen: Die Wissenschaft.

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Anmerkung

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II. Kunst S, Wolloscheck T, Grether M, Trunsch P, Wolfrum U, Spessert R. (2015) Photoreceptor cells display a daily rhythm in the orphan receptor *Esrrβ.* Mol Vis, 21:173-84.

III. Kunst S, Wolloscheck T, Kelleher DK, Wolfrum U, Sargsyan SA, Iuvone PM, Baba K, Tosini G, Spessert R. (2015) *Pgc-1α* and *Nr4a1* are target genes of circadian melatonin and dopamine release in murine retina. Invest Ophthalmol Vis Sci, 56:6084-94.

Alle drei Publikationen sind dieser Arbeit beigefügt. Im Abschnitt "Zusammenfassung der Ergebnisse und Diskussion" werden die Ergebnisse der Publikationen und ihre wissenschaftliche Relevanz dargestellt und diskutiert.

Veröffentlichungen

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Publikationen

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Kongressbeiträge

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Abkürzungsverzeichnis

&	und
Aanat	*arylalkylamine N-acetyltransferase*
Adra1b	*adrenergic receptor, alpha 1b*
Atf3	*activating transcription factor 3*
Bmal1	*aryl hydrocarbon receptor nuclear translocator-like (ARNTL)*
bzw.	beziehungsweise
cAMP	Cyclisches Adenosinmonophosphat
C3H	Labormausstamm
C57BL/6	Labormausstamm
ccgs	*clock-controlled genes*
Clock	*Circadian locomotor output cycles kaput*
Cry1	*cryptochrome 1*
Cry2	*cryptochrome 2*
D_4	Dopamin-D4-Rezeptor
DD	Dauerdunkel
d.h.	das heißt
DNA	Desoxyribonukleinsäure
Drd4	*dopamine receptor D4*
E-Box	*Enhancer Box*
engl.	englisch
ERG	Elektroretinogramm
Esrrβ	*estrogen related receptor, beta*
et al.	*et alii*
FRZ	Fotorezeptorzelle
GCL	*ganglion cell layer*
Grk1	*G protein-coupled receptor kinase 1*
HAT	Histon-Acetyl-Transferase
INL	*inner nuclear layer*
IPL	*inner plexiform layer*
ipRGC	Intrinsisch photosensitive Ganglienzellen
Kcnv2	*potassium channel, voltage gated modifier subfamily V, member 2*
KO	*knock-out*
lat.	latein
LD	Licht Dunkel
LMPC	*laser microdissection and pressure catapulting*
mRNA	*messenger* Ribonukleinsäure
MT1	Melatonin Rezeptor 1
MT2	Melatonin Rezeptor 2
Nr4a1	*nuclear receptor subfamily 4, group A, member 1*
NSC	Nucleus suprachiasmaticus
ONL	*outer nuclear layer*
PCR	*polymerase chain reaction*
Pde8a	*phosphodiesterase 8A*
Per1	*period circadian clock 1*
Per2	*period circadian clock 2*
Pgc-1α	*peroxisome proliferator-activated receptor gamma, coactivator 1 alpha*
qPCR	*quantitative polymerase chain reaction*
rd	*retinal degeneration*
Rev-erbα	*nuclear receptor subfamily 1, group D, member 1 (Nr1d1)*
Rorα	*RAR-related orphan receptor alpha*
Rorβ	*RAR-related orphan receptor beta*
RORE	*retinoic acid-related orphan receptor response element*
ROS	*reactive oxygen species*
RPE	retinales Pigmentepithel
u.a.	unter anderem
vgl.	vergleiche
z.B.	zum Beispiel
ZNS	Zentralnervensystem

Zusammenfassung

Die Retina von Säugetieren und ihre Fotorezeptorzellen besitzen die Fähigkeit, sich an die tageszeitlich wechselnden Lichtintensitäten der Umwelt anzupassen. Die Adaptation umfasst unter anderem die Morphologie der Fotorezeptorzellen, die visuelle Signaltransduktion und den Energiemetabolismus der Retina. Sie wird durch retinale Uhren/Oszillatoren gesteuert, die durch die äußeren Lichtbedingungen synchronisiert werden.

Die Uhren-gesteuerte Anpassung von Retina und Fotorezeptorzellen an tageszeitliche Änderungen wird durch die Neuromodulatoren Melatonin und Dopamin vermittelt. Dabei regulieren Oszillatoren in den Amakrinzellen die Dopaminsynthese, während Oszillatoren in den Fotorezeptorzellen die Rhythmik der Melatoninsynthese steuern. Die Wirkung von Dopamin wird über Dopamin-D4-Rezeptoren vermittelt, die sich unter anderem in den Stäbchen befinden. Melatonin wirkt indes über die Melatonin-Rezeptoren MT1 und MT2, welche unter anderem in Stäbchen und Ganglienzellen der Retina lokalisiert sind. Melatonin und Dopamin fungieren bei der Kontrolle der retinalen Funktionalität als Gegenspieler: die Freisetzung von Melatonin ist nachts erhöht und ermöglicht die Dunkeladaptation der Retina, während die von Dopamin tagsüber erhöht ist und die Anpassung an Licht vermittelt.

Ziel dieser Arbeit war ein besseres Verständnis der tageszeitlichen Adaptation von Retina und Fotorezeptorzelle auf Ebene der Genexpression, wobei Ratten und Mäuse als Tiermodelle benutzt wurden. Es konnte mithilfe von Mikroarray-Analyse und qPCR gezeigt werden, dass die tageszeitliche Regulation der Fotorezeptorzellen Gene umfasst, die an der Fototransduktion (z.B. *Grk1*), am Metabolismus (z.B. *Pgc-1α, Esrrβ*), an der Regulation der Transkription (z.B. *Pgc-1α, Rorβ*) und an der Zellproliferation (z.B. *Atf3*) beteiligt sind. Weiterhin wurde beobachtet, dass die tageszeitliche Regulation der Genexpression in der Retina maßgeblich über die circadiane Uhr gesteuert wird (z.B. *Adra1b, Pde8a*). Mithilfe von Gen-defizienten Mausmodellen ($Drd4^{-/-}$, $MT1^{-/-}$) konnte darüber hinaus eine Beteiligung des dopaminergen (via *Nr4a1*) und des melatoninergen (via *Pgc-1α*) Systems an der circadianen Regulation der Genexpression der Retina nachgewiesen werden.

Zusammenfassend belegt die vorliegende Arbeit eine zentrale Rolle der Genregulation in der Uhren-, Melatonin- und Dopamin-abhängigen Adaptation der Retina.

1 Einleitung

1.1 Die Adaptation der Retina

Das Prinzip der Adaptation ist im gesamten neuronalen System ausgeprägt und zeugt von der Wichtigkeit der Gewebe sich an wechselnde Bedingungen anpassen zu können. Die Mechanismen der Anpassung sind sehr unterschiedlich und betreffen im Auge u.a. die Weite der Pupillenöffnung, den Gehalt an Fotopigmenten in den Fotorezeptorzellen (FRZ) [1, 2] oder die Intensität der synaptischen Weiterleitung [3, 4].

In der Retina können die Anpassungsvorgänge als Reaktion auf Veränderungen der äußeren Lichtbedingungen in Hell- und Dunkeladaptation unterteilt werden. Die Helladaptation beschreibt die Anpassungsvorgänge bei zunehmenden Lichtintensitäten, während die Dunkeladaptation bei abnehmen Lichtintensitäten auftritt. Diese Anpassungsmöglichkeiten sind notwendig, um die visuelle Signalverarbeitung und –weiterleitung während wechselnder Lichtbedingungen zu optimieren [2]. Stäbchen erfahren bereits bei geringen Lichtintensitäten eine Sättigung, die bis zu einer Stunde anhält. Während dieser Erholungsphase ist die Reaktion der Stäbchen auf Licht eingeschränkt, was dazu führt, dass sie bei Tageslicht zumeist nicht funktional sind [5]. Zapfen hingegen können ihre Sensitivität den Lichtbedingungen anpassen und innerhalb weniger Minuten verändern, wodurch sie auch bei sehr hellem Licht photosensitiv bleiben [6]. Dieses Zusammenspiel von Stäbchen und Zapfen und die Möglichkeit der Hell- und Dunkeladaptation ermöglicht es Säugetieren, auch bei stark wechselnden äußeren Lichtbedingungen ihre visuelle Wahrnehmung zu erhalten.

1.1.1 Tageszeitliche Rhythmik der Adaptation

Neben kurzfristigen Anpassungen an Hell und Dunkel, sind in der Retina auch tageszeitliche Adaptationsvorgänge bekannt. Die tagezeitliche Anpassung der Retina erfolgt über das Zusammenspiel von retinalen Oszillatoren und äußeren Lichtbedingungen [7, 8] und betrifft verschiedene Parameter, darunter die Synthese der Neuromodulatoren Melatonin und Dopamin [9, 10] (zur Übersicht, siehe [11]). Cameron et al. (2008) konnten zeigen, dass auch das Elektroretinogramm (ERG) der Mausretina 24-stündige Veränderungen aufweist, die eine tageszeitliche Rhythmik der visuellen Signalverarbeitung widerspiegeln [12]. Auch das sogenannte „disc shedding“ in Zapfen und Stäbchen der Retina unterliegt tageszeitlichen Schwankungen [13]. Das „disc shedding“ beschreibt die ständige Erneuerung der phototransduktiven Kompartimente im Außensegment der FRZ (Membranstapel, *discs*). Es ist notwendig, da es bei der Absorption von Photonen zu einem ständigen photooxidativen Stress innerhalb der FRZ kommt, welcher molekulare Zellbestandteile schädigen kann. Das „disc

shedding“ verhindert die Ansammlung geschädigter Komponenten und erhält die physiologischen Funktionen der Zellen aufrecht (zur Übersicht, siehe [14]).

Ein weiterer Adaptationsmechanismus der FRZ ist die Licht-abhängige Translokation von phototransduktiven Proteinen in das Außensegment der Zellen [15, 16] (zur Übersicht, siehe [14]). Diese Translokation der Proteine ermöglicht es den FRZ auch unter wechselnden äußeren Lichtbedingungen ihre Funktion aufrechtzuerhalten. Ein Beispiel hierfür ist die Translokation von Arrestin, einem Protein, welches durch Bindung an Rhodopsin zur Deaktivierung der Signaltransduktion führt. Während Arrestin in Dunkelheit hauptsächlich im Innensegment von Zapfen und Stäbchen vorkommt, translociert es unter Lichteinfluss ins Außensegment [17]. Diese Translokation wird dabei unter anderem durch Rhodopsin gesteuert, welches seinerseits durch Licht aktiviert wird [18-20] und erfolgt Energie-abhängig [16]. Die Translokation von Arrestin dient nicht nur der Aufrechterhaltung der Sensitivität von FRZ, sondern übt darüber hinaus eine neuroprotektive Rolle aus, indem sie die Lichtempfindlichkeit der Phototransduktion senkt (zur Übersicht, siehe [21]).

1.1.2 Metabolische Adaptation

FRZ benötigen Energie für verschiedene physiologische Prozesse wie Transduktion, elektrische Signalweiterleitung oder Synthese und Freisetzung von Neurotransmittern (zur Übersicht, siehe [22]). Ihr hoher Energiebedarf beruht vor allem auf der ständigen Aufrechterhaltung des Ruhepotentials, wobei ein Großteil der Energie für den Erhalt des Konzentrationsgradienten und des Membranpotentials benötigt wird [23, 24]. FRZ decken den Großteil ihres Energiebedarfs durch aerobe Glykolyse [25], weitere Energie wird durch oxidative Phosphorylierung gewonnen [25] (zur Übersicht, siehe [26]). Die Innensegmente der FRZ verfügen über eine besonders hohe Dichte an Mitochondrien, in denen der oxidative Stoffwechsel stattfindet (zur Übersicht, siehe [24, 27]).

Während der Dunkelphase haben FRZ einen zusätzlich erhöhten Energiebedarf, welcher durch eine Erhöhung des oxidativen Stoffwechsels gedeckt wird [28, 29]. Der nachts erhöhte Energiebedarf beruht auf der ständigen Öffnung von Ionenkanälen, darunter Natriumkanäle, die einen ständigen Einfluss der Ionen in die Zelle ermöglichen. Um den Konzentrationsgradienten aufrecht zu erhalten bzw. wiederherzustellen und eine Hyperpolarisation bei Lichtimpulsen zu ermöglichen, müssen daher fortwährend Natriumionen unter Energieaufwand aus der Zelle herausgepumpt werden [28]. Es konnte gezeigt werden, dass bis zu 41% des Sauerstoffverbrauchs einer Zelle für die Aufrechterhaltung des Membranpotentials in Dunkelheit benötigt wird [25]. Die tageszeitliche Rhythmik des Energiebedarfs der FRZ führt zu einer einhergehenden Rhythmik des oxidativen Stoffwechsels

und damit zu tageszeitlichen Veränderungen des oxidativen Stresses dem die Zellen ausgesetzt sind [30, 31]. So zeigen Messungen unterschiedliche Konzentrationen an Biomarkern für oxidativen Stress oder eine tageszeitliche Rhythmik der Konzentration von Antioxidantien [30, 31].

Oxidativer Stress entsteht durch die Bildung reaktiver Sauerstoffverbindungen (engl.: reactive oxygen species, ROS) im Rahmen metabolischer Prozesse (zur Übersicht, siehe [32]). Die tageszeitliche Rhythmik des oxidativen Stoffwechsels der Zellen führt ihrerseits zu einer Rhythmik in der Superoxidbildung, welche wiederum ein rhythmisches Auftreten von oxidativem Stress bedingt. Einen Schutz gegen oxidativen Stress bilden anti-oxidative Enzyme, die freie Sauerstoffradikale reduzieren und die Zelle dadurch vor Schäden durch oxidativen Stress schützen, darunter Glutathion Peroxidase (zur Übersicht, siehe [32]). Es gibt Hinweise, dass die Expression von Glutathion Peroxidase in neuronalem Gewebe eine Rhythmik mit erhöhten nächtlichen Werten zeigt [33-35]. Bei der erhöhten nächtlichen Expression könnte es sich um einen physiologischen Mechanismus zum Zellschutz handeln [36]. Demnach induzieren ein nächtlich erhöhter oxidativer Stoffwechsel und die damit verbundene Erhöhung der ROS-Bildung eine verstärkte Expression von anti-oxidativ wirkenden Enzymen. Die Bildung von oxidativem Stress in der Zelle zeigt eine Rhythmik, die ihrerseits zu einer Rhythmik der Schutzmechanismen der Zelle führt, wie die nachts erhöhte Expression von Glutathion Peroxidase zeigt (zur Übersicht, siehe [36]).

Ebenso könnte die tageszeitliche Regulation der Melatoninsynthese als Adaptation an tageszeitlich variierenden oxidativen Stress dienen (zur Übersicht, siehe [37]). So ist die Melatoninsynthese mit der Intensität des oxidativen Stresses negativ korreliert (zur Übersicht, siehe [37]). Dies unterstützt die Hypothese, dass die tageszeitliche Rhythmik von oxidativem Stress eine tageszeitliche Rhythmik von Schutzmechanismen bewirkt. Melatonin stimuliert zum einen die Synthese anti-oxidativer Enzyme (wie z.B. Glutathion Peroxidase), zum anderen kann es toxische Radikale direkt binden (darunter Hydroxyl- und Peroxyl-Radikale) [34, 37].

1.1.3 Die Rolle der Genexpression in der Adaptation

In Maus und Ratte konnten tageszeitliche Oszillationen der Genexpression beobachtet werden [38] (Publikation I), die die Uhren-abhängige Anpassung der retinalen Physiologie vermitteln könnten. So konnten Storch et al. (2007) mittels Mikroarray-Analyse zeigen, dass eine Vielzahl von Genen im Auge von Säugetieren eine Rhythmik aufweist und ein Teil dieser Gene circadian, d.h. durch endogene Oszillatoren, reguliert wird [39, 40]. Zu den circadian

regulierten Genen gehören auch solche, die den Kern der inneren Uhr bilden und deshalb als Uhrengene bezeichnet werden [41-43].

Ein Beispiel für ein Gen, dessen circadiane Expression zu einer korrespondieren Rhythmik einer physiologischen Funktion führt ist *Arylakylamine N-acetyltransferase* (*Aanat)*. Die tageszeitliche Regulation der *Aanat*-Genexpression reguliert die Expression des Aanat-Enzyms, welche die Melatonin-Synthese reguliert (vgl. Kapitel 1.4).

Darüber hinaus konnten tageszeitliche Veränderungen von Proteinen nachgewiesen werden, obwohl die entsprechenden Gene keine rhythmische Anpassung auf mRNA-Ebene zeigten. Dies zeigt, dass neben der Genexpression auch post-transkriptionelle Veränderungen für die tageszeitliche Regulation relevant sind [44] (zur Übersicht, siehe [45]).

1.2 Oszillatoren als molekulare Grundlage der Rhythmik

Bei Säugetieren bestehen molekulare Oszillatoren aus zwei autoregulatorischen Rückkopplungsschleifen, deren Zusammenspiel die neuronale Aktivität steuert (zur Übersicht, siehe [46]). Hierbei kann eine negative und eine positive Schleife unterschieden werden. Die negative Schleife besteht aus sechs Genen (Abbildung 1). Diese Gene sind die *Period*-Gene *Per1* und *Per2,* die *Cryptochrome*-Gene *Cry1* und *Cry2*, sowie *Clock* und *Bmal1*. Die Translationsprodukte von *Clock* und *Bmal1* bilden ein Heterodimer, das an die E-Box-Region im Promoter der *Period*- und *Cryptochrome*-Gene bindet und dadurch die circadiane Expression von *Per1* und *Per2,* sowie *Cry1* und *Cry2* steuert. Durch Transkription und Translation der *Per*- und *Cry*-Gene entstehen die PER- und CRY-Proteine. Diese bilden nun ebenfalls Heterodimere, welche dann zurück in den Zellkern translozieren und die CLOCK- und BMAL1-Proteine inhibieren. Hierdurch unterdrücken sie ihre eigene Transkription (zur Übersicht, siehe [11, 47, 48]).

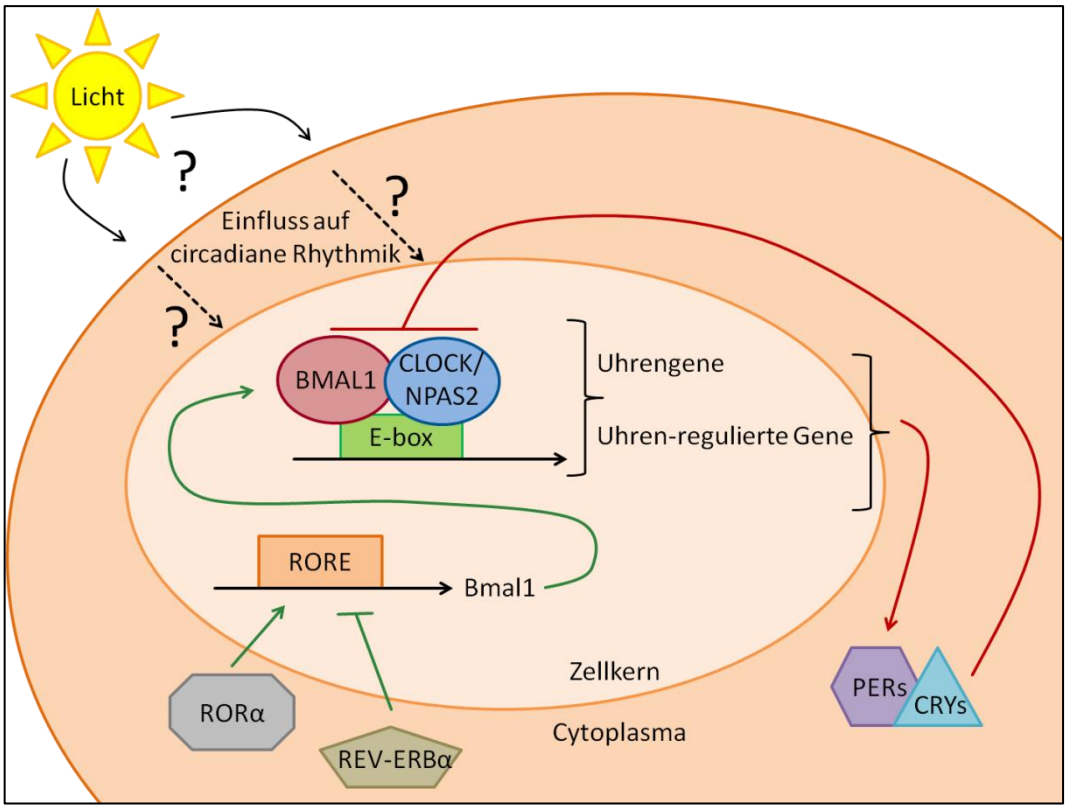

Abbildung 1: Der molekulare Mechanismus der circadianen Rhythmik
Erläuterungen, siehe Text. (verändert nach [48]).

In einer zweiten Rückkopplungsschleife erfolgt die Transaktivierung der Gene *Rev-Erbα* und *Rorα* (*retinoic acid-related orphan receptor*) durch den CLOCK/BMAL1-Proteinkomplex. Die Genprodukte REV-ERBα und RORα konkurrieren miteinander um die RORE-Bindungsstellen (RORE: *retinoic acid-related orphan receptor response element*) im Promoter des *Bmal1*-Gens. Durch Bindung des REV-ERBα-Proteins wird die Transkription des *Bmal1*-Gens unterdrückt, was wiederum auch die Synthese des REV-ERBα-Proteins inhibiert. RORα-Protein akkumuliert im Zytoplasma der Zelle und kann durch Translokation in den Zellkern die *Bmal1*-Transkription wieder aktivieren. Die Dauer beider Rückkopplungsschleifen beträgt in etwa 24 Stunden, wodurch sich die Periodendauer der Rhythmik erklärt (zur Übersicht, siehe [11, 47, 48]).

Neben den genannten Uhrengenen, die den Kern der Rhythmik bilden, gibt es weitere Gene, welche die Rhythmik von physiologischen Prozessen regulieren. Die Uhren-abhängige Transkription dieser Gene (engl.: *clock-controlled genes, ccgs*) erfolgt über E-Box oder RORE-Bindungsstellen in den jeweiligen Promotorregionen.

1.2.1 Circadiane Rhythmik

Eine Besonderheit der tageszeitlichen Rhythmik sind circadiane Rhythmen. Der Begriff „circadian“ (lat.: *circa* = ungefähr, *dies* = Tag) beschreibt die etwa 24-stündige Periodizität tageszeitlicher Rhythmen, die durch endogene Oszillatoren generiert wird und auch in Abwesenheit von externen Signalen bestehen bleibt. Die molekularen Oszillatoren werden von externen Signalgebern beeinflusst, unter denen Licht der wichtigste ist. Dieser synchronisiert die Rhythmik des Oszillators mit den äußeren Lichtbedingungen und fixiert seine Periodendauer auf genau 24 Stunden (zur Übersicht, siehe [48-51]).

Circadiane Rhythmen sind hinsichtlich unterschiedlicher Parameter relevant, unter ihnen Körpertemperatur, Schlaf oder Hormonhaushalt (zur Übersicht, siehe [48]). Bei Säugetieren werden die circadianen Rhythmen durch das circadiane System reguliert, das sich aus Nucleus suprachiasmaticus (NSC), Pinealorgan und Retina zusammensetzt und aus molekularen Oszillatoren besteht. Diese Oszillatoren steuern die endogene Rhythmik, wobei der Hauptschrittmacher der NSC im Hypothalamus ist (zur Übersicht, siehe [52, 53]). Zusätzlich zum circadianen System im Zentralnervensystem (ZNS), finden sich Oszillatoren auch in peripheren Geweben [54] (zur Übersicht, siehe [48, 55]). Diese peripheren Oszillatoren regulieren metabolische und physiologische Abläufe in ihren jeweiligen Geweben [56] während der NSC die peripheren Oszillatoren mit seinem eigenen Rhythmus synchronisiert (zur Übersicht, siehe [55, 57]).

1.3 Circadiane Oszillatoren in der Retina

In der Retina steuern Oszillatoren im Zusammenspiel mit äußeren Lichteinflüssen die Adaptation (Abbildung 2). Die Retina verfügt über eine endogene Uhr, die auch in isolierten Retina-Kulturen die circadiane Rhythmik aufrechterhält [58]. Die circadianen Oszillatoren der Retina regulieren die Melatonin- und Dopaminsynthese, die visuelle Sensitivität und den Augeninnendruck des Auges, wobei diese Funktionen jeweils bestimmten Schichten der Retina zugeordnet werden können (Abbildung 2; zur Übersicht, siehe [11]). Der molekulare Aufbau der Oszillatoren ist dabei zwischen der Retina und anderen neuronalen Geweben ähnlich [42, 43, 59].

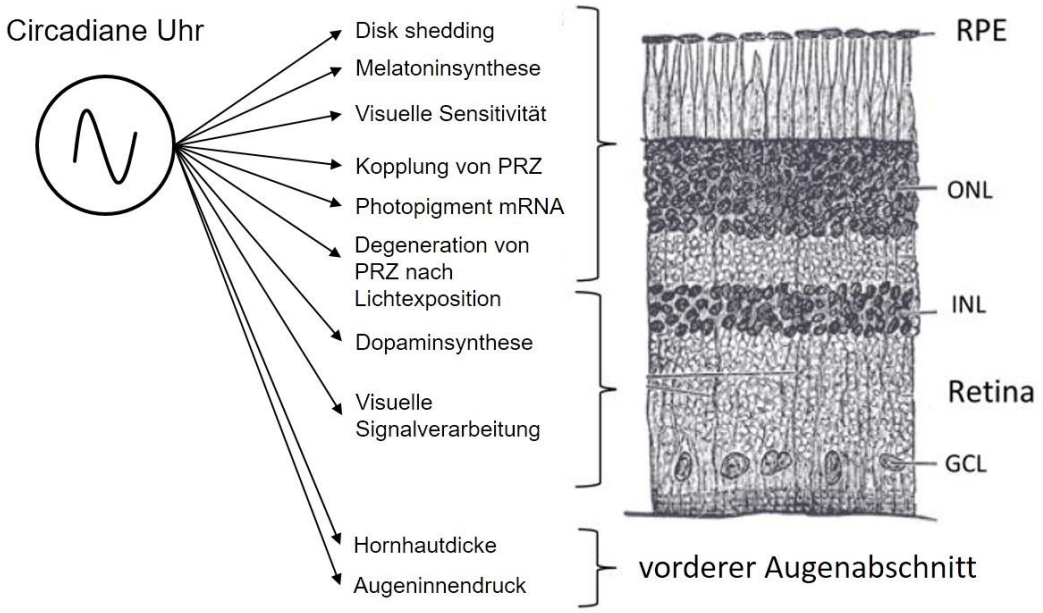

Abbildung 2: Circadiane Prozesse in der Säugetierretina
Viele Prozesse im Auge und in der Retina werden circadian gesteuert. Die Abbildung zeigt die Zuordnung circadianer Prozesse zur entsprechenden Schicht der Retina. RPE: retinales Pigmentepithel; ONL: äußere Körnerschicht; INL: innere Körnerschicht; GCL: Ganglienzellschicht. (verändert nach [11]).

Circadiane Oszillatoren konnten in verschiedenen Zelltypen der Retina nachgewiesen werden, unter ihnen FRZ [42, 43, 60], Neurone der inneren Retina [58, 61] und Pigmentepithelzellen [62]. So belegen Studien, dass isolierte FRZ in Kultur circadiane Rhythmen produzieren, z.B. hinsichtlich der Synthese von Melatonin [60]. Ebenso ist bekannt, dass die circadiane Uhr, welche die Synthese von Melatonin beeinflusst, bei Hühnern und Ratten in der äußeren Körnerschicht der Retina sitzt [60, 63]. Für einen FRZ-eigenen Oszillator spricht auch, dass die Rhythmik der Synthese erhalten bleibt, wenn die innere retinale Zellschicht entfernt wird [9, 10].

Uhrengene zeigen in verschiedenen Zelltypen der Retina Unterschiede in ihrer Expression. So konnte in der Ratte für *Per1*, *Bmal1* und *Clock* gezeigt werden, dass ihre Expression in Neuronen der inneren Retina im Vergleich zu FRZ erhöht ist [64, 65]. Die rhythmische Expression von Uhrengenen schwächt sich in FRZ unter DD-Bedingungen ab [42, 43]. Diese Ergebnisse zeigen, dass Licht die Oszillation der Uhrengene in FRZ verstärken kann. Der Einfluss von intrinsisch photosensitiven retinalen Ganglienzellen (ipRGCs) auf die circadiane Rhythmik der Retina unterstützt die These, dass die circadiane Uhr in der Retina durch ein externes Lichtsignal beeinflusst werden kann (zur Übersicht, siehe [11]).

Verschiedene Untersuchungen in der Mausretina kommen hinsichtlich der zellspezifischen Zuordnung der Uhrengene zu widersprüchlichen Ergebnissen. So wurde in einer Studie eine *Per1*-Expression in FRZ der Maus beobachtet [66], die andere Studien nicht bestätigen konnten [39, 58, 67, 68]. Nachdem Ruan et al. (2006) die Co-Expression aller sechs Uhrengene, welche den Kern der circadianen Uhr bilden, nur in Neuronen der inneren Retina nachweisen konnten, beobachteten Dkhissi-Benyahya et al. (2013) diese nur in FRZ, nicht aber in Neuronen der inneren Retina [58, 69]. Auf Proteinebene zeigten die Uhrengene sowohl in der inneren und äußeren Retina eine Rhythmik [70].

Das aktuelle Konzept postuliert, dass circadiane Uhren in den Zapfen der äußeren Retina und Neuronen der inneren Retina (dopaminerge Amakrinzellen und Müllerzellen) lokalisiert sind [58, 60, 63, 70].

1.4 Die Rolle von Melatonin und Dopamin in der circadianen Regulation der Retina

Neben Uhrengenen und Uhren-kontrollierten Genen, haben auch die Neuromodulatoren Melatonin und Dopamin einen Einfluss auf die circadiane Adaptation der Retina (zur Übersicht, siehe [48]). Sie zeigen in der Retina eine circadiane Rhythmik und ihre Sekretion beeinflusst die Synchronisation der retinalen Funktionen, darunter z.B. Hell- und Dunkeladaptation [9, 68-70] (zur Übersicht, siehe [52, 71]).

Die Sekretion von Dopamin zeigt in der Retina eine circadiane Rhythmik mit erhöhten Werten am Tag, sowie eine lichtstimulierte Sekretion [72]. Dopamin wird in den dopaminergen Amakrinzellen der inneren Körnerschicht (engl.: inner nuclear layer, INL) und inneren plexiformen Schicht (engl.: inner plexiform layer, IPL) gebildet (zur Übersicht, siehe [52, 73]). Die Wirkung von Dopamin wird in der Retina über verschiedene Dopamin-Rezeptoren vermittelt, vor allem aber über Dopamin-D4-Rezeptoren (D_4) in FRZ. Dadurch werden Helladaptation und verstärkte Kontrastsensitivität ermöglicht [74, 75] und die circadiane Uhr der FRZ [76] sowie deren Melatonin-Freisetzung beeinflusst [51]. Die lichtinduzierte Sekretion von Dopamin aus Amakrinzellen scheint für die Synchronisation der Uhren der FRZ relevant zu sein. So konnte gezeigt werden, dass lichtinduziertes Dopamin die Expression von *Period*-Uhrengenen in der Retina stimuliert [77]. Lichtinduzierte Dopamin-Antworten bleiben in Retinae, deren Stäbchen und Zapfen degeneriert sind, erhalten. Dies deutet darauf hin, dass für dopaminerge Neurone das Lichtsignal der ipRGCs eine stärkere Rolle spielt als das der Stäbchen und Zapfen [78-80].

Während die Ausschüttung von Dopamin am Tag erhöht ist, zeigt die Synthese von Melatonin eine circadiane Rhythmik mit erhöhten nächtlichen Werten (zur Übersicht, siehe [52]). Die Synthese von Melatonin erfolgt in der Retina von Säugetieren in den FRZ und wird durch die circadiane Uhr gesteuert (zur Übersicht, siehe [81]). Sie vermittelt die Dunkeladaptation der Retina und wird durch die Uhren-abhängige Regulation der Transkription des *Aanat*-Gens reguliert [82, 83]. Diese erfolgt über die Bindung des CLOCK/BMAL1-Heterodimer an die E-Box im *Aanat*-Promoter [84]. Somit vermittelt die transkriptionelle Regulation des *Aanat*-Gens die circadiane Rhythmik der Melatonin-Synthese (zur Übersicht, siehe [71, 85]).

Die folgenden Befunde lassen vermuten, dass die circadiane Rhythmik der retinalen Melatonin-Synthese von retinalen Uhren, und nicht vom NSC gesteuert wird. So bleibt bei Ratten die circadiane Rhythmik der *Aanat*-Transkription in der Retina auch nach Entfernung des NSC erhalten [57]. Des Weiteren konnten Tosini und Menaker Ende der 1990er Jahre zeigen, dass die circadiane Rhythmik der Melatonin-Freisetzung auch in explantierten Retinae in Kultur über mehrere Tage erhalten bleibt [9, 10].

Die Wirkung von Melatonin wird über die membranständigen G-Protein-gekoppelten Melatonin-Rezeptoren Typ 1 und Typ 2 (MT1 und MT2) vermittelt, die unter anderem in INL und IPL vorkommen (zur Übersicht, siehe [86]). Hierbei scheint Melatonin die visuelle Signalverarbeitung über MT1, die retinale Uhr sowie die Rhythmik der Uhrengene dagegen über MT2 zu steuern [82, 83]. Darüber hinaus können MT1 und MT2 auch heterodimere Rezeptoren bilden [87].

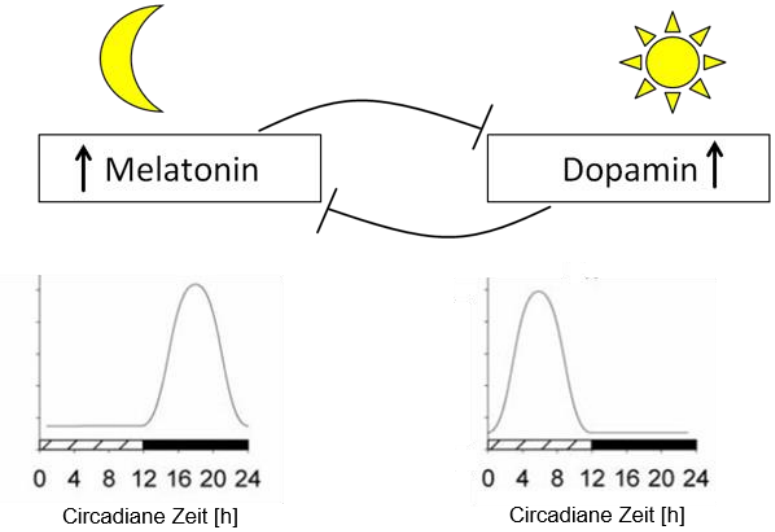

Abbildung 3: Melatonin und Dopamin in der Retina
Melatonin und Dopamin beeinflussen die retinale Physiologie antagonistisch. Während Dopamin für die lichtinduzierte Adaptation zuständig ist, vermittelt Melatonin die Dunkeladaptation. Die Synthese und Freisetzung beider Neuromodulatoren wird circadian reguliert und zeigt erhöhte Dopaminkonzentrationen bei Tag und erhöhte Melatoninkonzentrationen bei Nacht. Melatonin inhibiert die Freisetzung von Dopamin über Melatoninrezeptoren, während Dopamin die Synthese und Freisetzung von Melatonin über Dopaminrezeptoren an dopaminergen Neuronen der inneren Retina inhibiert. Dieser Rückkopplungsmechanismus steuert die circadiane Physiologie der Retina (zu Teilen aus: [52]).

Die Synthese von Melatonin und Dopamin ist durch eine negative Rückkopplungsschleife verbunden, so inhibiert Melatonin die Freisetzung von Dopamin und umgekehrt [63, 88] (Abbildung 3, zur Übersicht, siehe [52]).

Zusammenfassend kann gesagt werden, dass Dopamin und Melatonin die Uhren-gesteuerte Regulation von Retina und FRZ vermitteln und damit die funktionellen Anpassungen der Retina an tageszeitliche Änderungen unterstützen.

1.5 Zielsetzung der Arbeit

In der vorliegenden Arbeit wurde die tageszeitliche Adaptation der Retina auf Ebene der Genexpression analysiert. Hierzu wurden folgende Fragestellungen untersucht:

1. Welche Gene weisen tageszeitliche Schwankungen in ihrer Expression auf und vermitteln so die Anpassung von Retina und FRZ (bearbeitet in den Publikationen I-III)?

2. Wird die Expression dieser Gene durch Licht und/oder circadiane Oszillatoren reguliert (Publikationen I-III)?

3. Wird die Expression der circadianen Gene über Melatonin und/oder Dopamin bzw. die zugehörigen Rezeptoren reguliert (Publikationen II und III)?

Neben den drei Publikationen, die den Kern dieser Arbeit bilden, wurden auch im Rahmen weiterer Publikationen unserer Arbeitsgruppe und unserer Kooperationspartner tageszeitlich regulierte Gene identifiziert und die Regulation ihrer Expression untersucht (Hölter *et al*., 2012; Hiragaki *et al*., 2014; Wolloscheck *et al*., 2015).

Die Untersuchung der Genexpression erfolgte in Proben von FRZ (Stäbchen und Zapfen), Neuronen der inneren Retina (Ganglienzellen) und Gesamtretina-Gewebe. Mit Hilfe von Real-Time PCR und Western Blot wurde die Expression des jeweiligen Gens auf Transkript- bzw. Proteinebene analysiert. Zur Untersuchung des Einflusses von endogenen Oszillatoren und Licht wurde die Genexpression der Retina zwischen Lichtregimen mit 12 Stunden Licht und 12 Stunden Dunkelheit (LD12:12) und Dauerdunkel (DD) verglichen. Der Einfluss der Neuromodulatoren Melatonin und Dopamin wurde mithilfe von MT1- und D_4-Knockout Mäusen untersucht.

2 Publikationen

2.1. Kunst S, Wolloscheck T, Hölter P, Wengert A, Grether M, Sticht C, Weyer V, Wolfrum U, Spessert R. (2013) Transcriptional analysis of rat photoreceptor cells reveals daily regulation of genes important for visual signaling and light damage susceptibility. J Neurochem, 124(6):757-69.

2.2. Kunst S, Wolloscheck T, Grether M, Trunsch P, Wolfrum U, Spessert R. (2015) Photoreceptor cells display a daily rhythm in the orphan receptor *Esrrβ*. Mol Vis, 21:173-84.

2.3. Kunst S, Wolloscheck T, Kelleher DK, Wolfrum U, Sargsyan SA, Iuvone PM, Baba K, Tosini G, Spessert R. (2015) *Pgc-1α* and *Nr4a1* are target genes of circadian melatonin and dopamine release in murine retina. Invest Ophthalmol Vis Sci, 56:6084-94.

Publikation I

Kunst S, Wolloscheck T, Hölter P, Wengert A, Grether M, Sticht C, Weyer V, Wolfrum U, Spessert R. (2013) Transcriptional analysis of rat photoreceptor cells reveals daily regulation of genes important for visual signaling and light damage susceptibility. J Neurochem, 124(6):757-69.

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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.

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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.

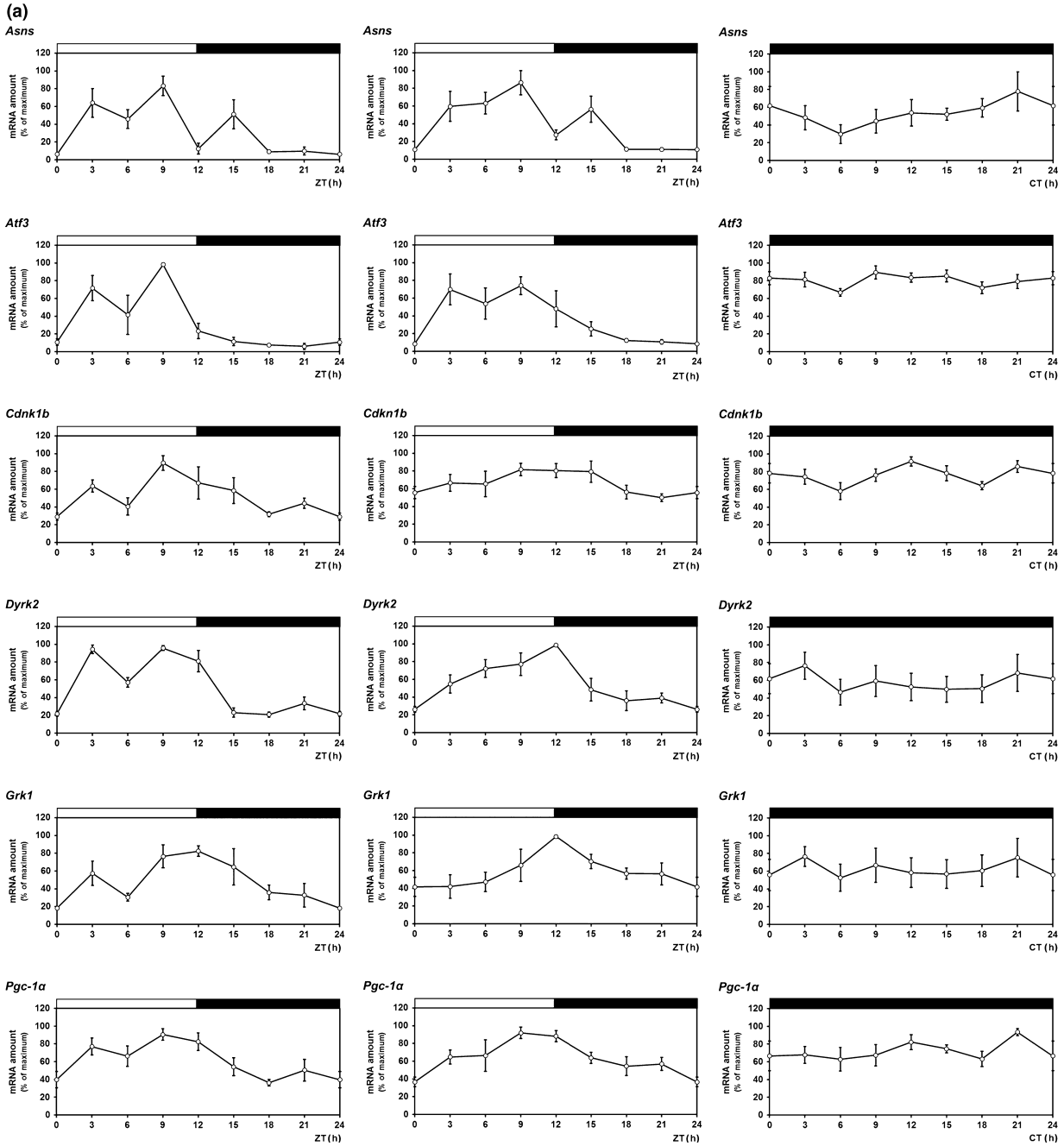

Fig. 1 24-h profiling of (a) genes predicted by microarray analysis to show enhanced expression during daytime, (b) genes predicted by microarray analysis to show enhanced expression at night, (c) reference genes. The mRNA levels are plotted both as a function of Zeitgeber time (ZT) and Circadian time (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

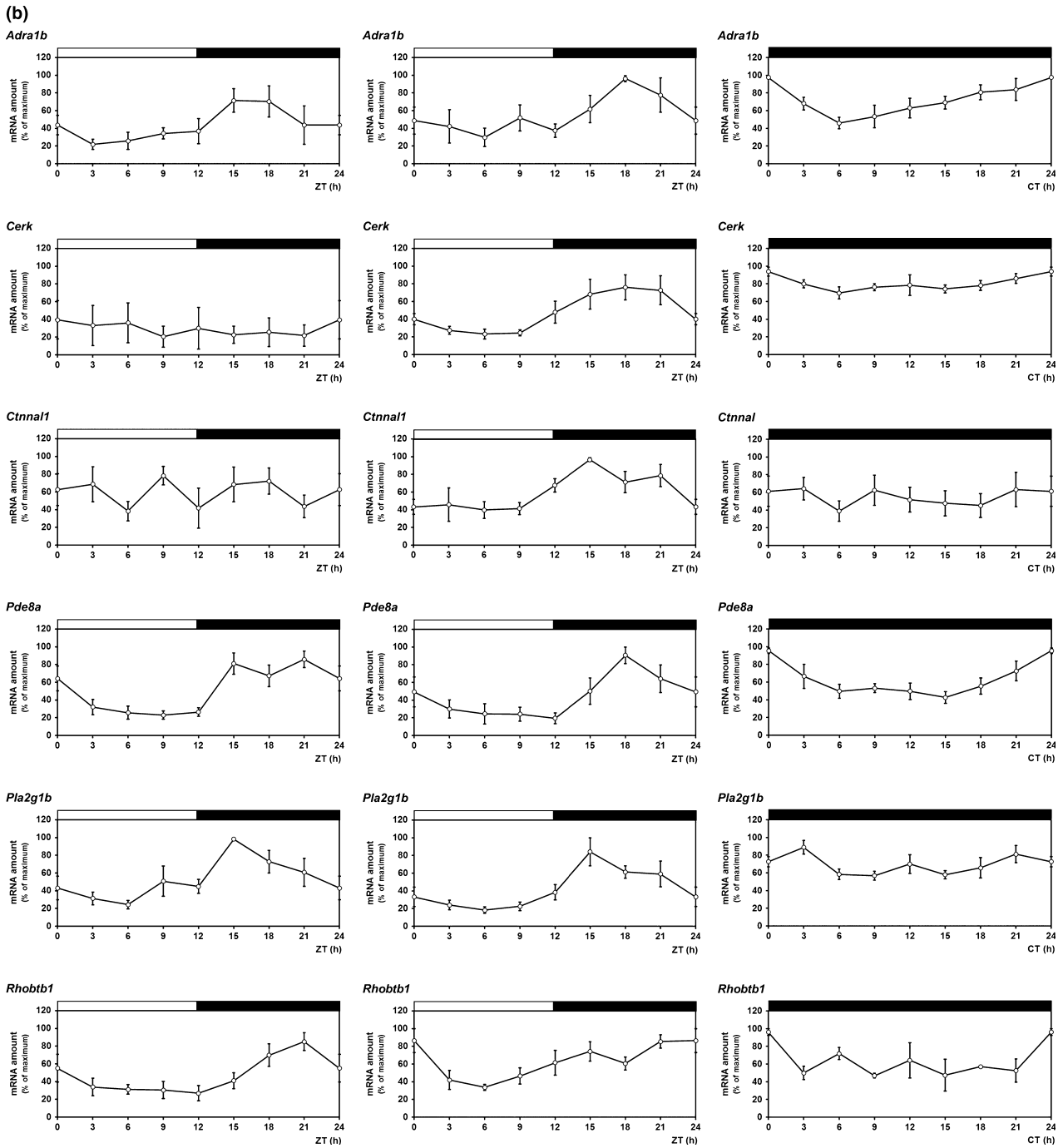

Fig. 1 Continued.

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.

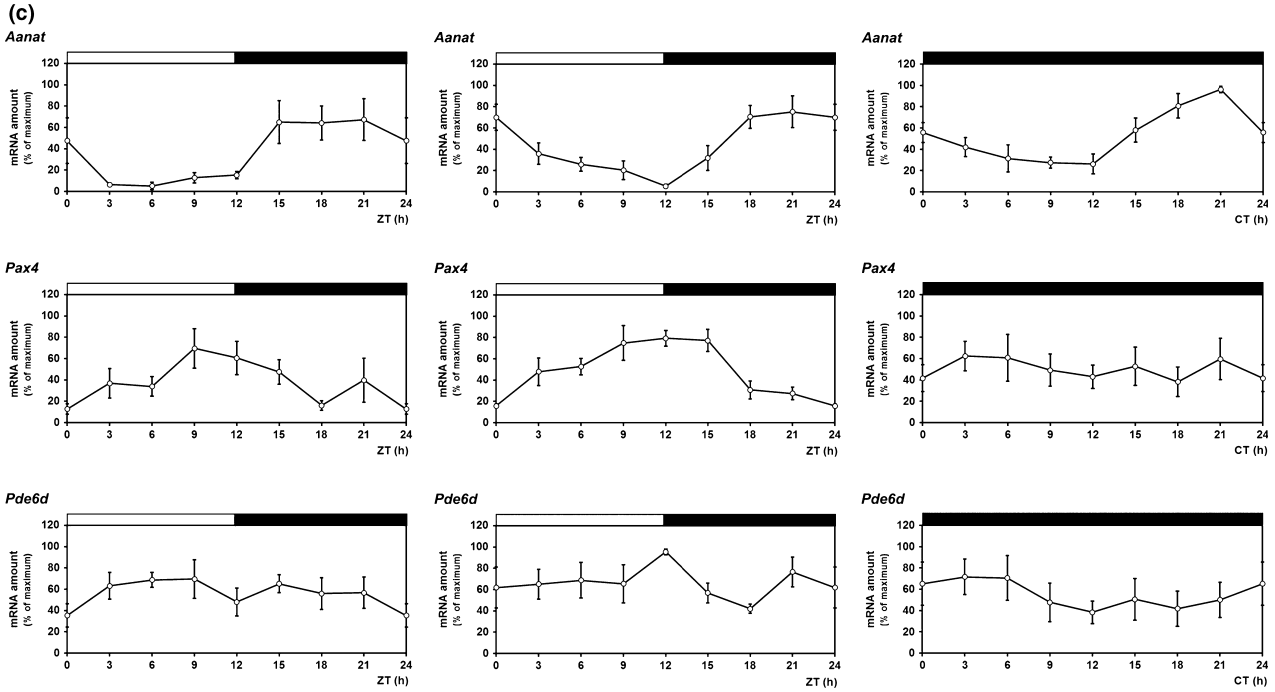

Fig. 1 Continued.

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

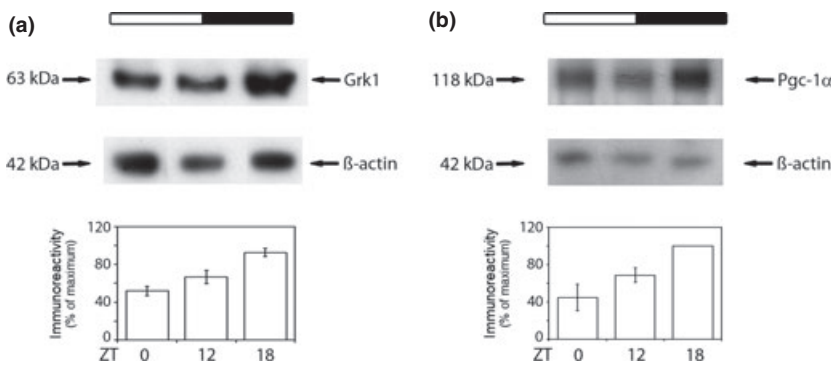

Fig. 2 Western blot analysis of Grk1 (a) and Pgc-1α (b) conducted at different Zeitgeber times (ZTs) during the 24-h cycle. The upper lanes show representative Western blots with Grk1- and Pgc-1α immunostaining at ~63 kDa and ~118 kDa. The lower lanes show the β-actin signal to which the Grk1 and Pgc-1α 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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Publikation II

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Photoreceptor cells display a daily rhythm in the orphan receptor *Esrrβ*

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Purpose: Nuclear orphan receptors are critical for the development and long-term survival of photoreceptor cells. In the present study, the expression of the nuclear orphan receptor *Esrrβ*—a transcriptional regulator of energy metabolism that protects rod photoreceptors from dystrophy—was tested under daily regulation in the retina and photoreceptor cells.
Methods: The daily transcript and protein amount profiles were recorded in preparations of the whole retina and micro-dissected photoreceptor cells using quantitative PCR (qPCR) and western blot analysis.
Results: *Esrrβ* displayed a daily rhythm with elevated values at night in the whole retina and enriched photoreceptor cells. Daily regulation of *Esrrβ* mRNA depended on light input but not on melatonin, and evoked a corresponding rhythm in the Esrrβ protein.
Conclusions: The data presented in this study indicate that daily regulation of *Esrrβ* in photoreceptor cells may contribute to their adaptation to 24-h changes in metabolic demands.

In the mammalian retina, nuclear receptors complement other classes of transcriptional regulators in determining the fate of cells [1,2], their function [3,4], and homeostasis [5]. The majority of nuclear receptors found in the retina are so-called orphan receptors that are nuclear receptors with unknown physiologic ligands. Retinal nuclear orphan receptors typically control photoreceptor development and differentiation, and mutations result in dysfunction and degeneration of rod and/or cone photoreceptors [5]. A prominent example is the *retinoic acid-related orphan receptor beta* (*Rorβ*; OMIM 601972; Gene ID mouse 225998, Gene ID rat 309288) gene that directs the cell fate of rods and short (s)-opsin-expressing cones (S-cones) and controls the terminal differentiation of photoreceptor cells. Accordingly, mice mutant for *Rorβ* lack rods and gain primitive S-cones and photoreceptors lacking outer segments [6-8]. Among the nuclear orphan receptors, *estrogen-related receptor* β (*Esrrβ*, *Nr3b2*, OMIM 602167, Gene ID mouse 26380, Gene ID rat 299210) plays a special role. This orphan receptor does not detectably impair development but the long-term survival of photoreceptor cells instead. Thus, mice deficient for *Esrrβ* show slow and selective degeneration of rod photoreceptors in later life, which is preceded by the loss of rod outer segments [9]. Whereas mutations in nuclear orphan receptor genes often result in photoreceptor or ocular phenotypes, thus far only a few are known to underlie inherited retinal degeneration in humans [10-14]. Mutations in the human *Esrrβ* gene are associated with autosomal-recessive deafness [15] although investigations regarding vision disturbances have not yet been reported.

Mammalian photoreceptor cells must maintain their function for a lifetime in the face of hazards such as oxidative stress [16] and metabolic/energy challenges [17,18] occurring during the day/night cycle. To avoid age-related dysfunction or death, this may require the ability to adapt the cellular defense mechanisms and metabolism to 24-h changes in the environment [16]. Daily adaptation of photoreceptor cells (and other retinal neurons) is driven by light input and retinal clocks [19-21] through the release of the neuromodulators melatonin and dopamine, both of which play opposing roles in retinal adaptation [22]. Whereas melatonin is released during the dark/night and promotes dark-adaptive mechanisms [23-25], dopamine is released during the light/day and contributes to light adaptation of the photoreceptor cells [26,27].

At the transcriptional level, 24-h changes in the nuclear orphan receptor *Rorβ* contribute to daily adaptation of the retina and photoreceptor cells [28-30]. The data included in the present study show that daily changes of the nuclear orphan receptor *Esrrβ* are evident in photoreceptor cells and may contribute to their ability to comply with metabolic demands and thus to the cells' long-term survival.

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METHODS

Animals: Animal experimentation was performed in accordance with the European Communities Council Directive (86/609/EEC). The study was approved by the German national investigation office and adhered to the ARVO Statement for Use of Animals in Research. Adult male and female rats (Sprague-Dawley) or mice (melatonin-proficient C3H/He, not carrying the *rd* mutation; melatonin-deficient C57BL/6Jb) were kept under standard laboratory conditions (illumination with fluorescent strip lights, 200 lux at cage level during the day and dim red light (<3 lux) during the night; 20±1 °C; water and food ad libitum) under 12 h:12 h light-dark (12:12 LD) for 3 weeks. When indicated, after LD treatment the animals were kept for one cycle under dim red light and killed during the next cycle. They were killed at the indicated time points by decapitation following anesthesia with 100% carbon dioxide for approximately 3 min. All dissections during the dark phase were performed under dim red light. The retinas were rapidly removed and immediately processed as follows.

Sample preparation: The sample size for all experiments was n=4, with each n deriving from 4 pooled retinas of 2 animals. The HEPES-glutamic acid buffer mediated organic solvent protection effect (HOPE; DCS, Hamburg, Germany) technique was applied to fix the retinas. Briefly, fixation started with the incubation of fresh retinas in an aqueous protection-solution HOPE I (DCS) for 48 h at 0–4 °C. Retinas were then dehydrated in a mixture of HOPE II solution (DCS) and acetone for 2 h at 0–4 °C, followed by dehydration in pure acetone for 2 h at 0–4 °C (repeated twice). Tissues were then embedded in low-melting paraffin (T_m=52–54 °C). Tissue sections (10 µm) from HOPE-fixed and paraffin-embedded retinas were prepared on membrane-mounted slides (DNase/RNase free PALM MembraneSlides, P.A.L.M., Bernried, Germany). Three sections were placed on each slide. The sections were deparaffinized with isopropanol (2 × 10 min each, at 60 °C). All sections were stained with cresyl violet (1% w/v cresyl violet acetate in 100% ethanol) for 1 min at room temperature, washed briefly in 70% and 100% ethanol, and then air-dried [31].

Laser microdissection and pressure catapulting: To isolate photoreceptor cells and inner retinal neurons from the stained sections in a contact- and contamination-free manner, the laser microdissection and pressure catapulting (LMPC) technique was applied [32]. The LMPC technique was performed using a PALM MicroBeam system (Zeiss MicroImaging, Munich, Germany) with PALM RoboSoftware (P.A.L.M.). Under the 10X objective, the outer nuclear layer bearing the photoreceptor cells and the tissue between the inner part of the outer plexiform layer and the inner ganglion cell layer bearing the inner retinal neurons 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 using a pulsed ultraviolet-A (UV-A) nitrogen laser. Smaller areas of the sections were pooled to reach total average sample sizes of 4 million square microns per tube. Alternatively, the whole retina was excised with a scalpel and collected in a 0.5 ml microfuge tube. Cell lysis for RNA preparation was performed immediately after the sample was collected. To verify the purity of the preparations, they were subjected to molecular analysis with rhodopsin (*Rho*) and neural retina leucine zipper (*Nrl*) as markers for photoreceptor cells and tyrosine hydroxylase (*Th*) and metabotropic glutamate receptor 6 (*mGluR6*) as markers for inner retinal neurons.

RNA extraction: RNA of the laser-microdissected tissue samples was isolated using the RNeasy Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, 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 with centrifugation, diluted with ethanol, and applied to a 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 performed as described by the manufacturer. Isolated RNA was eluted in the final volume of 12 µl RNase-free water. The amount of extracted RNA was determined by measuring the optical density at 260 and 280 nm.

Reverse-transcription and quantitative PCR: 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 supplied with the kit in a final volume of 20 µl. cDNA was then diluted 1:3 in RNase-free water, and aliquots of 5 µl were used for PCR. Quantitative PCR was performed in a total volume of 25 µl containing 12.5 µl ABsolut 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 a CFX96 (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 performed 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. The

transcript amount of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was constitutively expressed over the 24-h period in preparations of the whole retina, photoreceptor cells, and inner retinal neurons. Values were then normalized according to the amount of GAPDH mRNA present.

Western blot analysis: For western blot analysis, samples were loaded on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen, Carlsbad, CA), separated, and blotted onto polyvinylidene fluoride (PVDF) membrane (Westran S, Whatman Inc., Sanford, ME). For immunodetection, membranes were

Table 1. Primer sequences.

Gene	Accession number	Primer sequence 5′ to 3′	Product length of product [bp]PCR
r-Crx	NM_021855	F: GGCTGTCCCATACTCAAGTG	107
		R: GGTACTGGGTCTTGGCAAAC	
r-Esrrα	AY280663.1	F:GTGGCCGACAGAAGTACAAG	144
		R: CAACCACCAGCAGATGAGAC	
r-Esrrβ	NM_001008516	F: CAAGAGACGGCTGGATTCG	105
		R: TCGGCCACCAGTAGATACG	
m-Esrrβ	NM_011934	F: CCCTCGCCAACTCAGATTC	104
		R: GCGCTGACTCAGCTCATAG	
r-Esrrγ	AY341057	F: CTTGATCCTGGGTGTTGTG	101
		R: GGCCTGCTAATTTGGACTG	
r-Gapdh	NM_017008	F: ATGACTCTACCCACGGCAAG	89
		R: CTGGAAGATGGTGATGGGTT	
m-Gapdh	BC082592	F: GTCATCCCAGAGCTGAAC	144
		R: CTCAGATGCCTGCTTCAC	
r-mGluR6	NM_022920	F: ACAACCGCAGAAACATCTGG	113
		R: TTCCTCACCTGTGCATTTCC	
r-NeuroD	AF107728	F: AGACGAGTGCCTCAGTTC	136
		R: CTCCTCCAGATCCTCATCTTC	
r-Nr2c1	NM_145780	F: CGGAGAGGAGGAAGTTGTTG	126
		R: CGGGACTGAAGAGAACGATG	
r-Nr2e1	NM_001113197	F: GAGTTGACCGCTGTGTCTG	139
		R: CTGATTCGCACACGGACTC	
r-Nrl	NM_001106036	F: AGGCCTGGAGGAGCTATAC	134
		R: CATGAGGCCCTTCTACAGAG	
r-Nupr1	NM_053611	F: GCCTGGCCCAATCTTATGTC	116
		R: CTTGGTCAGCAGCTTCCTC	
r-Otx2	NM_001100566	F: CAGTCAATGGGCTGAGTC	112
		R: GCCCTCGTGAAAGTAGTC	
r-Rho	NM_033441	F: ACTCAGAAGGCAGAGAAGG	638
		R: CCTAGACACTTAGCCGTAAGC	
r-Rorβ	NM_001270958	F: GCCTGGCTGTTAGAACCAAG	147
		R: GTTGCAGACTGCCGTGATAG	
m-Rorβ	NM_001043354	F: CCTGGCTGATCGAACCAAG	144
		R: TGCAGACTGCCGTGATAG	
r-Th	NM_012740	F: CCAGGGCCTTTCCCAAAGTC	186
		R: CATGGAGGGCAGGAGGAATG	

blocked in 5% skimmed milk powder, and anti-Esrrβ polyclonal antibody (1:500; Abcam, Cambridge, MA; ab19331) was applied overnight at 4 °C. The horseradish-peroxidase-coupled secondary antibodies (goat anti-rabbit-HRP 1:5000; Sigma-Aldrich, St. Louis, MO; A0545) were visualized using an enhanced chemiluminescence (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).

Statistical analysis: All PCR data are expressed as the mean ± standard error of the mean (SEM) of four independent experiments including eight time points. Transcript levels from each sample (consisting of four pooled retinas from two animals) were calculated relative to the average expression of each data set throughout 24 h to plot the temporal expression. Quantitative data were analyzed with ANOVA (one-way ANOVA) to evaluate variations among the groups. Cosinor analysis was used to fit sine-wave curves to the circadian data to mathematically estimate the time of peaking gene expression (acrophase) and to assess the amplitude [33,34]. The model can be expressed according to the following equation: $f(t)=A + B \cos [2\pi (t + C) / T]$. The f(t) indicates the relative expression levels of the target genes, t specifies the sampling time (h), A represents the mean value of the cosine curve (mesor; midline estimating statistic of rhythm), B indicates the amplitude of the curve (half of the sinusoid), and C indicates the acrophase (point of time, when the function f(t) is maximum). T gives the time of the period, which was fixed at 24 h for this experimental setting. The significance of the daily regulation was defined by showing $p<0.05$ in the ANOVA and Cosinor analysis.

RESULTS

Esrrβ mRNA and protein levels are under daily regulation in the retina: Nuclear orphan receptors important for the survival of photoreceptor cells or protective against photoreceptor dystrophy [5,35] were tested under daily regulation. Of the nuclear receptors considered under daily regulation (Figure 1, Table 2), only *Esrrβ* displayed a daily change in both statistical analyses applied (Figure 2, Table 3). The 24-h pattern of *Esrrβ* mRNA levels showed peak expression at ZT18.7 and an amplitude of 32.9%. Consistent with the validity of the results, the daily profile of the reference gene *Rorβ* was similar to those found in previous reports [28,30] (Figure 2, Table 3).

In the context of a putative role of *Esrrβ* in the protection of the retina against daily changes in the environment, the question of whether daily regulation of the *Esrrβ* gene results in daily changes in the protein product was addressed using western blotting (Figure 3). The anti-Esrrβ antibody recognizes a band of about 52 kDa, a molecular mass predicted from the *Esrrβ* gene. The intensity of Esrrβ immunoreactivity tended to increase during the light phase ($p=0.041$ in one-way ANOVA). This suggests that the daily rhythm in *Esrrβ* transcript levels evokes corresponding variations in the protein amount, with the temporal lag reflecting the time necessary to translate mRNA into protein.

Daily regulation of Esrrβ depends on illumination: Daily regulation of *Esrrβ* may be promoted by light input and/or a circadian clock. To investigate and compare the influence of both parameters between *Esrrβ* and *Rorβ*, rats adapted to the 12 h:12 h light-dark cycle were kept in constant darkness (DD) for one cycle and recorded during the subsequent cycle (Figure 2, Table 3). Under these conditions, the daily rhythm in the transcript levels was not detected for *Esrrβ*. As expected for a component of a circadian clock [23] and as reported previously [30], the amount of *Rorβ* mRNA persisted to the cycle under DD even though the cycling amplitude was clearly decreased (Figure 2, Table 3).

Daily regulation of Esrrβ evoked by photoreceptor cells: To compare daily regulation of *Esrrβ* and *Rorβ* between photoreceptor cells and inner retinal neurons, the LMPC technique was applied [32]. The purity grades of the preparations obtained were verified by using specific gene markers of photoreceptor cells, namely, *Rho* and *Nrl* (as markers for rods [36,37]) and of inner retinal neurons, namely, *Th* (as a marker for amacrine cells [38]) and *mGluR6* (as a marker of ON-bipolar cells [39]). Compared to whole retina preparations, in the photoreceptor cells collected with LMPC, the ratio of *Rho* to *Th* and *mGluR6* increased 34-fold and 85-fold, respectively, and that of *Nrl* to *Th* and *mGluR6* increased 170-fold and 43-fold, respectively. For the inner retina sample, the ratio of *Th* to *Rho* and *Nrl* increased 23-fold and 29-fold, respectively, and that of *mGluR6* to *Rho* and *Nrl* increased 15 fold and 18 fold, respectively.

To match the expression of the nuclear orphan receptors in the photoreceptor cells and the inner retinal neurons, mRNA levels were compared at the ZT of respective peak expression (Table 4). Both genes displayed higher transcripts in photoreceptor cells than in inner retinal neurons (*Esrrβ*: 23-fold; *Rorβ*: 15-fold). A comparison of the genes within the same tissue revealed that *Esrrβ* displayed higher transcript levels in photoreceptor cells (30-fold) and inner retinal neurons (15-fold).

To define and compare daily regulation of *Esrrβ* and *Rorβ* in photoreceptor cells and inner retinal neurons, the transcript levels were profiled in microdissected preparations of photoreceptor cells and inner retinal neurons. Both nuclear orphan receptors displayed daily rhythms in photoreceptor cells (Figure 4, Table 5) with similar 24-h profiles observed in preparations of the whole retina (Figure 2, Table 3). In contrast, the nuclear orphan receptors showed non-rhythmic expression in the inner retinal neurons (Figure 4, Table 5). These data suggest that the daily regulation of both genes in the retina is evoked primarily by photoreceptor cells.

Daily regulation of Esrrβ does not depend on melatonin: To investigate the contribution of melatonin to daily changes in the expression of *Esrrβ*, the 24-h course of the transcript levels of *Esrrβ* were compared between melatonin-proficient (C3H/He, not carrying the *rd* mutation) and melatonin-deficient mice (C57BL/6Jb; Figure 5, Table 6). The daily profiles of *Esrrβ* were similar in both mice strains and resembled that of rats (Figure 2, Table 3). As with *Esrrβ*, the reference gene *Rorβ* cycled in a similar manner in melatonin-proficient and -deficient mice. These observations suggest that daily regulation of *Esrrβ* and *Rorβ* does not require a pulsatile melatonin signal.

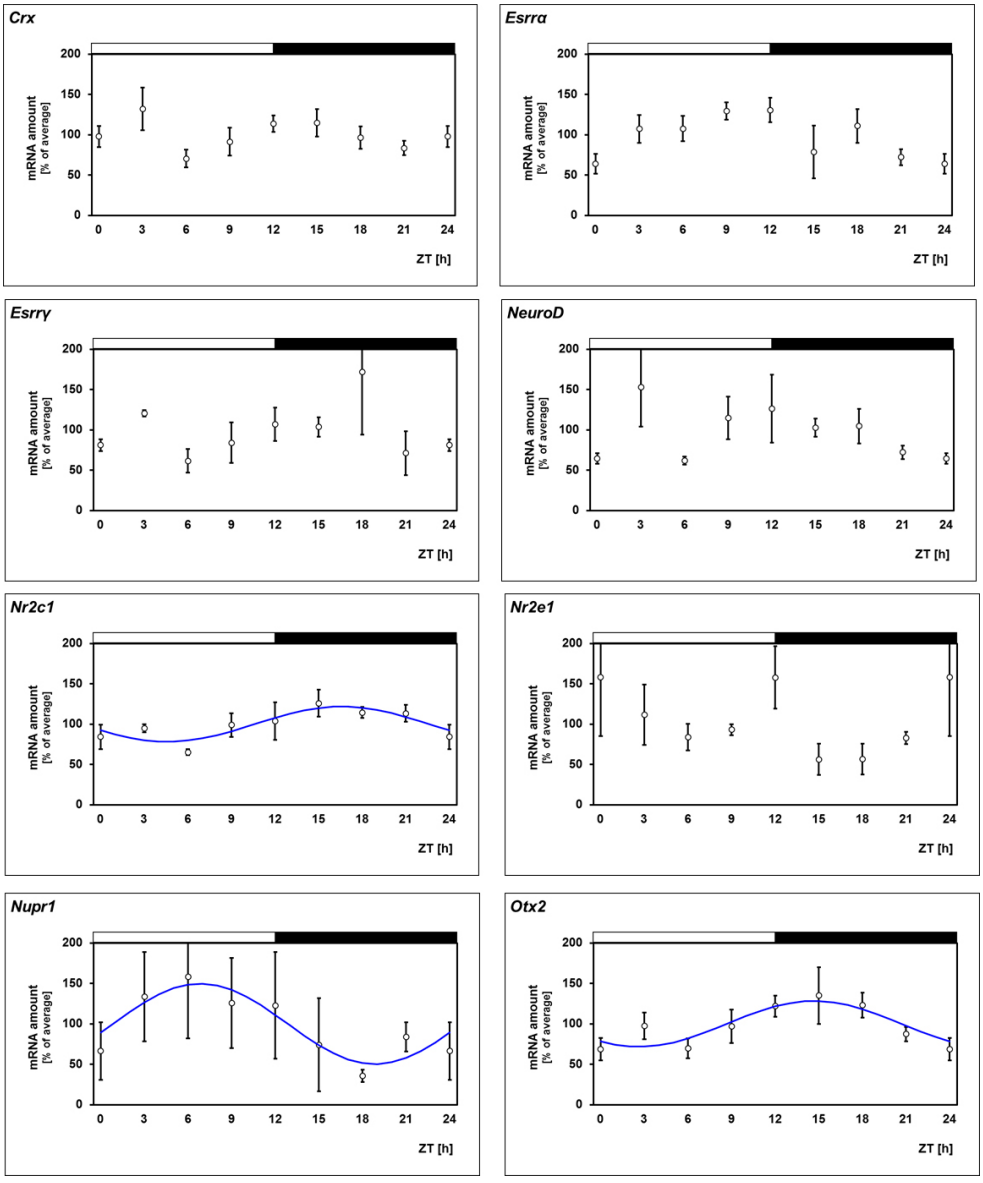

Figure 1. Twenty-four-hour profiling of various nuclear orphan receptors in preparations of the whole rat retina under 12 h:12 h light-dark cycles. The mRNA levels are plotted as a function of Zeitgeber time (ZT). The blue lines represent the periodic sinusoidal functions (illustrated only for $p<0.05$ in cosinor analysis). The solid bars indicate the dark period. Data represent a percentage of the average transcript amount during the 24-h period. The value of ZT0 was plotted twice at ZT0 and ZT24. Each value represents mean ± standard error of the mean (SEM; n=4). Statistical analysis of transcriptional profiling is provided in Table 2.

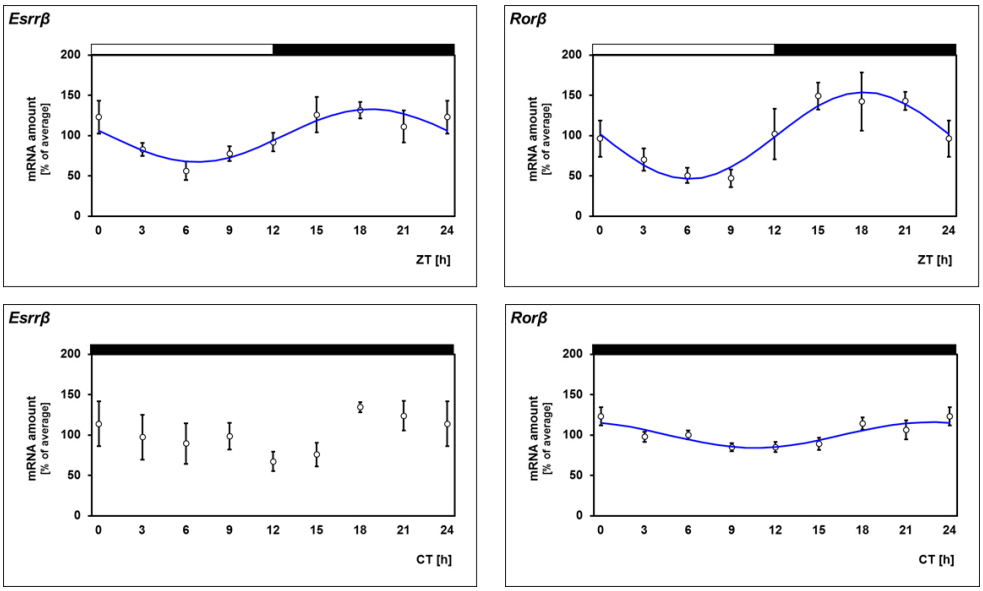

Figure 2. Twenty-four-hour profiling of *Esrrβ* and the reference gene *Rorβ* in preparations of whole rat retina under 12 h:12 h light-dark cycles (LD; upper line) or constant darkness (DD; lower line) using qPCR. The mRNA levels are plotted as a function of Zeitgeber time (ZT) and circadian time (CT). The blue lines represent the periodic sinusoidal functions determined with cosinor analysis (only for $p<0.05$). The solid bars indicate the dark period. Data represent a percentage of the average value of the transcript amount during the 24-h period. The value of ZT0 was plotted twice at ZT0 and ZT24. Each value represents mean ± standard error of the mean (SEM; n=4). Statistical analysis of transcriptional profiling is provided in Table 3. Note that daily rhythmicity of *Esrrβ* does not persist under DD.

Table 2. Statistical analysis of transcriptional profiling illustrated in Figure 1.

Gene	Whole retina under LD 12:12			
	One-way Anova	Cosinor analysis		
	P value	P value	Acrophase [h]	Amplitude [%]
Crx	=0.91	>0.05	-	-
Esrrα	=0.90	>0.05	-	-
Esrrγ	=0.86	>0.05	-	-
NeuroD	=0.90	>0.05	-	-
Nr2c1	=0.85	<0.05	16.6	21.8
Nr2e1	=0.95	>0.05	-	-
Nupr1	=0.72	<0.05	6.8	49.8
Otx2	=0.14	<0.05	14.7	28.3

Statistical analysis using one-way Anova and Cosinor analysis are shown for each gene under LD 12:12. (LD 12:12: light/dark 12:12)

Table 3. Statistical analysis of transcriptional profiling illustrated in Figure 2.

Gene	Whole retina under LD 12:12				Whole retina under DD			
	One-way Anova	Cosinor analysis			One-way Anova	Cosinor analysis		
	P value	P value	Acrophase [h]	Amplitude [%]	P value	P value	Acrophase [h]	Amplitude [%]
Esrrβ	=0.016	<0.05	18.7	32.9	=0.28	>0.05	-	-
Rorβ	=0.006	<0.05	18.1	53.9	=0.021	<0.05	22.6	15.9

Statistical analysis using one-way Anova and Cosinor analysis are shown for each gene under LD 12:12 and DD. (LD 12:12: light/dark 12:12; DD: constant darkness)

Table 4. Comparison of peak mRNA levels between photoreceptor cells (PRC) and the inner retina neurons.

Gene	PRC	Inner retina	PRC/inner retina ratio
Esrrβ	143,000	6.290	22.73
Rorβ	4530	296	15.30

Transcript amount was determined by qPCR and represents the number of transcripts in relation to *Gapdh* x 10^5 (mean ± SEM with n=4). (PRC: photoreceptor cells)

DISCUSSION

In this study, *Esrrβ* expression was observed under daily regulation in the retina. This indicates that cyclicity of *Esrrβ* is evident not only in peripheral tissues (such as white adipose tissue, brown adipose tissue, the liver, and muscle [40]) but also in the retina and thus in an area of the brain. The temporal timing of *Esrrβ* expression in the retina is similar to that found in both types of adipose tissues and is phase-advanced to that observed in the liver and muscle [40]. The retina resembles the peripheral tissues with *Esrrβ* cyclicity in its high metabolic activity and energy demand [41]. Therefore, daily regulation of *Esrrβ* may be a characteristic feature of tissues with high metabolic demand or may even be unique to them.

In the retina, the daily cyclicity of *Esrrβ* is evoked by photoreceptor cells. This is evident from the finding that the microdissected photoreceptor cells showed a daily rhythm in *Esrrβ*, which is similar to that observed in preparations of the whole retina, whereas microdissected inner retinal neurons did not appear to display *Esrrβ* rhythmicity at all. Although the high-purity grade of the photoreceptor preparations used was proven by the enrichment of transcripts known to be

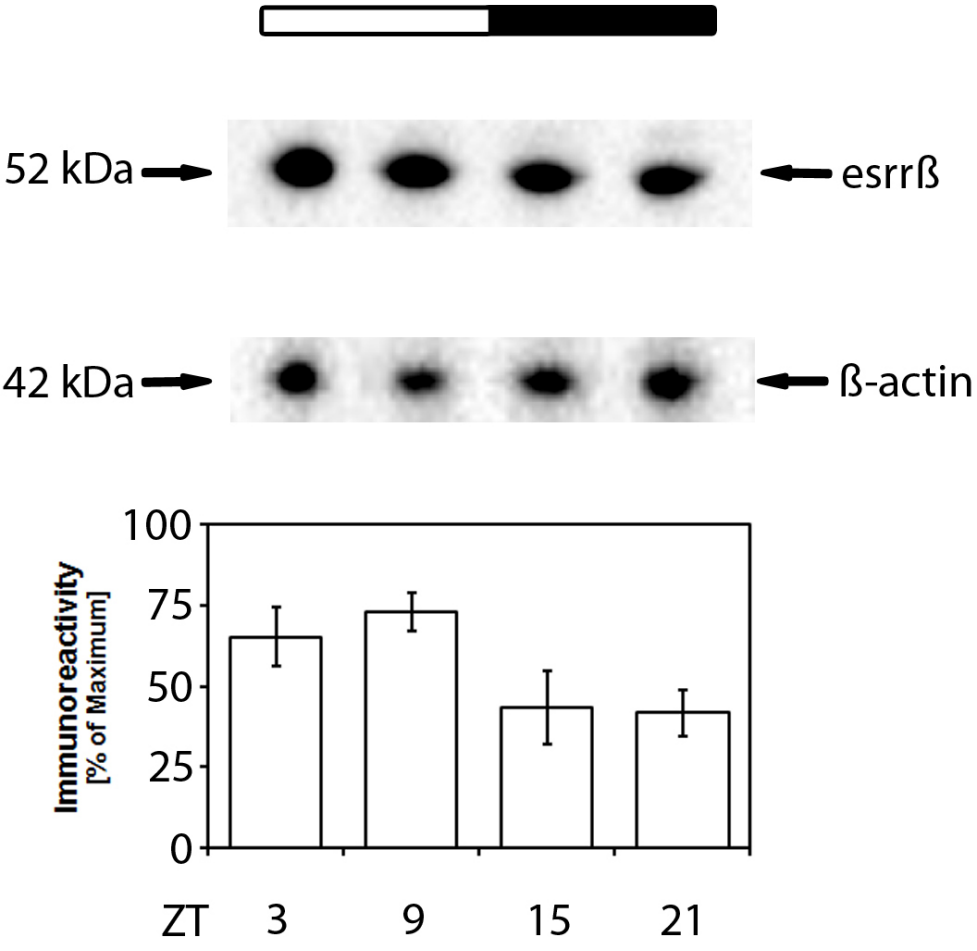

Figure 3. Western blot analysis of Esrrβ conducted at different ZTs (Zeitgeber times) during the 24-h cycle in the whole rat retina. The upper lanes show a representative western blot with Esrrβ immunostaining at about 52 kDa. The lower lanes show the β-actin signal to which the Esrrβ immunostaining was normalized. The diagram shows the quantification of immunoreactivity in relation to the corresponding β-actin signal. The solid bar indicates the dark period in the 12 h:12 h light-dark cycles. Data were obtained with densitometric measurement and represent percentages of the overall maximal value. Each value is the mean ± standard error of the mean (SEM; n=4). Note that Esrrβ immunoreactivity is elevated during the light phase (p=0.041 in one-way ANOVA).

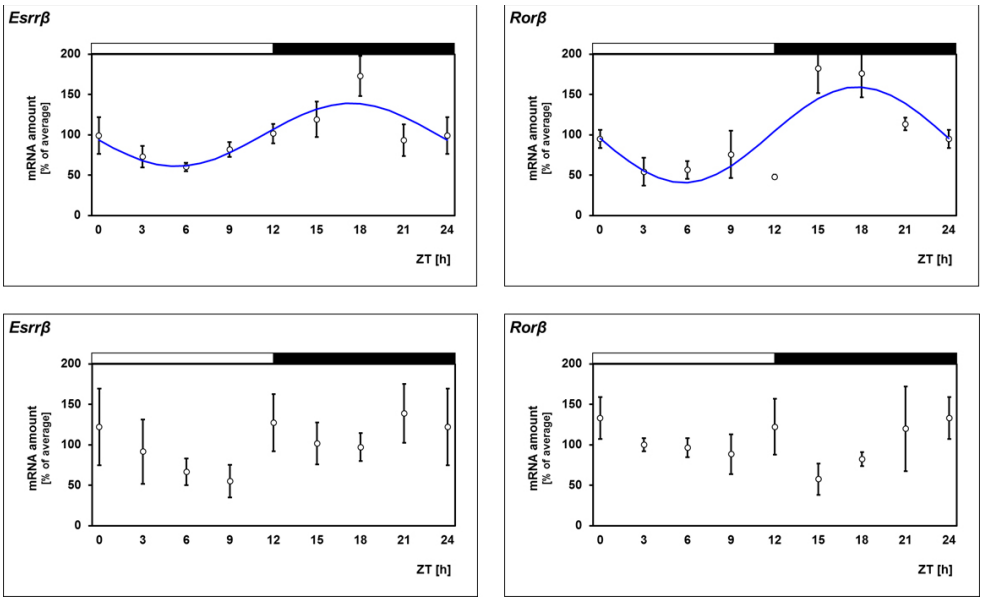

Figure 4. Comparative 24-h profiling of *Esrrβ* and the reference gene *Rorβ* in preparations of photoreceptor cells (upper line) and inner retinal neurons (lower line) of rats. The mRNA levels are determined by using quantitative PCR (qPCR), and both are plotted as a function of Zeitgeber time (ZT). The blue lines represent the periodic sinusoidal functions determined with cosinor analysis (only for $p<0.05$). The solid bars indicate the dark period. Data represent a percentage of the average transcript amount during the 24-h period. The value of ZT0 was plotted twice at ZT0 and ZT24. Each value represents mean ± standard error of the mean (SEM; n=4). Statistical analysis of transcriptional profiling is provided in Table 5. Note that daily variations of *Esrrβ* are evoked by photoreceptor cells.

specifically abundant in photoreceptor cells (*Rho*, *Nrl*), a limitation of this study is that the photoreceptor preparations were derived from rods and cones. Thus, the gene monitoring performed in the microdissected photoreceptors reflects the average rod and cone values and may not necessarily be valid for each photoreceptor type. However, *Esrrβ* was observed to be highly enriched in rod photoreceptors [42,43], and the *Esrrβ* mutation impairs only rod photoreceptors [9]. Therefore, daily regulation of *Esrrβ* probably occurs in rod photoreceptors.

Daily changes in *Esrrβ* require light-dark transitions and therefore depend on light input (this study) whereas that of *Rorβ* is truly circadian [30] (this study). This suggests that retinal nuclear orphan receptors on the whole mediate light-dependent and circadian adaptation. The rhythmicity of *Esrrβ* and *Rorβ* continued in melatonin-deficient mice and therefore does not require a pulsatile melatonin signal. As a result, neither the illumination-dependent regulation of *Esrrβ* nor the circadian regulation of *Rorβ* is mediated by melatonin.

Esrrβ influences the energy balance in the whole body [44] and is known to regulate key enzymes of fatty acid and carbohydrate metabolism in peripheral tissues [45] and photoreceptor cells [9]. Therefore, the cyclicity of *Esrrβ* may contribute to daily changes in the energy metabolism of the retina and photoreceptor cells and thus to their ability to adapt to 24-h changes in metabolic demands [17,18]. The fulfillment of metabolic demands appears to be a strong criterion in photoreceptor survival [46-48]. Thus, a role of *Esrrβ* in metabolic adaptation could (on a long-term basis) contribute to the survival of photoreceptor cells. This suggestion agrees with the observations that the loss of functional *Esrrβ* on a long-term basis leads to dysfunction and degeneration of rods and that enhanced *Esrrβ* activity rescues photoreceptor defects [9].

Table 5. Statistical analysis of transcriptional profiling illustrated in Figure 4.

Gene	PRCs under LD 12:12				IRN under LD 12:12			
	One-way Anova	Cosinor analysis			Oone-way Anova	Cosinor analysis		
	P value	P value	Acrophase [h]	Amplitude [%]	P value	P value	Acrophase [h]	Amplitude [%]
Esrrβ	=0.017	<0.05	17.3	39.0	=0.56	>0.05	-	-
Rorβ	=0.002	<0.05	17.7	59.3	=0.57	>0.05	-	-

Statistical analysis using one-way Anova and Cosinor analysis are shown for each gene in photoreceptor cells and inner retinal neurons. (LD 12:12: light/dark 12:12; PRC: photoreceptor cells; IRN: inner retinal neurons)

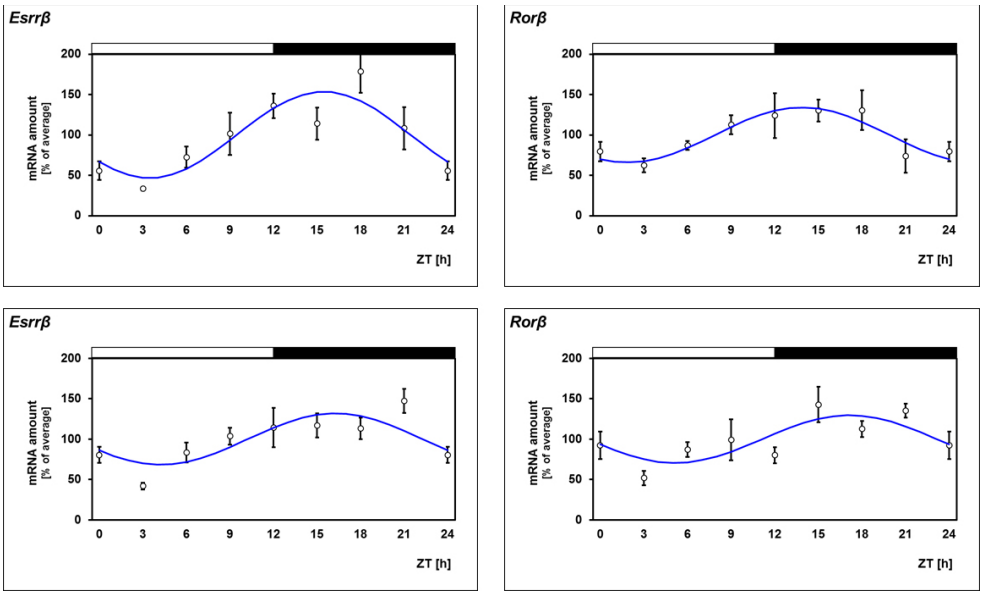

Figure 5. Comparative 24-h profiling of *Esrrβ* and *Rorβ* in the retina of melatonin-proficient (C3H/He, $rd^{+/+}$; upper line) and melatonin-deficient mice (C57BL/6J; lower line). The mRNA levels are determined by using quantitative PCR (qPCR), and both are plotted as a function of Zeitgeber time (ZT). The blue lines represent the periodic sinusoidal functions determined with cosinor analysis (only for $p<0.05$). The solid bars indicate the dark period. Data represent a percentage of the average value of the transcript amount during the 24-h period. The value of ZT0 was plotted twice at ZT0 and ZT24. Each value represents mean ± standard error of the mean (SEM; n=4). Statistical analysis of transcriptional profiling is provided in Table 6. Note that daily rhythmicity of *Esrrβ* does not depend on a pulsatile melatonin signal.

Similar to *Esrrβ*, the transcriptional coactivator *Pgc-1α* (i) protects photoreceptor cells from dystrophy [49], (ii) is under daily regulation in photoreceptor cells [50], and (iii) is important for metabolic homeostasis [51]. As a result, *Esrrβ* and *Pgc-1α* may act synergistically to increase photoreceptor survival through daily adjustment of its metabolism. Members of the *Esrr* family are coactivated by *Pgc-1α* to regulate energy metabolism in peripheral tissues [52]. If this occurs in photoreceptor cells, then *Esrrβ* and *Pgc-1α* may act jointly at the promoter level to maintain metabolic homeostasis of photoreceptor cells.

However, *Esrrβ* is known to target rod-specific genes including *Rho* [9]. Since *Rho* transcript levels are under daily regulation [28,53], this is consistent with the concept in which *Esrrβ* promotes the daily adaptation of rods by regulating rod-specific gene expression including that of *Rho*.

In conclusion, the data of the present study suggest a daily rhythm in *Esrrβ* expression that could promote metabolic adaptation of rods and in this way their long-term survival. Future investigations are required to investigate to what extent impairment of daily regulation of *Esrrβ* expression and energy metabolism may contribute to the various forms of photoreceptor dystrophy—in particular to those that are age-related.

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Table 6. Statistical analysis of transcriptional profiling illustrated in Figure 5.

Gene	Melatonin proficient (C3H/He, $rd^{+/+}$)				Melatonin deficient (C57BL/6J)			
	One-way Anova	Cosinor analysis			One-way Anova	Cosinor analysis		
	P value	P value	Acrophase [h]	Amplitude [%]	P value	P value	Acrophase [h]	Amplitude [%]
Esrrβ	=0.016	<0.05	15.5	53.5	=0.001	<0.05	16.3	31.7
Rorβ	=0.041	<0.05	13.9	34.0	=0.006	<0.05	17.1	29.6

Statistical analysis using one-way Anova and Cosinor analysis are shown for each gene from samples of melatonin proficient and melatonin deficient mice.

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Publikation III

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Pgc-1α and *Nr4a1* Are Target Genes of Circadian Melatonin and Dopamine Release in Murine Retina

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Purpose. The neurohormones melatonin and dopamine mediate clock-dependent/circadian regulation of inner retinal neurons and photoreceptor cells and in this way promote their functional adaptation to time of day and their survival. To fulfill this function they act on melatonin receptor type 1 (MT_1 receptors) and dopamine D4 receptors (D_4 receptors), respectively. The aim of the present study was to screen transcriptional regulators important for retinal physiology and/or pathology (*Dbp*, *Egr-1*, *Fos*, *Nr1d1*, *Nr2e3*, *Nr4a1*, *Pgc-1α*, *Rorβ*) for circadian regulation and dependence on melatonin signaling/MT_1 receptors or dopamine signaling/D_4 receptors.

Methods. This was done by gene profiling using quantitative polymerase chain reaction in mice deficient in MT_1 or D_4 receptors.

Results. The data obtained determined *Pgc-1α* and *Nr4a1* as transcriptional targets of circadian melatonin and dopamine signaling, respectively.

Conclusions. The results suggest that *Pgc-1α* and *Nr4a1* represent candidate genes for linking circadian neurohormone release with functional adaptation and healthiness of retina and photoreceptor cells.

Keywords: retina, circadian regulation, melatonin receptor type 1, dopamine D4 receptor, *Nr4a1*, *Pgc-1α*

The mammalian retina has the ability to adapt to the marked daily changes in the environment. This facilitates the retina, and in particular photoreceptor cells (PRCs), to comply with daily changes in metabolic and functional demands and on a long-term basis may contribute to their survival. Daily adaptation of retinal physiology[1–3] is driven by retinal clocks[4] partly through the rhythmic release of the hormones melatonin from PRCs and dopamine from amacrine cells (for review, see Ref. 5). Melatonin and dopamine play opposing roles in the control of retinal adaptation (for reviews, see Refs. 6, 7). Melatonin release occurs at night and mediates dark adaptation of retina through G-protein receptors named melatonin type 1 (MT_1) and type 2 (MT_2) receptors (for review, see Ref. 8) that appear to function as MT_1/MT_2 heteromers.[9] In this process it modulates visual processing and viability of PRCs.[10–12] On the other hand, dopamine release is higher during daytime[13,14] and promotes adaptation of visual function to light (for reviews, see Refs. 6, 15, 16). Dopamine exerts its effects on various types of dopamine receptors widespread through the retina, but in particular on dopamine D4 (D_4) receptors expressed primarily on PRCs to entrain light-adapted vision including contrast sensitivity function,[12,16,17] the photoreceptor circadian clock in terms of protein phosphorylation,[18] melatonin release,[19] and adenylyl cyclase 1/cAMP signaling.[20]

In the mammalian retina, transcriptional regulators are critical for visual function,[21,22] cell fate determination (for reviews, see Refs. 23, 24), and homeostasis.[25–28] They are also important for retinal cell health and viability. Thus, transcriptional regulators (1) underlie different forms of inherited retinal degeneration in humans, such as *Nr2e3*[29–31] and *Rorβ*,[32] and (2) are protective against retinal dystrophy, such as *Nr1d1*[33] and *Pgc-1α*,[27] or activate immune defense pathways in response to damage of the retina, such as *Egr-1* (*Krox24*; *Ngfi-a*; *Zif268*; Zenk[28,34,35]; *Fos*[34,36] and *Nr4a1* (*Ngfi-b*, *Nur77*[34]). Remarkably, numerous transcriptional regulators display a daily rhythm of expression in the retina (*Dbp*[37]; *Egr-1*[38]; *Fos*[39]; *Nr1d1*[40]; *Nr2e3*[41]; *Nr4a1*[42]; *Pgc-1α*[43]; *Rorβ*[44]). Therefore, their positive role in retinal health may be based on their ability to promote daily adjustment of the retina to comply with ambient demands.

The aim of the present study was to determine transcriptional regulators that may mediate retinal responses to circadian neurotransmitter release in retina of melatonin-proficient mice not carrying *rd* mutations. For this, daily regulation of the transcriptional regulators *Dbp*, *Egr-1*, *Fos*, *Nr1d1*, *Nr2e3*, *Nr4a1*, *Pgc-1α*, *Rorβ* was seen to be driven by a circadian clock, to take place in PRCs, and, most importantly, to depend on melatonin signaling/MT_1 receptors or dopamine signaling/D_4 receptors. As a result, *Pgc-1α* and *Nr4a1* were identified as

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Table 1. Primer Sequences Used for qPCR

Gene	Accession Number	Primer Sequence 5′ to 3′		Length of PCR Product, bp
Dbp	NM_016974.3	Forward	GGAGGTGCTAATGACCTTTG	146
		Reverse	GGACTTTCCTTGCCTTCTTC	
Egr-1	NM_007913.5	Forward	CCCTTCCAGTGTCGAATC	127
		Reverse	CACTCCTGGCAAACTTCC	
Fos	NM_010234	Forward	CTTGGAGCCAGTCAAGAG	141
		Reverse	TAGAAGGAACCGGACAGG	
Gapdh	BC082592	Forward	GTCATCCCAGAGCTGAAC	144
		Reverse	CTCAGATGCCTGCTTCAC	
Nr1d1	NM_145434	Forward	CTGCATCATCGTCATCCTCTTC	150
		Reverse	ACAGTAGCACCATGCCATTC	
Nr2e3	NM_013708	Forward	GCCCTGGTCCTCTTCAAAC	112
		Reverse	GGTGGTGAGCCTTGCTATG	
Nr4a1	NM_010444	Forward	TGATGTTCCCGCCTTTGC	126
		Reverse	GCCATGTGCTCCTTCAGAC	
Nrl	NM_008736	Forward	GTGGAGGAACGGTCCAGATG	149
		Reverse	GAACTGGAGGGCTGGGTTAC	
Pgc-1α	NM_008904	Forward	GTGTTCCCGATCACCATATTCC	101
		Reverse	GGTGTCTGTAGTGGCTTGATTC	
Rorβ	NM_00146095.3	Forward	CCTGGCTGATCGAACCAAG	144
		Reverse	TGCAGACTGCCGTGATAG	
Th	NM_009377.1	Forward	CAGCCCTACCAAGATCAAAC	129
		Reverse	GTACGGGTCAAACTTCACAG	

putative transcriptional targets of the circadian melatonin/dopamine system in murine retina.

Materials and Methods

Animals

Adult male and female mice with intact PRCs not carrying *rd* mutation were used in this study. As a rule the mice used were melatonin proficient (C3H/He (*rd*$^{+/+}$), C3H/f$^{+/+}$MT1$^{+/+}$, C3H/f$^{+/+}$MT1$^{-/-}$, C3H/f$^{+/+}$Drd4$^{+/+}$, and C3H/f$^{+/+}$Drd4$^{-/-}$) and where indicated melatonin deficient (C57BL/6Jb). Mice were genotyped by PCR analysis of genomic DNA. The mice 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 animals were then kept for one cycle under dim red light and killed during the next cycle. Animals (two for each time point) 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 (four for each time point) were quickly removed, pooled, and immediately frozen or processed for laser microdissection and pressure catapulting (LMPC). Each experiment was carried out four times independently. 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 and 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

To prepare the retinas for LMPC, the HOPE technique (HOPE, Hepes-glutamic acid buffer-mediated organic solvent protection effect; DCS, Hamburg, Germany) was applied for the fixation.[45] Photoreceptor cells were isolated from the stained sections in a contact- and contamination-free manner by using the LMPC technique as described previously.[46] The purity grades of the preparations obtained were verified by using a specific gene marker of PRCs, namely, *Nrl* (as markers for rods[47]), and of inner retinal neurons, namely, *Th* (as a marker for amacrine cells[48]). In comparison to whole-retina preparations, in PRCs collected by LMPC, the ratio of *Nrl* to *Th* was increased 84-fold.

RNA Extraction, Reverse Transcription (RT), and Quantitative Polymerase Chain Reaction (qPCR)

RNA was isolated and reverse transcribed as described previously.[43] In brief, PCR amplification and quantification were performed in an i-Cycler (BioRad, Munich, Germany) according to the following protocol: denaturation for 3 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 20 seconds at 60°C, and 20 seconds 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 an internal standard curve with 10-fold serial dilutions (10^1–10^8 copies/μL). The values were normalized with respect to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA present.

Statistical Analysis

All PCR data are expressed as the mean ± standard error of the mean (SEM) of four independent experiments including eight time points. Transcript levels were calculated relative to average expression of each dataset throughout 24 hours to plot temporal expression. Significance of daily regulation was defined by showing a $P < 0.05$ in ANOVA (one-way analysis of variance). Cosinor analysis was used to fit sine wave curves to the circadian data to mathematically estimate the time of peak gene expression (acrophase) and to assess the amplitude.[49,50] The model can be expressed according to the equation $f(t) = A$

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+ B cos [2π (t + C) / T] 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 is 24 hours for this experimental setting.

Results

Daily Profiling of the Transcriptional Regulators in Whole Retina of Melatonin-Proficient Mouse

With the exception of a recently published study,[37] daily profiling of gene expression in murine retina has so far been performed in mice strains that either are deficient for melatonin or carry *rd* mutation (resulting in damaged PRCs).[51–55] The objective of this study—to investigate melatonin-dependent regulation of genes in the retina including PRCs—requires melatonin-proficient mice with intact PRCs (C3H/He (*rd*$^{+/+}$)). It was seen that in these mice all transcriptional regulators under investigation display daily changes with peak expression during the day (*Dbp*, *Nr1d1*, *Nr2e3*, *Pgc-1α*) or at night (*Egr-1*, *Fos*, *Nr4a1*, *Rorβ*) (Fig. 1, blue lines; for statistical analysis, see Table 2).

Daily Profiling of the Transcriptional Regulators in PRCs of Melatonin-Proficient Mouse

In order to compare daily regulation of the transcriptional regulators between preparations of the whole retina and PRCs, the LMPC technique was applied.[46] The transcript levels of the genes were observed to exhibit daily rhythms in PRCs (Fig. 1, red lines; for statistical analysis, see Table 2) with profiles resembling those obtained from preparations of the whole retina (Fig. 1, blue lines; for statistical analysis, see Table 2). The findings obtained suggest that daily rhythmicity of the transcriptional regulators also occurs in PRCs. Interestingly, the relative strength of cycling of the genes (indicated in terms of the height of the amplitude) was different in PRCs (*Nr4a1* > *Fos* > *Dbp* > *Nr2e3* > *Pgc-1α* > *Rorβ*) and whole retina (*Nr4a1* > *Fos* > *Rorβ* > *Dbp* > *Nr2e3* > *Pgc-1α* > *Rorβ*). This might reflect different circadian output in outer and inner retina.

Daily Profiling of the Transcriptional Regulators in Retina of Melatonin-Proficient Mice in Constant Darkness

To gain a view of transcriptional control of the retina by a circadian clock, light input, or both, mice (C3H/He (*rd*$^{+/+}$)) adapted to LD 12:12 were kept in constant darkness (DD) for one cycle and monitored during the subsequent cycle (Fig. 1, black lines; for statistical analysis, see Table 2). Under these conditions, the daily rhythm in mRNA amount continued for all transcriptional regulators investigated (*Dbp*, *Egr-1*, *Fos*, *Nr1d1*, *Nr2e3*, *Nr4a1*, *Pgc-1α*, *Rorβ*). This observation suggests that daily rhythmicity of the transcriptional regulators investigated requires a circadian clock. As far as assessable ($P < 0.05$ in cosinor analysis), the retained rhythmicity of the transcriptional regulators occurred with reduced amplitude under DD conditions when compared with LD conditions with the exception of *Nr1d1*. Therefore clock-dependent rhythmicity of the transcriptional regulators appears to be enhanced by light/dark transitions.

Daily Profiling of the Transcriptional Regulators in Melatonin-Deficient Mice

The retinal clock system—probably in terms of a clock within PRCs[40,46]—evokes a daily rhythm in melatonin release (for review, see Ref. 5). Therefore clock-driven rhythmicity of the transcriptional regulators may be conveyed by melatonin signaling. To test this assumption, daily profiling of the transcriptional regulators in melatonin-proficient mice C3H/He (*rd*$^{+/+}$) was compared with that of melatonin-deficient C57BL/6Jb mice (Fig. 2, blue versus red lines; for statistical analysis, see Table 2). This revealed that *Pgc-1α* rhythmicity was prevented in melatonin-deficient mice. Conversely, the other transcriptional regulators under investigation (*Dbp*, *Egr1*, *Fos*, *Nr1d1*, *Nr2e3*, *Nr4a1*, *Rorβ*) were found to display daily patterns similar to those being observed in melatonin-proficient mice. These observations suggest that melatonin signaling is a prerequisite for the *Pgc-1α* rhythmicity.

Daily Profiling of the Transcriptional Regulators in Melatonin-Proficient $MT_1^{-/-}$ Mice

To verify and specify the response of the transcriptional regulators to melatonin signaling, their 24-hour profiles were compared in wild-type (WT) and MT_1 receptor-deficient retina (Fig. 3, blue versus red lines; for statistical analysis, see Table 3), with both genotypes deriving from a melatonin-proficient mice strain with intact PRCs (C3H/f$^{+/+11}$). Consistent with the results obtained from melatonin-deficient mice, the loss of MT_1 receptors prevented the cycling of *Pgc-1α*. As far as assessable ($P < 0.05$ in cosinor analysis), it was seen to slightly dampen the rhythmicity of the other transcriptional regulators under investigation (*Dbp*, *Nr1d1*, *Nr2e3*, *Nr4a1*, *Rorβ*). This observation suggests that MT_1 receptors couple the pulsatile melatonin signal to the expression of *Pgc-1α* and possibly play a general role in amplifying circadian regulation of retinal transcription.

Daily Profiling of the Transcriptional Regulators in $Drd4^{-/-}$ Mice

Clock-dependent regulation of the transcriptional regulators may also involve dopamine signaling via dopamine D_4 receptors (for review, see Ref. 5). To investigate this possibility, 24-hour profiling of the transcriptional regulators was performed in *Drd4*-deficient mice (C3H/f$^{+/+}$, melatonin-proficient mice with intact PRCs) (Fig. 4, blue versus red lines; for statistical analysis, see Table 3). The rhythmicity of *Nr4a1* was seen to be dampened in $Drd4^{-/-}$ mice, with a decrease in amplitude of 84%, whereas the other transcriptional regulators under investigation were seen to display similar daily profiles in both the genotypes. This finding suggests that *Nr4a1* links the clock-driven dopamine signaling with retinal gene expression.

Discussion

The retinal clocks exert their influence on visual processing and survival of the retina through the neurohormones melatonin and dopamine and their respective action on MT_1 and D_4 receptors (for review, see Ref. 5). The main objective of this study was to identify candidate genes for linking clock-dependent neurohormone release with daily adaptation and healthiness of the retina. For this purpose, transcriptional regulators were screened for dependence on melatonin signaling/MT_1 receptors or dopamine signaling/D_4 receptors

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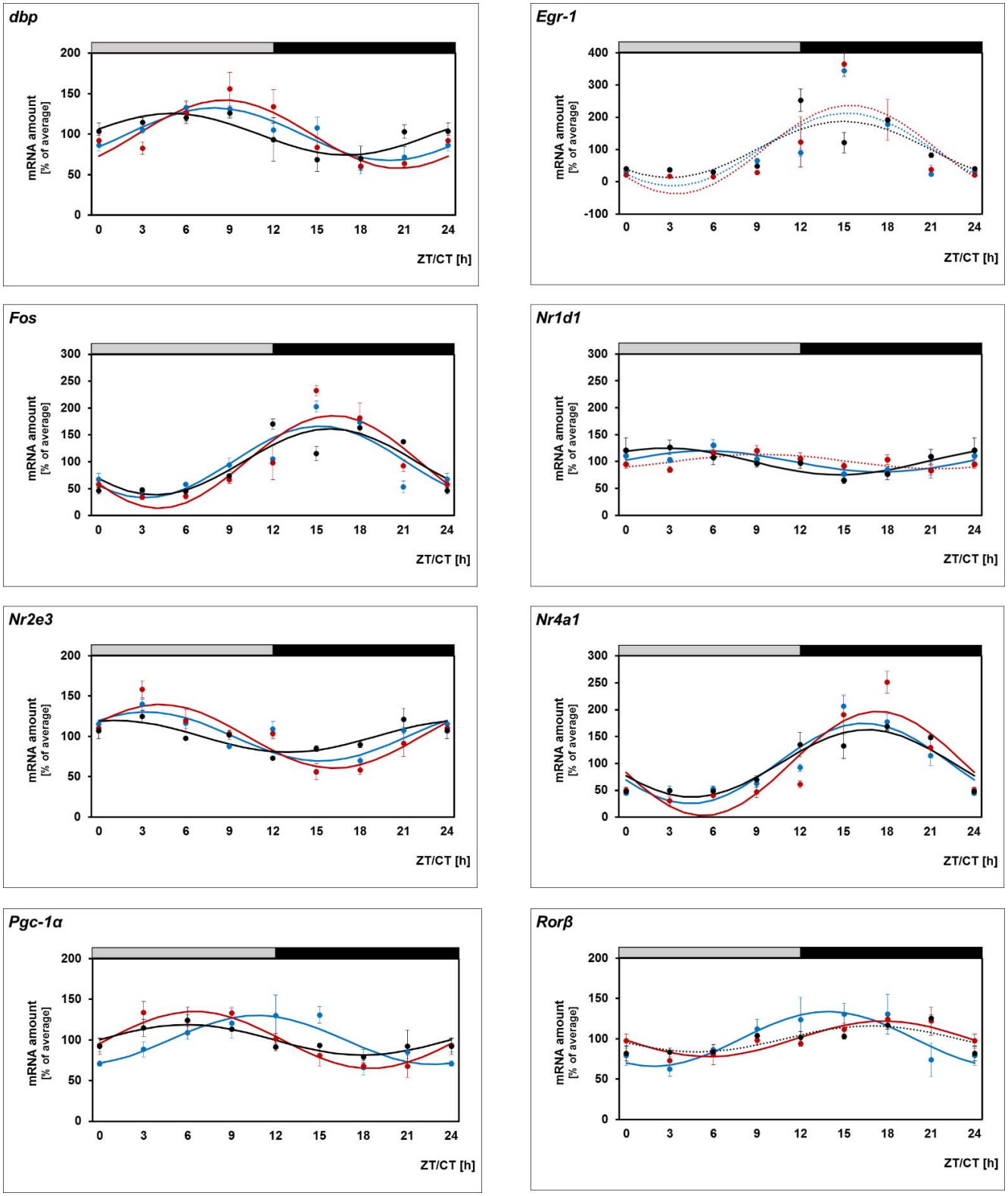

Figure 1. Twenty-four-hour profiling in whole retina and photoreceptor cells. 24-hour profiling of the transcriptional regulators *Dbp*, *Egr-1*, *Fos*, *Nr1d1*, *Nr2e3*, *Nr4a1*, *Pgc-1α*, *Rorβ* in preparations of the whole retina under light/dark (LD) 12:12 (*blue lines*), in microdissected photoreceptor cells under light/dark (LD) 12:12 (*red lines*), and in preparations of the whole retina of mice under constant darkness (DD) (*black lines*). The mRNA levels are plotted as a function of Zeitgeber time (ZT), and the *lines* represent the periodic sinusoidal functions (*solid* and *dotted lines* for $P < 0.05$ and $P > 0.05$ in cosinor analysis). The *solid bars* indicate the dark period. Data represent a percentage of the average value of transcript amount during the 24-hour period. The value of ZT0 was plotted twice at both ZT0 and ZT24. Each value represents mean ± SEM ($n = 4$). Statistical analysis of transcriptional profiling is provided in Table 2.

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Table 2. Statistical Analysis of Transcriptional Profiling Illustrated in Figures 1 and 2

	Retina of Mouse, C3H/He (rd^{++})				PRCs of Mouse, C3H/He (rd^{++})				Retina of Mouse, C3H/He (rd^{++}); DD				WT C57BL/6Jb; LD			
	1-Way ANOVA	Cosinor Analysis			1-Way ANOVA	Cosinor Analysis			1-Way ANOVA	Cosinor Analysis			1-Way ANOVA	Cosinor Analysis		
Gene	*P* Value	*P* Value	Acrophase, h	Amplitude, %	*P* Value	*P* Value	Acrophase, h	Amplitude, %	*P* Value	*P* Value	Acrophase, h	Amplitude, %	*P* Value	*P* Value	Acrophase, h	Amplitude, %
Dbp	< 0.001	< 0.05	7.9	32.4	< 0.001	< 0.05	8.6	42.1	= 0.039	< 0.05	5.0	25.7	< 0.001	< 0.05	8.5	40.5
Egr1	< 0.001	> 0.05	-	-	< 0.001	> 0.05	-	-	< 0.001	> 0.05	-	-	= 0.041	> 0.05	-	-
Fos	= 0.041	< 0.05	15.2	66.5	< 0.001	< 0.05	16.1	86.1	< 0.001	< 0.05	15.9	58.5	= 0.001	< 0.05	17.2	44.4
Nr1d1	= 0.041	< 0.05	5.5	19.6	= 0.032	> 0.05	-	-	= 0.043	< 0.05	2.8	24.8	= 0.047	< 0.05	4.6	26.2
Nr2e3	< 0.001	< 0.05	3.3	30.4	< 0.001	< 0.05	4.2	40.0	< 0.001	< 0.05	1.1	19.7	= 0.036	< 0.05	5.3	23.9
Nr4a1	< 0.001	< 0.05	16.4	77.6	< 0.001	< 0.05	17.4	105.3	< 0.001	< 0.05	16.6	62.7	< 0.001	< 0.05	18.2	55.5
Pgc-1α	= 0.005	< 0.05	10.8	30.0	< 0.001	< 0.05	6.5	35.1	= 0.047	< 0.05	5.9	18.7	= 0.477	> 0.05	-	-
Rorb	= 0.041	< 0.05	13.9	34.0	= 0.020	< 0.05	17.7	22.1	< 0.001	> 0.05	-	-	= 0.006	> 0.05	-	-

Significance of daily regulation was tested by ANOVA. The acrophase (time of peak gene expression) and the amplitude of daily profiles were obtained from cosinor analysis.

Table 3. Statistical Analysis of Transcriptional Profiling Illustrated in Figures 3 and 4

	$C3H/f^{+/+}MT_1^{+/+}$				$C3H/f^{+/+}MT_1^{-/-}$				$C3H/f^{+/+}Drd_4^{+/+}$				$C3H/f^{+/+}Drd_4^{-/-}$			
	1-Way ANOVA	Cosinor Analysis			1-Way ANOVA	Cosinor Analysis			1-Way ANOVA	Cosinor Analysis			1-Way ANOVA	Cosinor Analysis		
Gene	*P* Value	*P* Value	Acrophase, h	Amplitude, %	*P* Value	*P* Value	Acrophase, h	Amplitude, %	*P* Value	*P* Value	Acrophase, h	Amplitude, %	*P* Value	*P* Value	Acrophase, h	Amplitude, %
Dbp	= 0.014	< 0.05	6.7	32.1	< 0.001	< 0.05	6.7	28.8	= 0.013	< 0.05	4.4	43.8	= 0.010	> 0.05	-	-
Egr1	< 0.001	> 0.05	-	-	< 0.001	> 0.05	-	-	< 0.001	> 0.05	-	-	< 0.001	> 0.05	-	-
Fos	< 0.001	> 0.05	-	-	< 0.001	> 0.05	-	-	< 0.001	> 0.05	-	-	< 0.001	< 0.05	14.4	20.5
Nr1d1	< 0.001	< 0.05	7.0	25.0	< 0.001	< 0.05	5.1	21.9	< 0.001	< 0.05	4.6	22.5	< 0.001	< 0.05	3.3	16.5
Nr2e3	< 0.001	< 0.05	3.4	23.5	< 0.001	< 0.05	2.0	21.9	= 0.005	> 0.05	-	-	< 0.001	> 0.05	-	-
Nr4a1	< 0.001	< 0.05	14.1	61.8	< 0.001	< 0.05	14.3	48.7	= 0.040	< 0.05	15.3	46.9	= 0.040	< 0.05	16.5	9.9
Pgc-1α	= 0.001	< 0.05	7.3	25.8	= 0.066	> 0.05	-	-	= 0.001	< 0.05	6.7	16.4	= 0.003	< 0.05	10.6	16.6
Rorb	= 0.017	< 0.05	15.9	23.9	< 0.001	< 0.05	15.3	22.2	= 0.011	> 0.05	-	-	= 0.041	< 0.05	18.0	27.4

Significance of daily regulation was tested by ANOVA. The acrophase (the time of peak gene expression) and the amplitude of daily patterns were obtained from cosinor analysis.

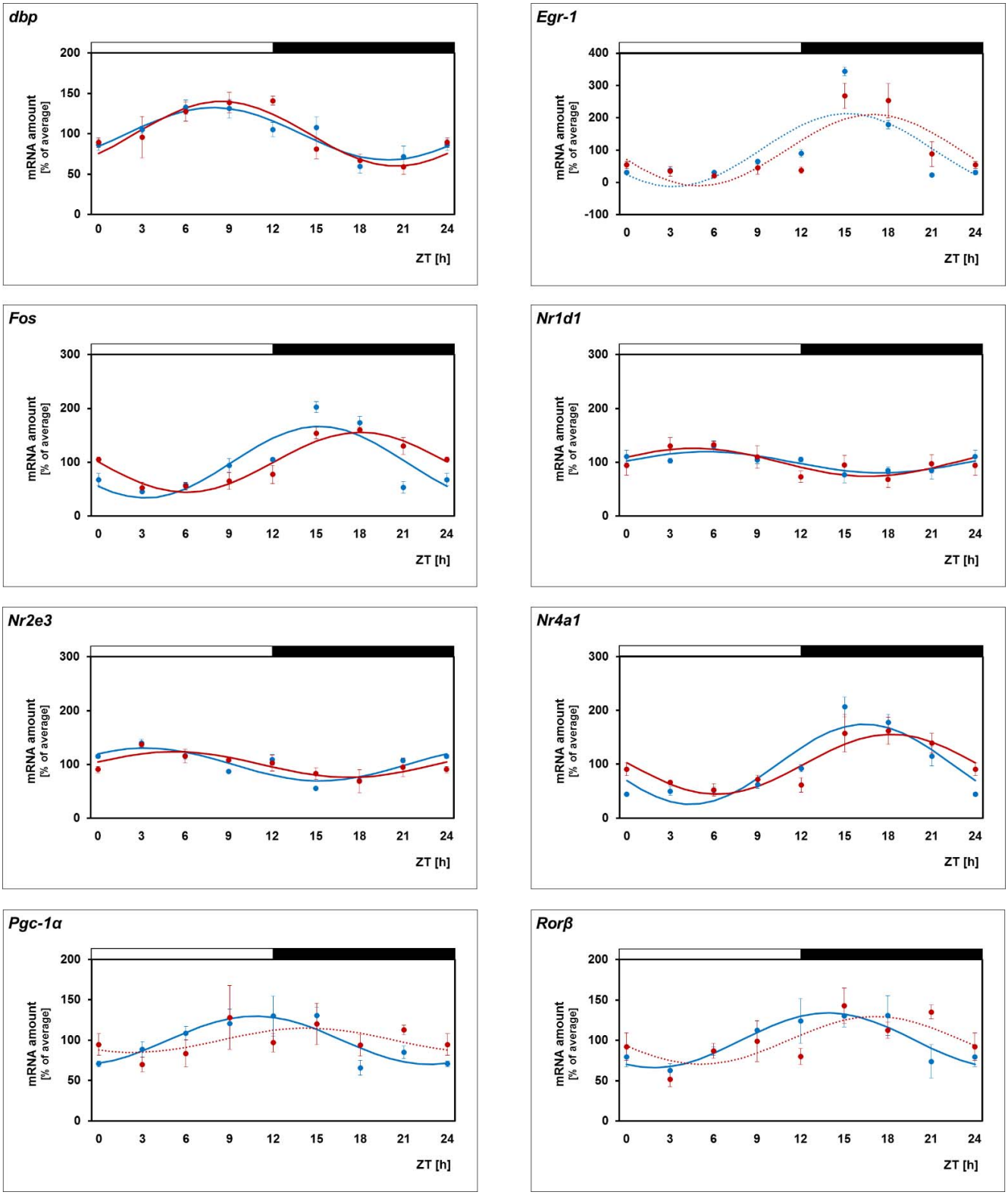

Figure 2. Daily profiling in melatonin-proficient and -deficient mice. Daily profiling of the transcriptional regulators *Dbp*, *Egr-1*, *Fos*, *Nr1d1*, *Nr2e3*, *Nr4a1*, *Pgc-1α*, *Rorβ* in retina of melatonin-proficient C3H/He (*rd*$^{++}$) mice (*blue lines*, corresponding to those in Fig. 1) and melatonin-deficient C57BL/6Jb mice (*red lines*). The mRNA levels are plotted as a function of Zeitgeber time (ZT), and the *lines* represent the periodic sinusoidal functions (*solid* and *dotted lines* for $P < 0.05$ and $P > 0.05$ in cosinor analysis). The *solid bars* indicate the dark period. Data represent a percentage of the average value of transcript amount during the 24-hour period. The value of ZT0 was plotted twice at both ZT0 and ZT24. Each value represents mean ± SEM ($n = 4$). Statistical analysis of transcriptional profiling is provided in Table 2.

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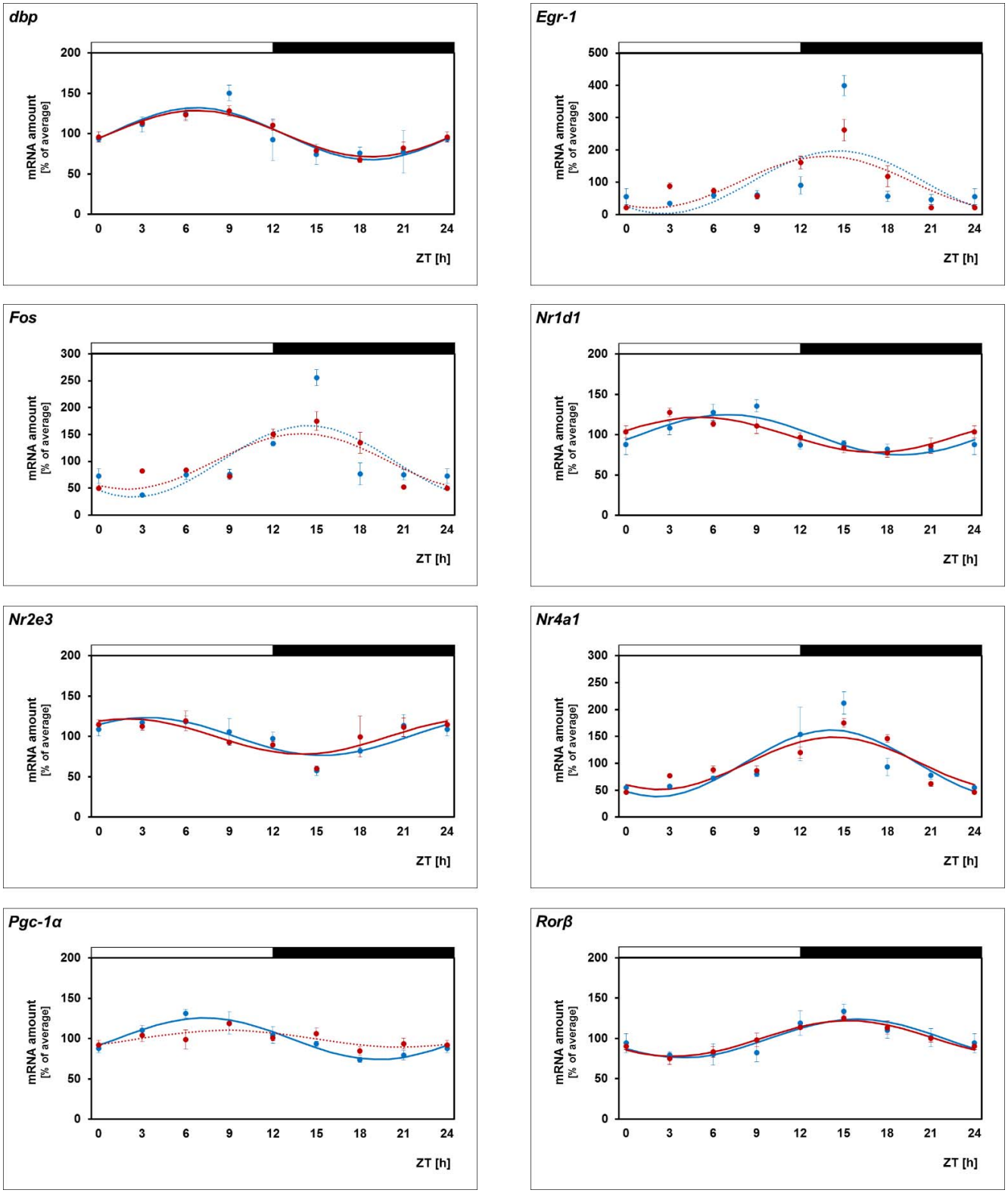

FIGURE 3. Twenty-four-hour profiling in WT and $MT_1^{-/-}$ mice. 24-hour profiling of the transcriptional regulators *Dbp*, *Egr-1*, *Fos*, *Nr1d1*, *Nr2e3*, *Nr4a1*, *Pgc-1α*, *Rorβ* in retina of WT (*blue lines*) and $MT_1^{-/-}$ mice (*red lines*). The mRNA levels are plotted as a function of Zeitgeber time (ZT), and the *lines* represent the periodic sinusoidal functions (*solid* and *dotted lines* for $P < 0.05$ and $P > 0.05$ in cosinor analysis). The *solid bars* indicate the dark period. Data represent a percentage of the average value of transcript amount during the 24-hour period. The value of ZT0 was plotted twice at both ZT0 and ZT24. Each value represents mean ± SEM ($n = 4$). Statistical analysis of transcriptional profiling is provided in Table 3.

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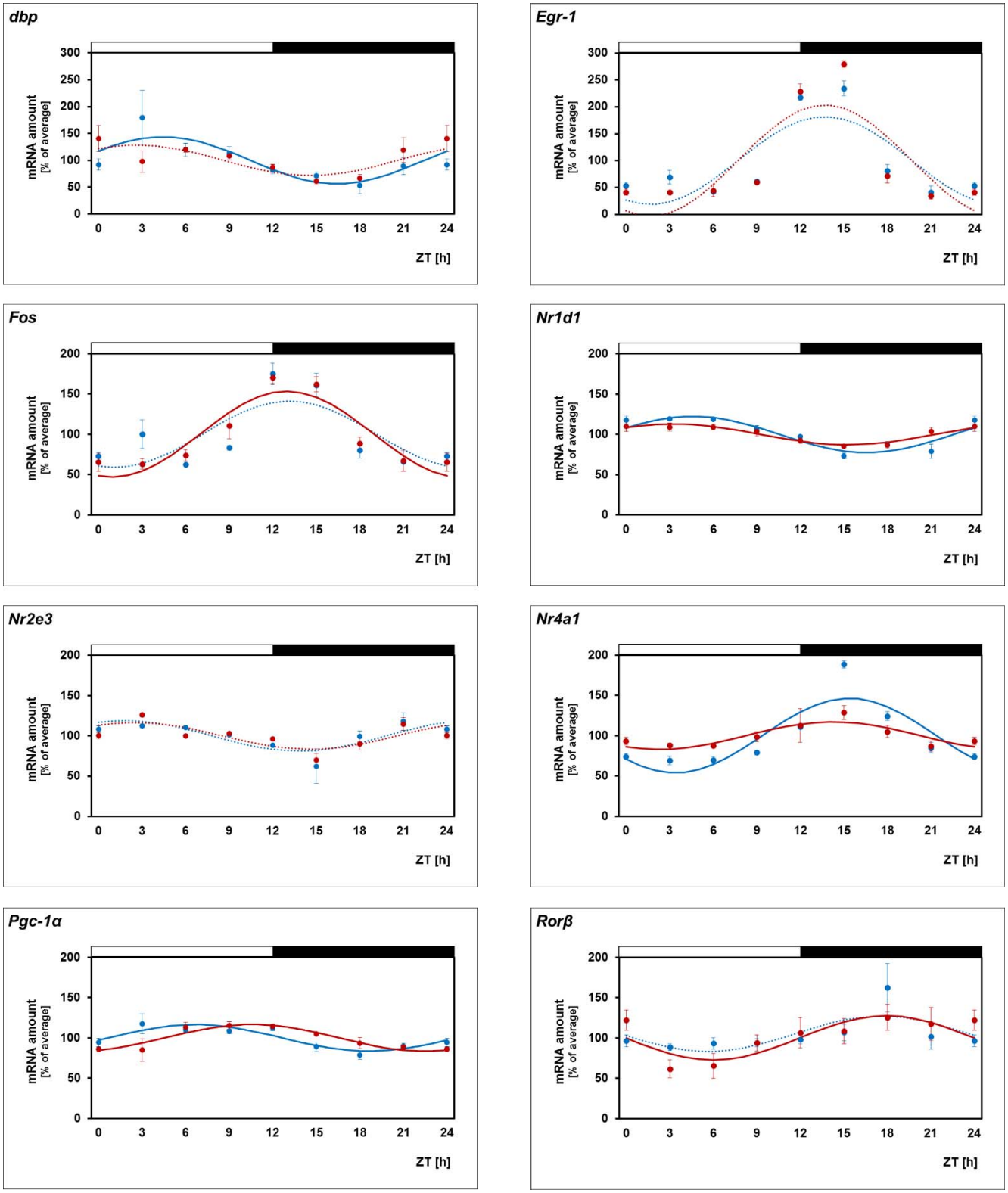

FIGURE 4. Twenty-four-hour profiling in WT and Drd4$^{-/-}$ mice. 24-hour profiling of the transcriptional regulators *Dbp*, *Egr-1*, *Fos*, *Nr1d1*, *Nr2e3*, *Nr4a1*, *Pgc-1α*, *Rorβ* in retina of WT (*blue lines*) and Drd4$^{-/-}$ mice (*red lines*). The mRNA levels are plotted as a function of Zeitgeber time (ZT), and the *lines* represent the periodic sinusoidal functions (*solid* and *dotted lines* for $P < 0.05$ and $P > 0.05$ in cosinor analysis). The *solid bars* indicate the dark period. Data represent a percentage of the average value of transcript amount during the 24-hour period. The value of ZT0 was plotted twice at both ZT0 and ZT24. Each value represents mean ± SEM ($n = 4$). Statistical analysis of transcriptional profiling is provided in Table 3.

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that fulfill the requirements to (1) cycle in PCRs (*Dbp*[37]: Fig. 1; *Egr-1*: Fig. 1; *Fos*, *Nr1d1*[40]: Fig. 1; *Nr2e3*: Fig. 1; *Nr4a1*: Fig. 1; *Pgc-1α*[43]: Fig. 1; *Rorβ*[40]: Fig. 1), (2) be driven by a circadian clock (*Dbp*[37]: Fig. 1; *Egr-1*[39]: Fig. 1; *Fos*[39]: Fig. 1; *Nr1d1*[40]: Fig. 1; *Nr2e3*: Fig. 1; *Nr4a1*: Fig. 1; *Pgc-1α*: Fig. 1; *Rorβ*[32]: Fig. 1), and (3) influence retinal physiology or pathology (*Egr-1*, *Fos*[34,36]; *Nr1d1*[33]; *Nr2e3*[29–31]; *Nr4a1*[34]; *Nr1d1*: *Pgc-1α*[27]; *Rorβ*[32]). As our experimental system we used mice with targeted deletion of MT_1 receptors and D_4 receptors, respectively, that we have crossed onto the $C3Hf^{+/+}$ background.[56,57] Thus the mouse model used in this study combines physiological melatonin formation and intact photoreceptors.

The data obtained determine the transcriptional coactivator *Pgc-1α* as a transcriptional target of circadian melatonin/MT_1 receptor signaling. This becomes evident from the finding that the daily rhythmicity of *Pgc-1α* mRNA amount was dampened in both melatonin-deficient (C57BL/6Jb) and $MT_1^{-/-}$ mice.

Melatonin/MT_1 receptor-dependent control of *Pgc-1α* may take place in PRCs. This follows from the observation that PRCs combine a prominent rhythmicity of *Pgc-1α* (this study) with high density of MT_1 receptors.[11,12] Of interest is that, in PCRs, melatonin appears to act as an autocrine signal.[11] Therefore PRCs may regulate their own *Pgc-1α* expression by melatonin release and its subsequent action on MT_1 receptors located at their cell membrane. Recent studies have shown that the action of melatonin in the PRCs is mediated by MT_1/MT_2 heteromers.[9] Therefore, MT_1 receptor deficiency may result in nonfunctional MT_1/MT_2 heterodimers, and MT_2 knockout mice would have produced similar results from those obtained in MT_1.

Pgc-1α is a master regulator of glucose and energy metabolism in various tissues (for review, see Ref. 58) including retina.[27] Therefore the observed melatonin/MT_1 receptor-dependent regulation of *Pgc-1α* may contribute to daily changes in the energy metabolism of the retina and thus to its ability to comply with 24-hour changes in metabolic demands.[59]

Remarkably, *Pgc-1α* knockout mice reportedly suffer from increased light damage susceptibility, overexpression of *Pgc-1α* has antiapoptotic effects, and *Pgc-1α* expression is reduced in different mouse models of retinitis pigmentosa.[27] This suggests that *Pgc-1α* provides protection against light damage and is therefore a promising candidate gene for transferring the positive effect of melatonin/MT_1 receptors on the viability of retinal PRCs and ganglion cells.[11] Accordingly, melatonin/MT_1 receptor-dependent control of *Pgc-1α* could contribute to the protection of retinal cells against light damage and on a long-term basis to the survival of retinal cells during aging.

Outside the retina, MT_1 receptor-dependent expression of *Pgc-1α* could play a role in the pathogenesis of type 2 diabetes. This becomes evident from the observations that the removal of MT_1 receptors[60] or *Pgc-1α* (for review, see Ref. 61) equally leads to increased insulin resistance in peripheral tissues.

As with *Pgc-1α* in the melatonergic system, the nuclear orphan receptor *Nr4a1* (Nur77, Ngfi-b) was identified to respond to the dopaminergic system. This is evident from the finding that the daily rhythmicity of *Nr4a1* is reduced in $Drd4^{-/-}$ mice. Dopaminergic regulation of retinal *Nr4a1* probably occurs in PRC since this cell type combines circadian regulation of *Nr4a1* (this study) with the abundance of D_4 receptors.[62,63] In the retina, clock-dependent dopamine release occurs from the unique population of cells in the inner nuclear layer that are either amacrine or interplexiform neurons.[15] The dopamine neurons in retina express a full complement of core circadian clock genes.[64,65] Therefore circadian/dopaminergic control of *Nr4a1* in PRCs appears to be driven by these cell types. Furthermore, *Drd4*, the gene that encodes the D_4 receptor, is itself a circadian clock-controlled gene in retina,[3,20,42,66] and this too may contribute to circadian regulation of *Nr4a1*.

Dopaminergic regulation of *Nr4a1* was until now believed to be confined to the motor/motivation system (for reviews, see Refs. 67, 68). Therefore its concurrent occurrence in the visual (this study) and the motor/motivation system suggests that it appears to be a more general feature of different functional brain systems affected by dopamine.

Furthermore, *Nr4a1* is a promising candidate gene for being responsible for dysfunction of the retinal dopaminergic system and its subsequent visual defects in type I diabetes mellitus.[69] This becomes evident from the observations that *Nr4a1* expression on one hand influences the functioning of the dopaminergic system (for reviews, see Refs. 67, 68) and on the other hand depends on glucose metabolism.[70–72]

Compelling experimental evidence indicates that *Nr4a1* not only contributes to dopaminergic responses but also is a decisive factor in dopamine-related neuroadaptation (for reviews, see Refs. 67, 68). If this is also valid in retina, circadian/dopaminergic regulation of *Nr4a1* expression (this study) may feed back to the dopaminergic system of the retina to promote its reported circadian plasticity.[42]

It is important to note that—at variance with previous studies dealing with the role of melatonin in retina[51,52]—the present investigation was performed in mouse strains/genotypes that combine melatonin proficiency with intact PRCs and thus in mouse models that reflect retinal physiology as closely as possible. On the other hand, a general limitation of the present study is that the observed phenotypes of the mouse models used may reflect not only a direct action of the neurotransmitters/receptors, but also the secondary effects downstream of them. Since melatonin signaling[73–75] and dopamine signaling[76–78] make a cellular feedback loop in which melatonin inhibits the release of dopamine through melatonin receptors,[73–75] whereas dopamine downregulates melatonin formation, probably through dopamine D_4 receptors,[76,77] this includes a possibility that genetic disturbance of melatonin signaling affects dopamine signaling and vice versa.

In conclusion, the data of the present investigation determined candidate transcriptional regulators for linking the circadian neurohormone systems to adaptation, function, and healthiness of the retina. They could provide a reliable basis for further investigations that may achieve a better understanding of how clock-dependent neurotransmitter release influences visual processing and survival of the retinal neurons.

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3 Zusammenfassung der Ergebnisse und Diskussion

In früheren Arbeiten anderer (z.B. [39, 43, 59, 89]) und unserer Arbeitsgruppe ([38, 42, 90]) konnten Gene identifiziert werden, deren Expression in der Retina tageszeitlichen Schwankungen unterliegt. Mittels Mikroarray-Analyse und konsekutiver Analyse des tageszeitlichen Expressionsprofils mittels qPCR (Publikation I-III) konnten in der vorliegenden Arbeit weitere Gene identifiziert werden, deren Expression tageszeitlich reguliert wird (Publikation I). Weiterhin wurde für einige der untersuchten Gene (*Grk1*, *Pgc-1α* (Publikation I), *Esrrβ* (Publikation II)) eine korrespondierende Rhythmik auf Proteinebene nachgewiesen. Die Analyse ihrer molekularen und biologischen Funktionen zeigte, dass sie sich verschiedenen funktionellen Gruppen zuordnen lassen und einige für die Entwicklung (z.B. *Rorβ*), visuelle Funktion (z.B. *Grk1*) und Überleben von FRZ (z.B. *Pgc-1α*) von Bedeutung sind. Der Vergleich der Expressionsprofile der Gene zwischen Gesamtretinagewebe und isolierten FRZ zeigte, dass die tageszeitliche Regulation der Mehrzahl der Gene auch in FRZ stattfindet (Publikation I-III). Darüber hinaus wurde der Einfluss von Licht und circadianen Oszillatoren, sowie der Neuromodulatoren Melatonin und Dopamin auf die Regulation der Genexpression untersucht (Publikationen I-III). Dabei konnte gezeigt werden, dass die tageszeitliche Regulation der Genexpression über molekulare Uhren, sowie die Neuromodulatoren Melatonin und Dopamin erfolgt.

3.1 Die Identifikation tageszeitlich regulierter Gene und ihre Bedeutung für die Anpassung von Retina und Fotorezeptorzelle

Die tageszeitliche Anpassung der Retina ist auf Transkriptionsebene nur unzureichend erforscht. Die Identifikation weiterer tageszeitlich regulierter Gene ist aber Grundlage für ein besseres Verständnis der funktionellen Anpassung von Retina und FRZ, die sich unter anderem in 24-stündigen Veränderungen im ERG und in der Erneuerung der phototransduktiven Membran im Außensegment der FRZ manifestieren (zur Übersicht, siehe [14]). Im Rahmen dieser Arbeit konnten mittels Mikroarray-Analyse insgesamt 12 Gene identifiziert werden, deren tageszeitliche Regulation bisher unbekannt war (Publikation I). Für diese Gene wurden tageszeitliche Expressionsprofile erstellt, wobei 6 Gene eine erhöhte Expression während der Lichtphase (*Asns, Atf3, Cdkn1b, Dyrk2, Grk1, Pgc-1α*) und 6 Gene eine erhöhte Expression während der Dunkelphase (*Adra1b, Cerk, Ctnnal1, Pde8a, Pla2g1b, Rhobtb1*) zeigten. Die tageszeitliche Regulation des Referenzgens *Rorβ* bestätigte die Validität des experimentellen Systems [43].

Das Gen *Grk1* gehört zur Familie der G-Protein-gekoppelten Rezeptor-Kinasen (zur Übersicht, siehe [91]), und hemmt durch Phosphorylierung von Rhodopsin die Phototransduktion und

trägt damit zur Aufrechterhaltung der Licht-Sensitivität der Stäbchen bei (zur Übersicht, siehe [92, 93]). In unseren Untersuchungen konnte für *Grk1* eine tageszeitliche Rhythmik mit erhöhten Werten in der zweiten Taghälfte nachgewiesen werden (Publikation I). Dies deutet auf eine Licht-abhängige Aktivierung des Gens hin und korreliert mit seiner Schutzfunktion vor Licht-bedingter Dystrophie.

Die Genexpression von *Peroxisome proliferator-activated receptor (PPAR)-gamma coactivator 1 alpha (Pgc-1α)* zeigt in Retina und FRZ der Ratte eine erhöhte Expression während der zweiten Taghälfte (Publikation I). Das Gen *Pgc-1α* kodiert für einen transkriptionellen Coaktivator, dessen Einfluss auf die Koordination des Stoffwechsels in vielen unterschiedlichen Geweben nachgewiesen werden konnte. Coaktivatoren fungieren dabei zumeist als Komplexe verschiedener Proteine [94]. In braunem und weißem Fettgewebe konnte ein Einfluss von *Pgc-1α* auf die Expression nachgeschalteter Gene in der Thermogenese gezeigt werden [95]. Der Anstieg der *Pgc-1α*-Expression in neonatalem murinen Herzgewebe spiegelt den erhöhten Energiebedarf der kardialen Myocyten wider [94]. Durch die Regulation von Genen der Elektronentransportkette, der mitochondrialen Biogenese und der Fettsäuren-Oxidation wird der Einfluss von *Pgc-1α* auf den Zellstoffwechsel deutlich. So konnte unter anderem gezeigt werden, dass die *Pgc-1α*-Expression in Lebergewebe durch Fasten erhöht ist [96]. In der Retina könnte *Pgc-1α* die Adaptation des Stoffwechsels regulieren und darüber eine Anpassung an unterschiedlichen Energiebedarf ermöglichen. Außer Fett-, Muskel- und Lebergewebe konnte die Expression von *Pgc-1α* auch in retinalen Strukturen nachgewiesen werden und Egger et al. (2012) konnten zeigen, dass Pgc-1α die Expression von Genen, die in die Phototransduktion involviert sind, beeinflusst [97]. So führt die Expression von *Pgc-1α* unter anderem zur Erhöhung der Expression von *Grk1*. Des Weiteren konnte gezeigt werden, dass *Pgc-1α* als Coaktivator einen Einfluss auf die Transkription von Uhrengenen ausübt [98]. Die Initiierung der Transkription erfolgt dabei über die indirekte Aktivierung der Promoterregion der nachgeschalteten Gene. Die N-terminale Domäne von *Pgc-1α* bindet an DNA-bindende Proteine und aktiviert dadurch andere Coaktivator-Proteine mit Histon-Acetyl-Transferase (HAT) Domänen, durch deren Bindung die Transkription gestartet wird [94].

Ein weiteres Gen, dessen Einfluss auf den Energiestoffwechsel verschiedener Gewebe bekannt ist und welches unter anderem durch *Pgc-1α* coaktiviert wird, ist *Esrrβ* (zur Übersicht, siehe [99]). Die Expression des nukleären Orphan-Rezeptors *Esrrβ* geht unter DD-Konditionen verloren und zeigt damit eine lichtinduzierte tageszeitliche Schwankung, welche auch auf Proteinebene nachweisbar ist (Publikation II). *Esrrβ* ist ein transkriptioneller Regulator des Stoffwechsels von Zellen und seine tageszeitlich schwankende Expression wird als Folge von wechselnden metabolischen Anforderungen über den 24-stündigen Zyklus angesehen.

Tageszeitliche Schwankungen in der Expression von *Esrrβ* konnten bisher nur in peripheren Geweben nachgewiesen werden, darunter weißes und braunes Fettgewebe, Leber- und Muskelgewebe [100]. Die detektierten Schwankungen der Expression in der Retina ähneln denen im Fettgewebe, was darauf schließen lässt, dass die tageszeitliche Regulation der *Esrrβ*-Expression für Gewebe charakteristisch ist, die einen hohen Energiebedarf und eine hohe Stoffwechselrate aufweisen [100]. Der Einfluss von *Esrrβ* auf den Energiestoffwechsel von Geweben lässt vermuten, dass die tageszeitliche Rhythmik der *Esrrβ*-Expression eine Anpassung an den tageszeitlich wechselnden Energiebedarf repräsentiert [22, 101]. Der Einfluss von *Esrrβ* auf die Adaptation des Stoffwechsels kann dadurch zur Aufrechterhaltung der Fotorezeptorfunktion beitragen und ein Überleben der FRZ ermöglichen. Ähnlich wie *Pgc-1α* fungiert *Esrrβ* dabei als expressioneller Regulator anderer Gene. Die tageszeitliche Rhythmik der Genexpressionen von *Pgc-1α* und *Esrrβ* könnten synergistische Funktionen auf die Adaptation des Zellstoffwechsels der Retina ausüben.

Das Referenzgen *Rorβ* kodiert für einen nukleären Orphan-Rezeptor, dessen Regulation zur tageszeitlichen Anpassung von Retina und FRZ beiträgt [43, 59, 102] und der u.a. die Differenzierung der FRZ in Zapfen oder Stäbchen kontrolliert [103]. Die rhythmische Expression dieses Gens ist in Gesamtretina-Proben und isolierten FRZ, nicht aber in anderen inner-retinalen Neuronen nachweisbar (Publikation II).

Für *Atf3* konnte eine tageszeitliche Rhythmik mit erhöhten Werten während der ersten Tageshälfte nachgewiesen werden, die unter DD-Konditionen ausblieb (Publikation I). *Atf3* wird bei zellulärem Stress und degenerativen Prozessen in der Retina erhöht exprimiert [104, 105]. Die Gene *Adra1b* und *Pde8a* zeigten dagegen eine erhöhte Genexpression während der zweiten Nachthälfte und die tageszeitliche Rhythmik ihrer Expression blieb auch unter DD-Konditionen erhalten (Publikation I). *Adra1b* kodiert für einen Adrenozeptor, der Heterodimere mit D_4-Rezeptoren bilden kann, deren Funktionalität eine tageszeitliche Rhythmik zeigt [106]. Das *Phosphodiesterase 8A*-Genprodukt ist unter anderem in die Regulation der Transkription und die Phototransduktion involviert [89] und kann als cAMP-spezifische Phosphodiesterase extrazelluläre Signale umwandeln und weiterleiten [107].

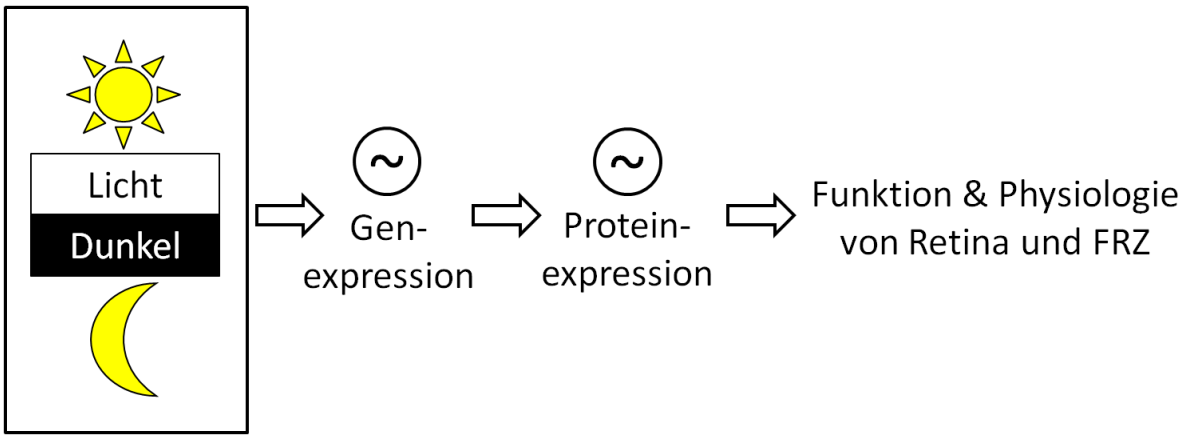

Abbildung 4: Schematische Darstellung der tageszeitlichen Adaptation
Die Abbildung zeigt schematisch, dass ein Wechsel von Licht und Dunkel die tageszeitliche Rhythmik der Genexpression beeinflusst. Dies wiederum führt zu einer korrespondierenden Rhythmik in der Proteinexpression und beeinflusst darüber die Funktion und Physiologie von Retina und FRZ.

Die Bedeutung der Untersuchung der tageszeitlichen Adaptation der Genexpression beruht unter anderem auf dem postulierten Zusammenhang zwischen fehlerhafter Adaptationsfähigkeit retinaler Zellen und ophthalmologischen Erkrankungen. So werden viele Gene, die an der visuellen Signalverarbeitung der FRZ beteiligt sind, tageszeitlich reguliert (z.B. *Pgc-1α, Grk1, Kcnv2, Esrrβ*; zur Übersicht, siehe Publikation I). Mutationen in diesen Genen können eine fehlerhafte Adaptationsfähigkeit bedingen, die in pathologischen Veränderungen der Retina resultiert. Dies zeigt sich unter anderem in Untersuchungen des Gens *Kcnv2*, welches in den FRZ Uhren-abhängig reguliert wird und dessen Mutation ursächlich für eine Form der retinalen Dystrophie ist [38, 108].

Für das Gen *Esrrβ* konnte gezeigt werden, dass eine Überexpression die Degeneration von Stäbchen vermindern kann [109] und für das Gen *Grk1* konnte ein Zusammenhang zwischen einer verminderten Expression und einer Verschlechterung des Krankheitsbildes der altersbedingten Zapfen-Dystrophie gezeigt werden [110]. *Grk1* spielt eine Schlüsselrolle in der tageszeitlichen Anpassung der visuellen Wahrnehmung und beeinflusst die retinale Degeneration [111] (zur Übersicht, siehe [112]). So bedingt das Fehlen eines funktionalen *Grk1*-Gens die Ausbildung der Oguchi-Form von kongenitaler stationärer Nachtblindheit [111]. *Grk1* konnte in Stäbchen und Zapfen der Retina nachgewiesen werden und ein funktionaler Ausfall des Gens führt zu einem lichtabhängigen Fortschreiten von altersbedingter Zapfen-Dystrophie [110]. Die Expression von *Grk1* wird durch die Licht-abhängige Aktivierung von Rhodopsin beeinflusst und eine fehlerhafte Deaktivierung von Rhodopsin führt zur Beeinträchtigung der Dunkeladaptation [113].

Pgc-1α verringert die Anfälligkeit von lichtinduzierten Schäden in Retina und FRZ. Zudem konnte in Mausmodellen für Retinitis Pigmentosa eine Abnahme der *Pgc-1α*-Expression

gezeigt werden [97]. Dies untermauert den protektiven Effekt dieses Gens gegen lichtinduzierte Schäden und unterstützt die Arbeitshypothese, dass die tagsüber erhöhte *Pgc-1α*-Expression eine Schutzfunktion ausübt (Publikation I).

Ein möglicher Zusammenhang zwischen ophthalmologischen Erkrankungen und fehlerhafter circadianer Regulation wird für unterschiedliche Krankheitsbilder diskutiert, darunter Katarakt, Glaukomerkankungen, Retinitis Pigmentosa oder diabetische Retinopathie [114, 115] (zur Übersicht, siehe [48, 116]), wobei sich diese Untersuchungen auf das circadiane System des Gesamtorganismus beziehen. Für die diabetische Retinopathie und das Glaukom konnte ein direkter Einfluss auf das circadiane System bereits gezeigt werden. So fanden Wang et al. (2014) heraus, dass die chronische Inflammation bei diabetischer Retinopathie zu einer Dysregulation der circadianen Rhythmik führt, welche sich in Veränderungen von Amplitude und Phase der Oszillationen zeigt [115]. Bei der Glaukomerkrankung resultiert der Einfluss auf das circadiane System aus der Degeneration und dem Verlust der retinalen Ganglienzellen und ihrer Axone [117] (zur Übersicht, siehe [48]). Es wird vermutet, dass die hierbei beobachtete Desynchronisation der circadianen Rhythmik unter anderem durch den Verlust von Melanopsin entsteht, welches am Erhalt der Lichtsensitivität der Retina beteiligt ist (zur Übersicht, siehe [116, 118]).

Diese Befunde zeigen, dass das Verständnis der tageszeitlichen Regulation der Genexpression und der Regulation der Adaptationsfähigkeit von Retina und FRZ für ein Verständnis retinaler Pathologien relevant ist. Ein Beitrag der vorliegenden Dissertation hierzu ist der Nachweis einer tageszeitlichen Regulation von Genen mit Bedeutung für die Pathologie und/oder Protektion von Retina und FRZ.

3.1.1 Lokalisation der Rhythmus-generierenden Zellen innerhalb der Retina

Neben der Identifikation von Genen, deren Expression eine tageszeitliche Rhythmik zeigt, ist auch die Lokalisation der Rhythmus-generierenden Zellen von Interesse. So konnten wir für eine Vielzahl der untersuchten Gene zeigen, dass ihre Rhythmik auch in FRZ auftritt. Zu diesen Genen gehören *Grk1* und *Pgc-1α* die beide eine erhöhte Transkriptmenge tagsüber zeigen (Publikation I). Die Expression beider Gene wird Licht-abhängig reguliert und trägt zum Schutz der FRZ vor Licht-induzierten Schäden bei (vgl. Kapitel 3.1).

Ganz analog zeigen die Gene *Esrrβ* und *Nr4a1* eine FRZ-spezifische Rhythmik, wobei hier die Transkriptmengen nachts erhöht sind (Publikation II und III). Unsere Untersuchungen lassen keine Differenzierung der FRZ in Stäbchen und Zapfen zu, aber Blackshaw et al. (2001, 2004) konnten zeigen, dass die Expression von *Esrrβ* Stäbchen-spezifisch [119, 120] und Mutationen im *Esrrβ*-Gen Dystrophien in den Stäbchen verursachen [109]. Dies lässt vermuten, dass auch

die tageszeitliche Regulation von *Esrrβ* spezifisch in diesem Zelltyp der Retina auftritt. Die Differenzierung der Rhythmus-generierenden Zellen bzw. die Zuordnung der rhythmischen Regulation der Genexpression zur Fotorezeptorzellschicht der Retina ist besonders im Hinblick auf fehlerhafte Adaptationsvorgänge von Interesse. Viele retinale Erkrankungen treten Zelltyp-spezifisch auf und Dystrophien von Stäbchen oder Zapfen der Retina sind die Ursache vieler ophthalmologischer Erkrankungen (vgl. Kapitel 3.1).

3.2 Regulation der Genexpression: Licht versus circadianer Oszillator

Die tageszeitliche Rhythmik der Genexpression und ihr Einfluss auf die Funktionalität von Retina und FRZ kann Uhren- und/oder Lichtgesteuert sein [39]. Die Adaptation der Physiologie und Funktionsfähigkeit der Retina erfolgt durch eine circadiane Regulation, die durch molekulare Uhren innerhalb der Retina selbst reguliert wird (zur Übersicht, siehe [11]). Ein besseres Verständnis der circadianen Regulation der retinalen Gentranskription ist hilfreich, um die Anpassungsfähigkeiten retinaler Funktionen zu erfassen.

In Publikation I konnten wir zeigen, dass die Expression der Gene *Grk1* und *Pgc-1α* in Retina und FRZ der Ratte direkt lichtgesteuert ist. Die rhythmische Regulation dieser Gene ging unter DD-Konditionen verloren, was impliziert, dass zur Aufrechterhaltung dieser Rhythmik ein Lichtsignal notwendig ist (Publikation I).

Auffällig ist, dass die Untersuchungen für *Pgc-1α* in Proben von Ratten und Melatonin-profizienten Mäusen unterschiedliche Ergebnisse lieferten. So geht die gemessene Rhythmik in Ratten unter DD verloren, während sie in C3H-Mäusen mit geringerer Amplitude bestehen bleibt. Der Erhalt der Rhythmik suggeriert eine Steuerung der Expression durch einen molekularen Oszillator, dessen Rhythmik durch den Wechsel von Licht und Dunkel verstärkt wird.

Im Unterschied zur lichtinduzierten Rhythmik von *Esrrβ*, zeigte das Gen *Nr4a1* auch unter DD eine rhythmische Regulation (Publikation III). Das Gen *Nr4a1* ist, wie *Esrrβ*, ein nukleärer Orphan-Rezeptor und trägt bei Schädigung der Retina zur Aktivierung von Mechanismen der Immunabwehr bei [121]. Auch für die Gene *Adra1b* und *Pde8a* konnte unter DD weiterhin eine Rhythmik gemessen werden, was zeigt, dass auch ihre Expression unter Kontrolle der circadianen Uhr steht (Publikation II und III). Dies korrespondiert mit früheren Ergebnissen unserer Arbeitsgruppe, die eine circadiane Regulation des Gens *Kcnv2* nachweisen [38].

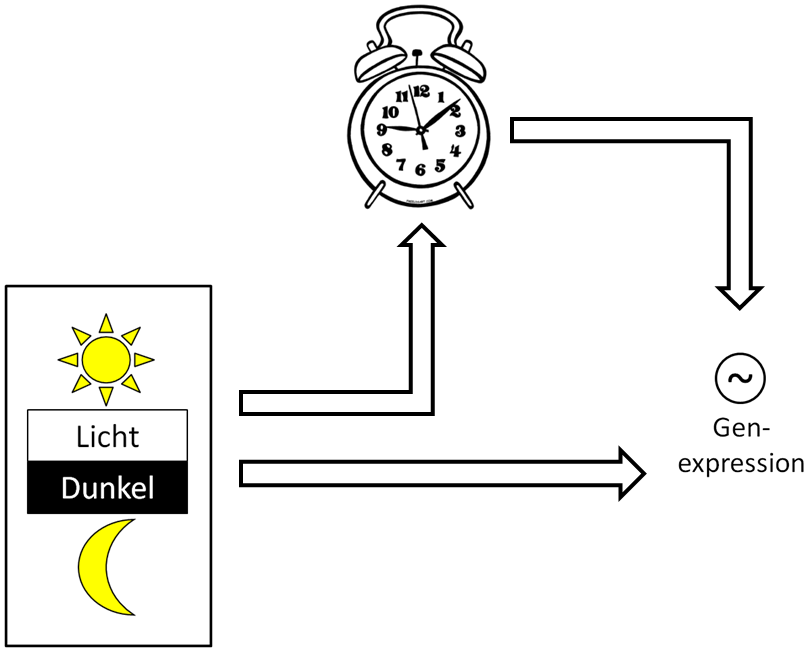

Abbildung 5: Licht versus Oszillator
Das dargestellte Schema zeigt das Zusammenspiel von Licht & Dunkel und molekularen Oszillatoren bei der Regulation der Genexpression. Eine rhythmische Genexpression kann einerseits durch den Wechsel von Licht und Dunkel und andererseits durch eine circadiane Regulation durch einen molekularen Oszillator möglich werden. Der molekulare Oszillator, hier als Uhr dargestellt (Clipart Kid), wird durch die äußeren Lichtbedingungen synchronisiert.

Die Gene *Esrrβ* und *Nr4a1* repräsentieren nukleäre Orphan-Rezeptoren. Insofern zeigen unsere Untersuchungen, dass nukleäre Orphan-Rezeptoren die Adaptation der Retina sowohl Licht-gesteuert als auch circadianen vermitteln.

3.3 Regulation der Genexpression: Die Rolle der Neuromodulatoren Melatonin und Dopamin

Die Neuromodulatoren Melatonin und Dopamin sind für die Regulation der retinalen Physiologie von zentraler Bedeutung. Allerdings sind nicht alle Modellorganismen der circadianen Grundlagenforschung in der Lage Melatonin zu synthetisieren. Während Ratten Melatonin-profizient sind, gibt es bei den verschiedenen Mausstämmen Unterschiede. Tiere, die einen genetischen C57BL6-Hintergrund haben, produzieren kein Melatonin, während Tiere mit C3H-Hintergrund Melatonin-profizient sind [66]. In bisherigen Studien wurden jedoch meist Tiere mit C3H-Hintergrund genutzt, die eine *rd*-Mutation (engl.: *retinal degeneration*) tragen und deshalb eine Dystrophie der äußeren Körnerschicht der Retina aufweisen [122, 123]. Erst kürzlich gelang die Etablierung eines Mausstammes, der aufgrund seines C3H-Hintergrunds Melatonin-profizient ist und gleichzeitig keine *rd*-Mutation aufweist [83, 87]. Dieses Tiermodell ermöglicht die Untersuchung des Einflusses von Melatonin auf die FRZ.

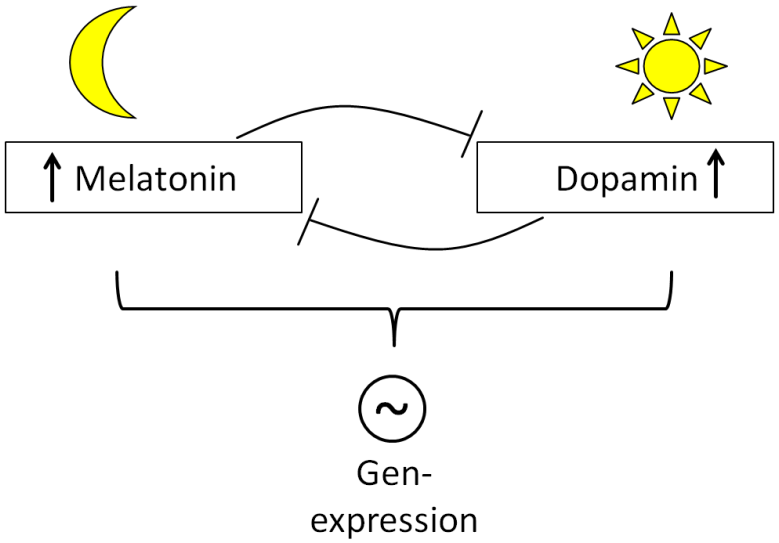

Abbildung 6: Melatonin und Dopamin im circadianen System
Zwischen der circadianen Ausschüttung der Neurohormone Melatonin und Dopamin besteht eine negative Rückkopplungsschleife. Die Ausschüttung von Melatonin ist nachts erhöht, während Dopamin tagsüber vermehrt freigesetzt wird. Das Zusammenspiel dieser Hormone beeinflusst die circadiane Regulation der Genexpression.

In Publikation III wurde der Einfluss der circadian gesteuerten Ausschüttung von Melatonin und Dopamin auf die Expression von Transkriptionsfaktoren untersucht. Ziel dieser Untersuchung war die Identifikation von Genen, welche die uhrengesteuerte Sekretion von Melatonin und Dopamin mit der tageszeitlichen Anpassung der Retina vernetzen. Für die Untersuchung wurden dabei Gene herangezogen, deren Expression in FRZ eine circadiane Rhythmik zeigt und die einen Einfluss auf Physiologie und Funktion der Retina haben. Für den transkriptionellen Coaktivator *Pgc-1α* konnte eine tageszeitliche Rhythmik in Retina und FRZ von Ratten und Melatonin-profizienten Mäusen mit erhöhten Transkriptmengen tagsüber gemessen werden. Die Untersuchung des tageszeitlichen Profils des Gens in Melatonin-defizienten Mäusen zeigte ein Ausbleiben der Rhythmik. Ebenso konnte in MT1$^{-/-}$ Mäusen keine Rhythmik gemessen werden, während die Expressionsprofile für *Pgc-1α* in *Drd4*$^{-/-}$ Mäusen und dem entsprechenden Wildtyp mit intakten D_4-Rezeptoren eine gleiche Rhythmik zeigten (Publikation III). Dies impliziert, dass die Expression von *Pgc-1α* Melatonin-abhängig reguliert wird.

Die Rhythmik der Expression von *Nr4a1* zeigt in Ratten und Melatonin-profizienten Mäusen nachts erhöhte Transkriptmengen. Die gemessene Rhythmik bleibt auch in Melatonin-defizienten Mäusen und Mäusen ohne MT1-Rezeptoren erhalten [102] (Publikation III). Das Melatoninsignal beeinflusst demnach die rhythmische Expression von *Nr4a1* nicht. Im Gegensatz dazu zeigte sich für *Nr4a1* eine deutliche Reduktion der Amplitude in *Drd4*$^{-/-}$ Mäusen im Vergleich zum Wildtyp (Publikation III).

Diese Ergebnisse implizieren, dass der transkriptionelle Coaktivator *Pgc-1α* Melatonin-abhängig reguliert wird, während die Expression des nukleären Orphanrezeptors *Nr4a1* durch das dopaminerge System kontrolliert wird. Zu beachten ist dabei die Möglichkeit, dass durch den Rückkopplungsmechanismus der Melatonin- und Dopaminsynthese eine Defizienz des Melatoninsystems die dopaminerge Signalübertragung beeinflussen kann und umgekehrt. Zusammenfassend konnten *Pgc-1α* und *Nr4a1* als mögliche Verbindung zwischen der circadianen Ausschüttung von Neuromodulatoren und der funktionellen Adaptation und Vitalität von Retina und FRZ identifiziert werden.

Dinet und Korf (2007) konnten einen möglichen Einfluss von Melatonin auf die Regulation von Uhrengenen in der Retina von Mäusen zeigen [124]. Ihre Untersuchungen beschränken sich jedoch auf Neurone der inneren Retina, da das genutzte Tiermodell aufgrund einer *rd*-Mutation keine intakten FRZ aufweist. Hiragaki et al. (2014) untersuchten die Expression von Uhrengenen in FRZ von Melatonin-profizienten Mäusen [82]. Ihre Untersuchungen an MT1- und MT2-Rezeptor-defizienten Mäusen zeigten, dass der Einfluss von Melatonin nicht die FRZ betrifft, sondern nur in Schichten der inneren Retina zu beobachten ist [82].

3.4 Selbstkritische Betrachtung der Untersuchungen und Ergebnisse

Die vorgestellten Studien und Ergebnisse bringen Limitierungen mit sich, die hier selbstkritisch betrachtet werden sollen. So ist zu beachten, dass die Studien an unterschiedlichen Versuchsmodellen durchgeführt wurden. In Publikation I wurden alle Untersuchungen an Ratten durchgeführt, welche Melatonin-profizient sind und über intakte FRZ verfügen. In Publikation II wurden die Versuche neben Ratten auch an Melatonin-profizienten und -defizienten Mäusen durchgeführt.

Am Beispiel von *Pgc-1α* werden die Unterschiede in der Genexpression zwischen den unterschiedlichen Tiermodellen deutlich (vgl. Kapitel 3.3). So konnte in Ratten und Melatonin-profizienten Mäusen eine Rhythmik gemessen werden, die in Melatonin-defizienten Mäusen ausblieb. Die Unterschiede in der Expression von *Pgc-1α* in Ratten und Melatonin-profizienten Mäuse deuten darauf hin, dass neben der Regulation der Rhythmik durch Melatonin auch ein externes Lichtsignal Einfluss auf die Rhythmik hat und beide Faktoren im Zusammenspiel die circadiane Rhythmik generieren.

In Publikation III wurden Mäuse mit einem knock-out der *MT1*- bzw. D_4-Rezeptoren eingesetzt. Es ist denkbar, dass gemessene bzw. nicht gemessene Effekte in knock-out Tieren auf kompensatorischen Effekten beruhen. Für Uhrengene ist bereits bekannt, dass der knock-out eines Gens durch die (verstärkte) Expression eines anderen Uhrengens kompensiert werden kann. Die verwendeten KO-Modelle zeichnen sich durch den Verlust der Rezeptoren bei

intakter Melatonin- bzw. Dopamin-Synthese aus. Da die Wirkung von Melatonin und Dopamin über verschiedene membranständige Rezeptoren vermittelt wird, ist es möglich, dass der knock-out eines Rezeptor-Typs durch andere Typen der Rezeptorfamilie kompensiert wird. Der knock-out eines Rezeptors kann darüber hinaus auch indirekt wirksam werden. So könnte z.B. ein knock-out des MT1-Rezeptors die Dopamin-Synthese beeinflussen und umgekehrt.

Des Weiteren wurden Untersuchungen zur Lokalisation der Rhythmus-generierenden Zellen an isolierten FRZ durchgeführt. Mit der hier angewandten Methode zur Isolierung wurde mittels LMPC (*laser microdissection and pressure catapulting*) die gesamte äußere Körnerschicht ausgeschnitten. Die eingesetzten Proben enthalten somit die Zellkerne von Zapfen und Stäbchen. Dies führt dazu, dass die Effekte unserer Messungen an FRZ nicht spezifisch einer FRZ-Art zugeordnet werden können. Auch bei der Erstellung der tageszeitlichen Profile von Gesamtretina-Proben repräsentieren die Ergebnisse natürlich nur Durchschnittswerte aus einem Pool verschiedener Zelltypen. Es ist daher nicht möglich zu sagen auf welchen Zelltyp der Retina der gemessene Effekt zurückzuführen ist.

4 Fazit und Ausblick

Der vorliegenden Dissertation liegt die Arbeitshypothese zugrunde, dass die funktionelle Anpassung von Retina und FRZ an die tageszeitlich wechselnden Lichtbedingungen durch eine tageszeitliche Regulation der Genexpression ermöglicht wird. Im Einklang mit der Arbeitshypothese wurde gefunden, dass Gene, die für die visuelle Verarbeitung (z.B. *Grk1*), die Protektion (z.B. *Pgc-1α*) und den Energiestoffwechsel retinaler Zellen (z.B. *Esrrβ*) von Bedeutung sind, tageszeitlich reguliert werden.

Die vorliegenden Ergebnisse zeigen, dass die retinale Genexpression von unterschiedlichen Parametern beeinflusst wird. Für die Entstehung und den Erhalt einer tageszeitlichen Rhythmik können, je nach Gen, die äußeren Lichtbedingungen oder intrinsische Oszillatoren entscheidend sein. Diese Oszillatoren können dabei ihrerseits wiederum durch äußere Lichteinflüsse beeinflusst werden. Die tageszeitliche Regulation der retinalen Genexpression erfolgt in Abhängigkeit von Melatonin und Dopamin. Ein zusammenfassendes Konzept zur tageszeitlichen Regulation der retinalen Gene zeigt Abbildung 7. Die Regulation der Rhythmik erfolgt über ein Netz von veränderlichen Einflüssen und involviert krankheitsassoziierte Gene, so dass die Pathogenese ophthalmologischer Erkrankungen möglicherweise auf eine fehlerhafte Adaptation der Gentranskription zurückzuführen ist.

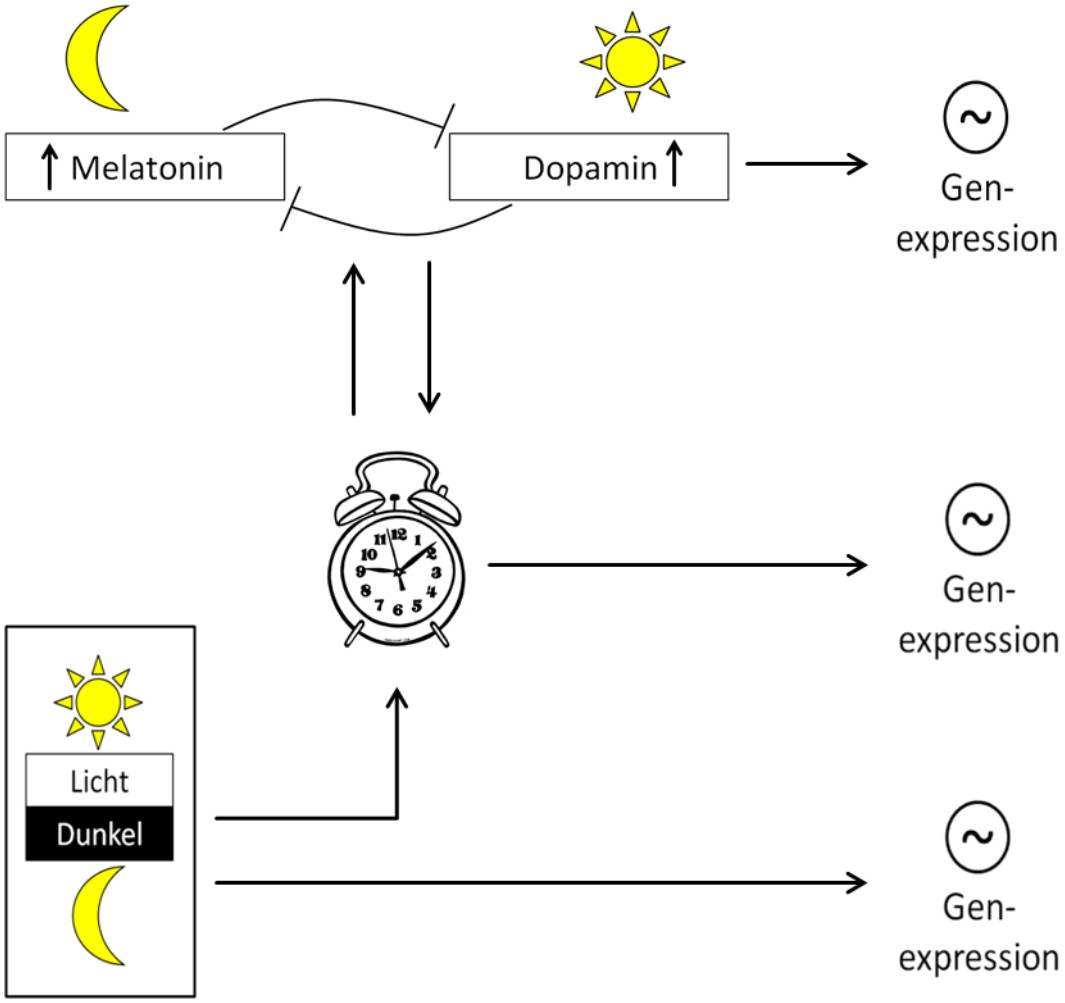

Abbildung 7: Schematische Darstellung der tageszeitlichen Regulation der Genexpression
Dieses Schema zeigt ein vereinfachtes Modell zur tageszeitlichen Regulation retinaler Gene über verschiedene Wege. So haben sowohl Licht & Dunkel, als auch der molekulare Oszillator und die Neuromodulatoren Melatonin und Dopamin Einfluss auf die Rhythmik der Genexpression. Der Oszillator kann dabei von Licht & Dunkel oder Melatonin & Dopamin beeinflusst werden. Andererseits wird die Sekretion von Melatonin und Dopamin direkt über die äußeren Lichtbedingungen und retinale Oszillatoren reguliert. (Clipart Kid)

Die Charakterisierung der tageszeitlich und circadian regulierten Gene hinsichtlich ihrer molekularen und biologischen Funktionen offenbart, dass einige Gene für die retinale Physiologie von hoher Relevanz sind. Sie sind involviert in die visuelle Verarbeitung, die Entwicklung der FRZ, sowie die Degeneration neuronaler Zellen. Des Weiteren sind sie an Zellproliferation, DNA-Bindung und/oder der Regulation der Transkription beteiligt. Die Möglichkeit eines pathologischen Effekts fehlerhafter Adaptation macht diese Gene zu interessanten Kandidaten für weiterführende Untersuchungen. Dabei spielen nicht nur mögliche Licht-induzierte Schäden eine Rolle, sondern auch ein möglicherweise altersbedingter Verlust der Adaptationsfähigkeit, der unter anderem aufgrund altersbedingter Degeneration retinaler Zellen auftreten kann.

In einem ersten Schritt könnte hierzu untersucht werden, inwieweit die Pathogenese retinaler Erkrankungen zu einer beeinträchtigten circadianen, dopaminergen oder melatoninergen Regulation retinaler Gene führt.

Weiterhin könnte der Einfluss einer beeinträchtigten circadianen, dopaminergen oder melatoninergen Regulation auf die Pathogenese retinaler Erkrankungen untersucht werden. Hier zeigen erste Befunde, dass eine Beeinträchtigung der circadianen Regulation FRZ-Defekte hervorrufen kann und eine Dopamindefizienz ursächlich für visuelle Dysfunktionen bei diabetischer Retinopathie sein kann [125, 126].

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6 Anhang

Zuordnungen der geleisteten Beiträge zu den einzelnen Publikationen

Bei Publikation I war ich im Vorfeld an der Entwicklung des Studienkonzepts und an der Versuchsplanung beteiligt. Zu Beginn der Experimente führte ich die gesamte Probengewinnung und Probenaufbereitung in Zusammenarbeit mit und . Weiterhin habe ich die Isolation der FRZ, die RNA Extraktion, die Reverse Transkription und die qPCR durchgeführt. Bei diesen Schritten wurde ich von und unterstützt. Die Durchführung der Western Blot Analysen wurde von mir zunächst technisch erarbeitet und etabliert und dann angewandt. Darüber hinaus war ich am Primerdesign für die qPCR, an der statistischen Auswertung und der Ergebnisinterpretation beteiligt und habe bei der Erstellung von Abbildungen, Tabellen und dem Manuskript mitgearbeitet.

Für Publikation II habe ich an der Versuchsplanung mitgewirkt und habe die Probengewinnung und Probenaufbereitung in Zusammenarbeit mit durchgeführt. Die Isolation der Zellschichten, die Probenvorbereitung, sowie Primerdesign und qPCR Analysen wurden von mir durchgeführt, wobei ich von und unterstützt wurde. Weiterhin wurden die Western Blot Analysen von mir durchgeführt und ich war an der statistischen Auswertung und der Interpretation der Versuchsergebnisse beteiligt, habe die Abbildungen und Tabellen erstellt und an der Ausarbeitung des Manuskripts mitgewirkt.

Im Rahmen von Publikation III beteiligte ich mich an der Entwicklung des Studienkonzepts und an der Planung der Versuchsreihen. Die Probengewinnung wurde von unseren Kooperationspartnern in Atlanta, USA (Emory University School of Medicine und Morehouse School of Medicine) durchgeführt, die anschließende Probenaufbereitung wurde von mir ausgeführt. Die Isolation der FRZ, Vorbereitung der Proben, sowie qPCR habe ich mit Unterstützung von und durchgeführt, außerdem habe ich die Primer für die qPCR designt. Darüber hinaus habe ich an der statistischen Auswertung der Messdaten und Interpretation der Ergebnisse mitgearbeitet. Alle Abbildungen und Tabellen wurden von mir erstellt und das Manuskript unter meiner Beteiligung ausgearbeitet.

Daneben konnte ich weitere Beiträge zu Veröffentlichungen unserer Arbeitsgruppe bzw. unserer Kooperationspartner leisten. Bei der Publikation *Hölter et al., 2012* war ich an der Versuchsplanung beteiligt, habe die Probengewinnung und –aufbereitung durchgeführt und habe an der Durchführung der qPCR-Analysen und Western Blot Analysen mitgearbeitet. Für die Publikation *Hiragaki et al., 2014* habe ich die Isolationsmethode für FRZ und die nachfolgende RNA-Extraktion und qPCR-Analyse etabliert. Weiterhin war ich an der Entwicklung des Studienkonzepts, der Interpretation der Daten und der Erstellung des

Manuskripts beteiligt. Für die Publikation *Wolloscheck et al., 2015* habe ich die Probengewinnung und –aufbereitung durchgeführt. Daneben habe ich das Primerdesign für die qPCR und einige Western Blot Analysen ausgeführt, sowie die Tabellen und Abbildungen erstellt und an der Ausarbeitung des Manuskripts mitgewirkt.

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Lebenslauf

Danksagung

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich diese Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Ich habe keinen vorherigen Promotionsversuch unternommen.

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