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Circadian gene expression patterns of melanopsin and pinopsin in the chick pineal gland

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Abstract

The directly light-sensitive chick pineal gland contains at least two photopigments. Pinopsin seems to mediate the acute inhibitory effect of light on melatonin synthesis, whereas melanopsin may act by phase-shifting the intrapineal circadian clock. In the present study we have investigated, by means of quantitative RT-PCR, the daily rhythm of photopigment gene expression as monitored by mRNA levels. Under a 12-h light/12-h dark cycle, the mRNA levels of both pigments were 5-fold higher in the transitional phase from light to dark than at night, both in vivo and in vitro. Under constant darkness in vivo and in vitro, the peak of pinopsin mRNA levels was attenuated, whereas that of melanopsin was not. Thus, whereas the daily rhythm of pinopsin gene expression is dually regulated by light plus the intrapineal circadian oscillator, that of melanopsin appears to depend solely on the oscillator.

Keywords: Melanopsin; Pinopsin; Gene expression; Pineal gland; Circadian rhythm; Chick

Light applied in the scotophase has two distinct effects in the isolated chick pineal gland: it acutely inhibits melatonin synthesis and it phase-shifts the intrapineal circadian oscillator that drives melatonin synthesis (for a review see [1]). Inhibitory and phase-shifting effects appear to be mediated by two different transduction pathways [2], which are probably separated from their origin, suggesting that different photopigments are involved [3,4]. The photopigment(s) that start the respective cascade have not as yet been identified, although there are at least two candidates, viz. pinopsin and melanopsin.

Pinopsin is an opsin-like protein whose gene is exclusively expressed in the pineal gland [5,6]. Pinopsin mRNA levels of chickens kept under a 12-h light/12-h dark schedule (LD 12:12) undergo diurnal fluctuations with peaks during the second half of the day and troughs during the dark. The light-induced up-regulation of pinopsin gene expression has also been observed in pineal organs in vitro, although to a lesser degree than in vivo [7]. Pinopsin gene expression is supposed not to be under the control of the intrapineal circadian clock, since no rhythm in the amount of pinopsin mRNA in constant darkness has been demonstrated so far [7]. This behaviour is in contrast to the gene expression of several retinal photoreceptive molecules, that all show a clear circadian rhythm with peaks near the transition between the subjective day and the subjective night and troughs during the subjective night [8–11]. Although the precise role of pinopsin is unclear, several features make it likely that it contributes to mediating the acute effect of light [12,13].

Melanopsin was first isolated from the photosensitive melanophores of *Xenopus laevis* [14]. In non-mammals, melanopsin is expressed in photoreceptive structures such as the pineal [15,16]. In mammals, a homologue of melanopsin is expressed in a photosensitive subset of retinal ganglion cells that project to the suprachiasmatic nucleus (SCN), the central circadian pacemaker

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[17]. This tissue localization has raised the possibility that melanopsin mediates the entraining and hence phase-shifting effect of light. Various studies with melanopsin knockout mice have confirmed that melanopsin is an important molecule in the photoreceptive system for the entrainment of the circadian rhythm in mammals [18]. An avian homologue of melanopsin has been identified in the chick pineal gland [19]. Chicken melanopsin has a high homology (72% amino acid identity) to *Xenopus* melanopsin and is exclusively expressed in the parafollicular cells of the pineal and in non-photoreceptor cells of the retina [1].

In contrast to pinopsin, nothing is known about the behaviour of melanopsin gene expression in the chicken pineal gland under various lighting conditions. To fill this gap, we have investigated the changes in melanopsin mRNA levels under an LD 12:12 cycle and under constant darkness, both in vivo and in vitro, by utilizing quantitative reverse transcription-polymerase chain reaction (RT-PCR) and comparing these changes with those of pinopsin mRNA levels at the corresponding time points.

Materials and methods

Animals and organ cultures. One-day-old chickens (Leghorns) were purchased from Schwerk (Wistedt, Germany). They were maintained for 7–9 days with food and water ad libitum under a 12-h light/12-h dark (LD 12:12) schedule with lights on at *Zeitgeber* time (ZT) 0 and lights off at ZT 12 or transferred to constant darkness (DD) on day 8. Light was provided by using lumilux deluxe "daylight" fluorescent tubes (Osram, Munich, Germany), with an intensity of 400 lux near the cages.

For the in vivo experiments, pineal glands were dissected out on days 7–9 at the indicated time points under the various light conditions and frozen at −70 °C until RNA-isolation was carried out. During the dark phase, dissections were carried out under dim red light.

For the in vitro experiments, pineal glands were taken from chickens that had been kept under the LD-cycle for 6 days. The pineal glands were quickly dissected out under sterile conditions between ZT 8 and ZT 10, and collected in culture dishes containing Hanks' balanced salt solution (Sigma, Taufkirchen, Germany). The glands were cultured in Medium 199 (Gibco, Karlsruhe, Germany) supplemented with 2.2 g/L $NaHCO_3$, 25 mM Hepes, 1 g/L BSA, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Organ cultures were maintained in a CO_2 incubator (Heraeus, Hanau, Germany) at 37 °C under an atmosphere of 95% O_2 and 5% CO_2 under the same lighting schedule as that in vivo for 1 day. Light inside the incubator (700 lux) was provided by adjustable white-light-emitting diodes (Everlight Electronics Europe, Karlsruhe, Germany) with $\lambda_{max} = 483$ nm, which is close to the absorption maximum of pinopsin of 470 nm [5,6]. On day 2 in culture, the LD 12:12 lighting schedule was either maintained or glands were cultured under DD. Glands were collected throughout the second day at the indicated time points under the various light conditions and frozen at −70 °C until RNA-isolation was performed. During the dark phase, the collection was performed under dim red light.

RNA isolation. The RNA of 3–5 pooled pineals was isolated by using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. The amount of extracted RNA was determined by measurement of the optical density at 260 and 280 nm.

Reverse transcription. Extracted RNA (1 μg) was reverse-transcribed by using 4 U Omniscript reverse transcriptase (Qiagen, Hilden, Germany) in a total volume of 20 μl, containing 2.0 μl of 10× buffer (supplied with the transcriptase), 0.5 mM each deoxynucleotide triphosphate, 10 U ribonuclease inhibitor (Ambion, Huntingdon, United Kingdom), and 1 μM oligo(dT) primer (MWG Biotech, Ebersberg, Germany). A sample without added RNA was routinely included as a control. The reverse transcription (RT) mixture was incubated at 37 °C for 60 min to promote cDNA synthesis. The reaction was terminated by heating the sample at 95 °C for 5 min. cDNA was diluted 1:5 or 1:25 in RNAse-free distilled water and aliquots of 5 μl were used for the polymerase chain reaction (PCR).

Real-time PCR. Real-time PCR was carried out in a total volume of 25 μl containing 12.5 μl absolute QPCR SYBR Green Fluorescein Mix (Abgene, Hamburg, Germany), 0.75 μl primer (10 mM) each, 5 μl sample, and 6 μl distilled water. New primers were designed by utilizing the software Primer designer 5, version 5.1 (Scientific and Educational Software, Cary, USA). For amplification of pinopsin cDNA (*Gallus gallus* pinopsin cDNA, GenBank Accession No. U15762) the following primers were used: (forward) 5′-TGG TGA ATG GGC TGG TCA TC-3′ and (reverse) 5′-TCC TCC TGC CAA ACA CGA AG-3′. Melanopsin cDNA (*Gallus gallus* melanopsin cDNA, GenBank Accession No. AY036061) was amplified by using primers (forward) 5′-TAT GCA ATA ATT CAC CCG AGA-3′ and (reverse) 5′-CTT GGT CCT TCC AGC AAG AG-3′. PCR amplification and quantification was performed in an i-Cycler (Bio-Rad, Munich, Germany) as follows: activation of the enzyme at 95 °C for 15 min followed by 40 cycles of 30 s at 95 °C, 30 s at 62 °C, and 25 s at 72 °C. All amplifications were carried out in duplicate. The purity of the amplification products was confirmed by both the melting curve and gel electrophoresis. The amount of RNA was calculated from the measured threshold cycles (C_t) by a standard curve. Data were normalized by determination of the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (chicken GAPDH cDNA, GenBank Accession No. K01458). For the amplification of GAPDH cDNA, the following primers were used: (forward) 5′-ACC ACT GTC CAT GCC ATC AC-3′ and (reverse) 5′-TCC ACA ACA CGG TTG CTG TA-3′ [20].

Results

The mRNA levels of melanopsin in chickens maintained under a LD 12:12 lighting schedule underwent diurnal fluctuations similar to those of pinopsin mRNA levels (Fig. 1A). Both melanopsin mRNA levels and pinopsin mRNA levels were low in the early morning (between ZT 0 and ZT 6). They started to increase in the middle of the daytime (ZT 6) by about 5-fold and reached their maximum levels between ZT 10 and ZT 12. Melanopsin mRNA levels decreased after lights off and returned to low nocturnal levels within 4–6 h. The diurnal rhythm of both transcripts persisted in organ culture with nearly the same amplitude as that in vivo (Fig. 1B). In constant darkness, pinopsin mRNA levels showed only a small increase (about 2-fold) during the late subjective day, both in vivo and in vitro (Figs. 2A and B); this was also observed on the second day under DD (data not shown). Pinopsin mRNA levels increased again by about 5-fold upon exposure to light (data not shown). By contrast, the circadian rhythm of melanopsin mRNA levels with about 5-fold increases in the late

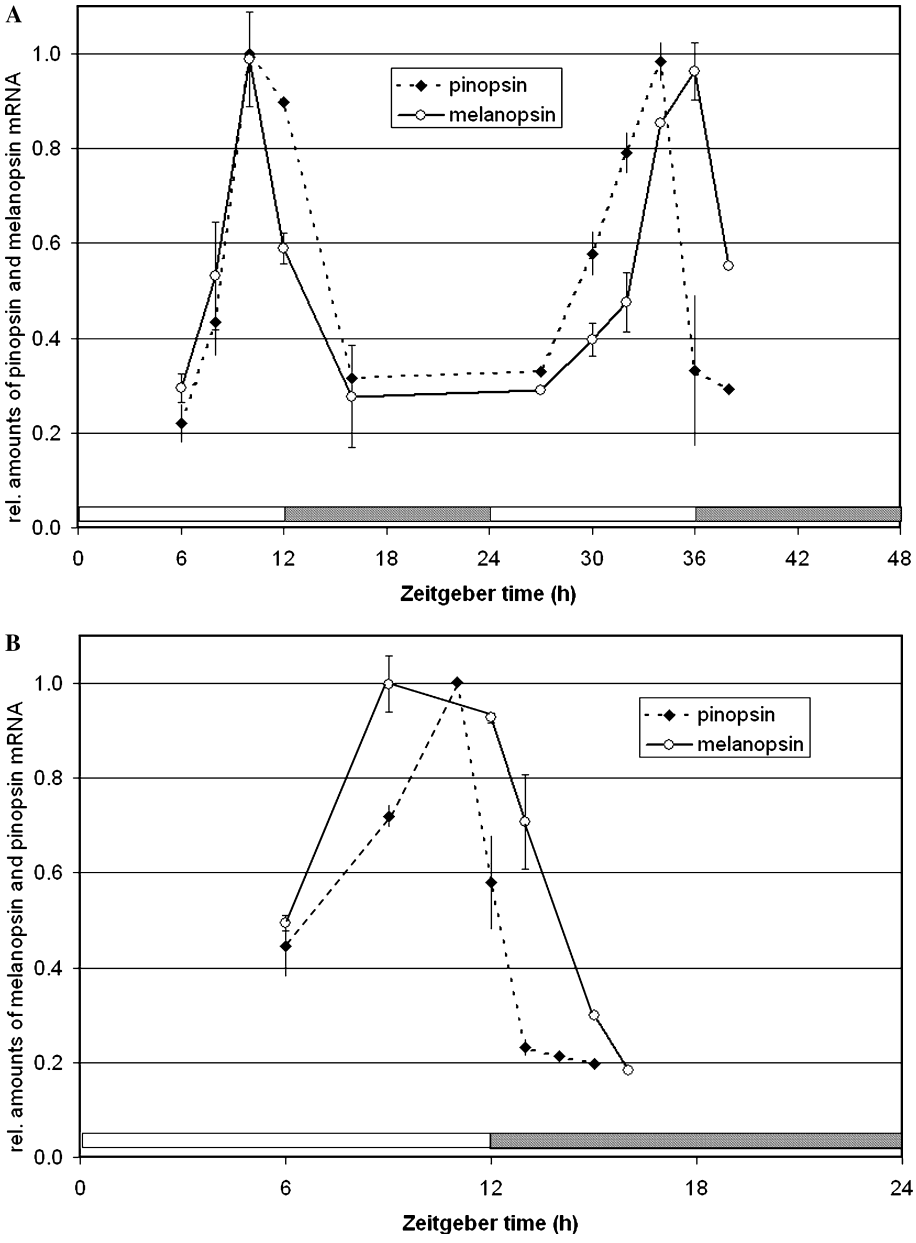

Fig. 1. Circadian variations of melanopsin and pinopsin mRNA levels in chick pineal glands under LD 12:12 in vivo (A) and in vitro (B). Note that identical results were obtained in vivo and in vitro: melanopsin and pinopsin mRNA formation increase about 5-fold during the second half of the light phase followed by troughs during the dark phase (open bars, light; filled bars, dark). Melanopsin and pinopsin mRNA amounts were normalized to GAPDH mRNA and expressed as relative amounts of the maximum value (means ± SEM).

subjective day and troughs during the subjective night persisted and was even slightly enhanced under constant darkness, both in vivo and in vitro (Figs. 2A and B).

Discussion

The present results confirm that, when chickens or their isolated pineal glands are kept under LD 12:12, pineal pinopsin mRNA formation increases during the second half of the light phase, followed by troughs during the dark phase [7]. However, the following differences have been noted. In our hands, pinopsin mRNA formation in pineal glands cultured in vitro under LD 12:12 exhibited distinctly greater oscillations than those observed by Takanaka et al. [7] (5-fold versus 1.5-fold) and, more importantly, under DD, a circadian rhythm was clearly detectable. The differences observed are probably explicable by the usage of different methodologies. Whereas we used the highly sensitive real-time RT-PCR, Takanaka et al. [7] measured pinopsin mRNA formation by Northern blot analysis, which, as shown for chick retinal cryptochrome 1 (Cry1) mRNA formation, yields smaller daily amplitudes [21]. Takanaka et al. [7]

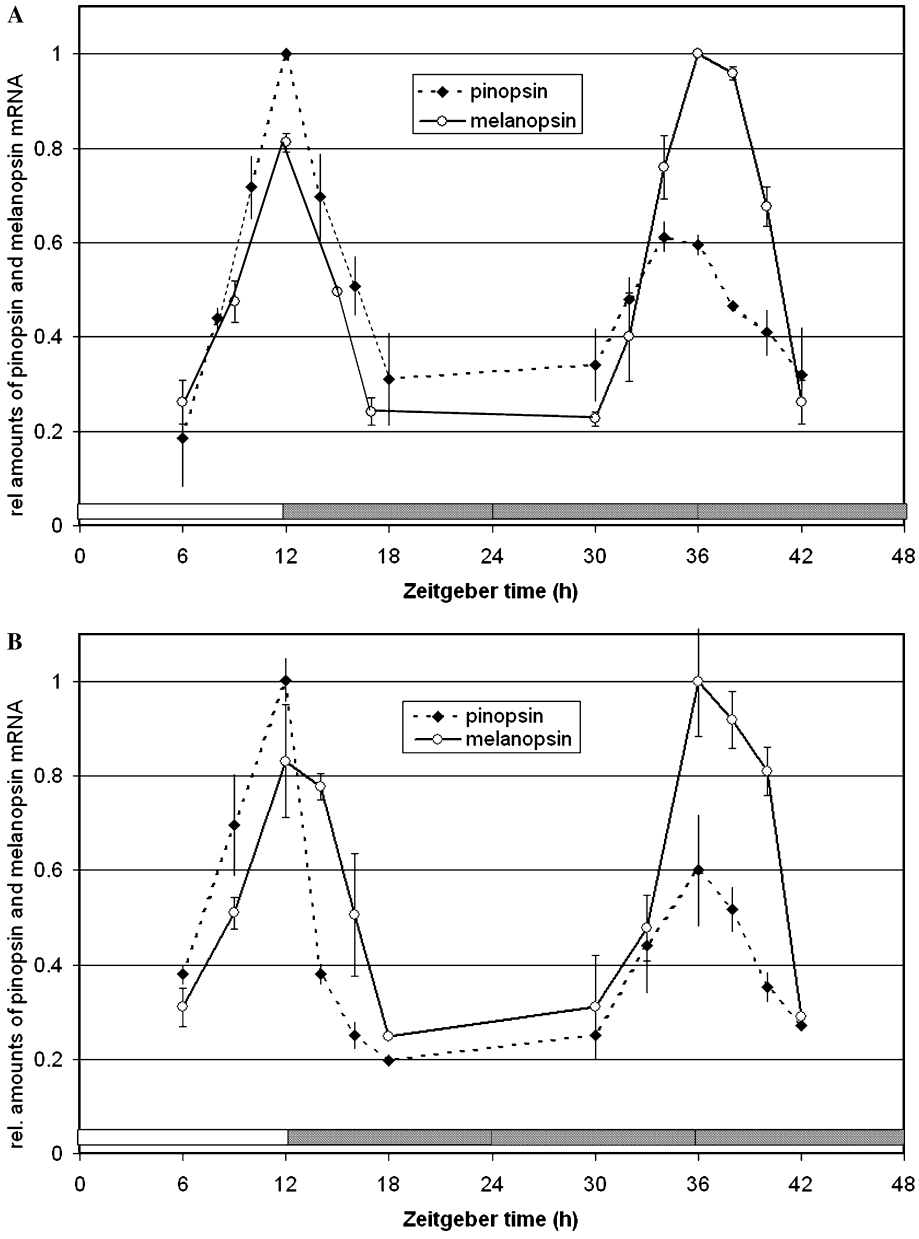

Fig. 2. Temporal changes of melanopsin and pinopsin mRNA levels in chick pineal glands under constant darkness in vivo (A) and in vitro (B). Note that the rhythmic expression of both transcripts with peaks in the late subjective day and troughs during the subjective night persists under constant darkness (open bars, light; filled bars, dark). Pinopsin mRNA levels increased only about 2-fold (versus 5-fold under LD 12:12). By contrast, the 5-fold increase of melanopsin mRNA levels persisted under constant darkness. Identical results were obtained in vivo and in vitro. Melanopsin and pinopsin mRNA amounts were normalized to GAPDH mRNA and expressed as relative amounts of the maximum value (means ± SEM).

have concluded from the absence of a circadian cycle of pinopsin mRNA formation under DD in vivo that pinopsin gene expression is regulated by light signals, independently of the circadian clock. As, in the present study, a clear circadian rhythm of the amount of formed pinopsin mRNA was demonstrable under DD, both in vivo and in vitro, the concept of a circadian clock-independent regulation of pinopsin gene expression [7] is no longer tenable. The picture that emerges is that, in addition to exogenous regulation by light, there is a distinct circadian component in pinopsin mRNA formation. Thus, pinopsin in the chick pineal does not differ from retinal photopigments, as far as their circadian rhythmicity is concerned [8–11]. In view of the presence of circadian oscillators in retina, suprachiasmatic nucleus, and the pineal gland, the question can be asked as to which of the oscillators is accountable for the presently observed circadian component. Because the circadian nocturnal increases are equally pronounced in vitro, when the gland is no longer under the control of the retina and/or the suprachiasmatic nucleus, as in vivo, we conclude that the intrapineal circadian oscillator is the prime mover.

The present study is the first to show that melanopsin transcript levels are demonstrable in the chick pineal gland, that they undergo diurnal fluctuations under LD 12:12, both in vivo and in vitro, and that the fluctuations are basically similar to those of pinopsin mRNA accumulation. In view of what has previously been found regarding the diurnal changes of photopigment transcript levels, these findings are not unexpected. However, a surprising finding is that, under DD, the daily amplitudes of melanopsin gene expression are not reduced, as is the case for pinopsin mRNA formation. We therefore suspect that the daily rhythm of melanopsin mRNA formation is not primarily regulated by light but by the intrapineal circadian oscillator.

In which way may the circadian pacemaker control the gene expression of melanopsin and pinopsin? The endogenous rhythmicity of the pacemaker is derived from cell-autonomous feedback loops. In the chick pineal gland, clock genes cClock, cBmal1/2, cPer2, and cCry1/2, and their protein products comprise feedback loops in which a cCLOCK-cBMAL1/2 heterodimer binds to a CACGTG E-box element in the promoter region of cPer2 thus activating its transcription [22]. cPER2-cCRY1/2 heterodimers in turn inhibit the cCLOCK-cBMAL1/2 complex, resulting in the suppression of cPer2 transcription. Recently, an E-box sequence has also been found in the promoter region of the pinopsin gene [23]. Therefore, the circadian regulation of pinopsin transcription might be mediated through the E-box. The presence of a corresponding E-box element in the promoter region of the melanopsin gene remains to be determined.

Finally, the question arises as to which of the two investigated pineal photopigments is the most likely candidate for mediating the circadian entrainment of melatonin synthesis in the chick pineal gland. In mammals, there is clear evidence that the acute inhibitory effect of light on melatonin synthesis involves the classical photoreceptors and their opsins [24]. The entraining effect of light appears to be mediated by the melanopsin present in retinal non-rod and non-cone cells [25]. Whether the expression of mammalian melanopsin in the retinal ganglion cells is light-induced or under the control of a circadian pacemaker is not yet known. In view of the situation in mammals, it is tempting to postulate that melanopsin also exerts an entraining function in the chick pineal gland. Perhaps, as presently shown, the expression of melanopsin is tightly coupled to the intrapineal circadian oscillator to allow melanopsin-mediated processes in the cell to feed back onto the oscillator.

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