

The expression of melanopsin and clock genes in *Xenopus laevis* melanophores and their modulation by melatonin

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Abstract

Vertebrates have a central clock and also several peripheral clocks. Light responses might result from the integration of light signals by these clocks. The dermal melanophores of *Xenopus laevis* have a photoreceptor molecule denominated melanopsin (OPN4x). The mechanisms of the circadian clock involve positive and negative feedback. We hypothesize that these dermal melanophores also present peripheral clock characteristics. Using quantitative PCR, we analyzed the pattern of temporal expression of *Opn4x* and the clock genes *Per1*, *Per2*, *Bmal1*, and *Clock* in these cells subjected to a 14-h light:10-h dark (14L:10D) regime or constant darkness (DD). Also, in view of the physiological role of melatonin in the dermal melanophores of *X. laevis*, we determined whether melatonin modulates the expression of these clock genes. These genes show a time-dependent expression pattern when these cells are exposed to 14L:10D, which differs from the pattern observed under DD. Cells kept in DD for 5 days exhibited overall increased mRNA expression for *Opn4x* and *Clock*, and a lower expression for *Per1*, *Per2*, and *Bmal1*. When the cells were kept in DD for 5 days and treated with melatonin for 1 h, 24 h before extraction, the mRNA levels tended to decrease for *Opn4x* and *Clock*, did not change for *Bmal1*, and increased for *Per1* and *Per2* at different Zeitgeber times (ZT). Although these data are limited to one-day data collection, and therefore preliminary, we suggest that the dermal melanophores of *X. laevis* might have some characteristics of a peripheral clock, and that melatonin modulates, to a certain extent, melanopsin and clock gene expression.

Key words: *Xenopus laevis*; Dermal melanophores; Melatonin; Melanopsin; *Per1*; *Per2*; *Bmal1*; *Clock*

Introduction

There are three structures in the core of the circadian system that regulate and control several forms of rhythmic expression in vertebrates, namely the retinas, the pineal complex (pineal body and the eye/parietal organ) and the suprachiasmatic nucleus (SCN) (1). The circadian system also includes hierarchically organized peripheral oscillators located outside the main oscillator itself (1). These peripheral oscillators can be coupled or not, even though they are linked to the SCN. Thus, their expression is connected and depends on the temporal structure of the organism, even though they are self-sustained and therefore independently capable of generating oscillation for the cell or tissue where they are located (2).

SCN and peripheral clocks work through a transcriptional circuit of clock genes, which generate the rhythmic patterns of protein expression during metabolism, and participate in several other functions (3-5). In mammals, the transcrip-

tion factors CLOCK and BMAL1 act as positive regulators, whereas the three PER proteins (PER1, PER2 and PER3) and the two CRY proteins (CRY1 and CRY2) operate as negative agents (3,6). The molecular mechanism of the circadian clock has also been confirmed in other organisms such as mice and humans (7).

Clock gene circadian oscillation controls the expression of genes involved in multiple cell functions, during a period of approximately 24 h by at least two mechanisms: 1) through direct interaction with *E-boxes* in the promoters of these genes, and 2) by the regulation of other clock-controlled genes, which also act as transcription factors (3,5).

The molecular mechanism involved in maintaining circadian rhythmicity is defined by transcription/translation in feedback loops, which are based on mRNA and protein rhythmic expression of circadian clock components. SCN synchronization by light induces expression of a group

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of clock genes, including *Per2* and *Per1* (8). Even though *Per* genes are critical elements for clock operation, their specific functions have not been well defined. For example, in *Drosophila*, a single *Per* gene is sufficient as a negative loop, whereas in the mammalian system there are three homologous genes (*Per1*, *Per2* and *Per3*), two of which (*Per1* and *Per2*) are essential for SCN operation. Apparently these three homologous genes possess distinct functions (9).

Peripheral clocks are widely distributed from fish to mammals (2,10-13). These peripheral clocks are reset by the central oscillator through neurohormonal signals, or by metabolic signals and food (13). The independence of these oscillators from the central oscillator has been demonstrated (2,10-13), suggesting the presence of their own synchronizing elements (13). Circadian expression observed in peripheral tissues can be directly synchronized by exposure to light/dark cycles, both in whole organisms and in various cell types in culture (14-17).

In the amphibian *Xenopus laevis*, an opsin was characterized in the dermal melanophores, and denominated melanopsin (*OPN4*) (14). Subsequently a melanopsin orthologous to that of *X. laevis* was identified in a small population of intrinsically photoreceptive cells (ipRGCs) in the retinas of mice (15).

OPN4 is expressed in all classes of vertebrates examined to date, from teleosts to mammals (2,18,19). Furthermore, melanopsin is also found in other encephalic regions, such as the pineal gland and the deeper brain regions of non-mammalian vertebrates in which circadian photoreception is manifest (2,18,20-23).

Melanopsin is also expressed in pigment cells and mediates the photo-response of melanophore proliferation (24) and melanosome dispersion (22,25,26). To date, little is known about the regulatory mechanisms of melanopsin or other opsins, such as rhodopsin, in vertebrate pigment cells. Data from previous studies from our laboratory demonstrated rhodopsin modulation by alpha-melanocyte-stimulating hormone (α -MSH) in pigment cells of *Carassius auratus* (GEM-81 erythrophoroma cells) (24). More recently, Farhat et al. (27) demonstrated the biphasic action of endothelin-1 on *Opn4x* expression in *Danio rerio* ZEM-2S cells, in which low concentrations of this hormone stimulated *Opn4x* expression and high concentrations inhibited it.

The pineal gland of non-mammalian vertebrates contains photoreceptors, which permit nocturnal production and release of melatonin, thereby synchronizing light/dark cycles. In amphibians, reptiles and birds, the pineal gland, together with the lateral eyes that are structures similar to retinal photoreceptors, efficiently assumes this function (28). In vertebrates, melatonin is released by the pineal gland during darkness (29). In unicellular animals, bacteria and invertebrates, it also marks this period (30). In vertebrates, melatonin is further synthesized by the retina, thereby participating in the adaptive processes of night vision (31).

It has been shown that the exogenous administration

of melatonin is capable of synchronizing circadian rhythms (32). The daily rhythms of *N*-acetyltransferase activity and melatonin secretion can be observed in pineal cell cultures. These rhythms can be initiated by light/dark cycles of 24 h in fish, lizards and birds (32).

In amphibian dermal melanocytes, melatonin induces the aggregation of melanosomes and is a potent skin-lightening agent (29). Embryonic dermal melanophores of *X. laevis* express *Opn4x* and *Opn4m*. Furthermore, when these melanophores are exposed to light, there is a dispersion of pigment granules throughout the cytoplasm (33). Considering the existence of *Opn4x* and clock genes in several types of cells in *X. laevis*, our hypothesis is that the dermal melanophores of this amphibian also present peripheral clock characteristics. As a preliminary approach to this hypothesis, we analyzed 24-h data of temporal expression of *Opn4x*, and the clock genes *Per1*, *Per2*, *Bmal1* and *Clock* in these cells, when subjected to a 14-h light:10-h dark (14L:10D) regime or constant darkness (DD). Also, in view of the physiological role of melatonin in the dermal melanophores of *X. laevis*, we determined whether expression of *Opn4x* and the clock genes in these cells would be modulated by melatonin under the experimental conditions utilized.

Material and Methods

Cell culture

X. laevis melanophores were kept in 60% L-15 medium supplemented with 10% fetal calf serum, 480 mg/L galactose, 5 mg/L insulin/transferrin/selenium, 4 mg/L uridine, 87.6 mg/L L-glutamine, 25 mg/L L-asparagine, 152 mg/L CaCl₂, 49.6 mg/L MgCl₂, 51.7 mg/L MgSO₄, 0.4X MEM non-essential amino acids, 0.2X MEM essential amino acids, 0.3X MEM vitamin solution, 2X hypoxanthine-thymidine supplement, 0.5% penicillin/streptomycin (all from Gibco, USA), and 0.1 nM α -MSH (Sigma Aldrich, USA), pH 7.5, at 25°C. The culture medium was replaced once a week and the cells were subcultured at confluence. In general, six to ten culture flasks were used in the experiments.

Assays monitoring *Opn4x* gene expression were carried out in the absence and presence of α -MSH, and with the reduction of bovine fetal serum from 10 to 2% for 1 to 8 days. The results of these tests (data not shown) demonstrated that removal of the hormone from the culture medium and/or reduction in serum led to an increase in *Opn4x* expression in the first days of DD. Nevertheless, 4 to 5 days after the removal of both components, mRNA levels stabilized. Based on these data, we chose to withdraw α -MSH from the culture medium prior to gene analysis.

Cell culture for total RNA extraction

In order to carry out experimental assays, the concentration of fetal bovine solution from cells maintained as described above was reduced to 2%, and the α -MSH

was withdrawn from the culture medium, which was then supplemented with 50 nM retinaldehyde. The cells were seeded onto 6-well dishes in duplicate (2×10^6 /well), and incubated for 9 days, either in a regime of DD or a photo-period of 14L:10D (light at 650 lux). On the 10th day, RNA was extracted every 3 h (0, 3, 6, 9, 12, 15, 18, and 21 Zeitgeber time, ZT) in such a way that 0 h of each experiment corresponded to the time the lights were turned on (9 am). All experiments were set up at the same time (3 pm). The same procedure was used for the analysis of all genes. During the periods when the cells were in darkness, medium changes and RNA extraction were performed under red light (Kodak GBX-2S Safelight filter).

Hormone assays

For hormone assays, the concentration of bovine fetal serum was reduced to 2% and α -MSH was withdrawn from the culture medium. The cells were seeded in duplicate onto 6-well dishes, and then incubated for 8 days in DD. The culture medium was aspirated on the 9th day, and the cells were washed twice with phosphate-buffered saline. The experimental culture medium containing 1.0 nM melatonin (Calbiochem, USA) and 1.0 mM stock solution in ethanol (Merck, USA) was added under red light. On the 10th day, RNA was extracted every 3 h as outlined above. All experiments were set up at the same time (3 pm). The same procedure was used for all gene analyses.

Extraction of total RNA, RT-PCR, and PCR

Total RNA was extracted with Trizol reagent (Invitrogen, USA) according to manufacturer instructions. The RNA pellet was resuspended in water containing diethylpyrocarbonate (DEPC; Ambion Inc., USA) and treated with DNase according to manufacturer instructions (turbo-DNA-free™, Ambion Inc.). RNA concentration was determined with a spectrophotometer (GeneQuant Pro, Biochrom Ltd., UK), and 1 μ g was used in the reverse transcriptase reaction (SuperScript III RT, Invitrogen).

Quantitative PCR was carried out with primer pairs specific for the genes (Table 1), designed with the Primer Express software (ABI, USA; synthesis by IDT, USA). Separate solutions containing the primers (300 nM for *Opn4x*, *Clock*, *Bmal1*, *Per1*, and *Per2*; 50 nM for RNA 18S), 2X iQ Sybr Green Supermix (100 mM KCl, 40 mM Tris-HCl, 1.6 mM dNTPS, 50 U/mL iTaq DNA polymerase, and 6 mM MgCl₂, BioRad Laboratories, USA) and autoclaved MilliQ water were prepared to a final volume of 23 μ L/well. Each solution was divided into Eppendorf tubes, each aliquot being sufficient for 2 wells and the cDNA of each sample being added to the corresponding tube. The solutions, already with cDNA, were then distributed onto 96-well slides in duplicate (23 μ L/well). Negative controls containing autoclaved MilliQ water instead of cDNA

were included in the assays. All assays were carried out in an iCycler (BioRad Laboratories) thermal cycler, under the following conditions: 1X 95°C for 7 min, 50X 95°C for 10 s, 60°C for 1 min, followed by the melting curve.

Data analysis

Data analysis was carried out using the $\Delta\Delta C_T$ method. The values were obtained by tracing the so-called threshold line, which intercepts the log phase of the amplification curves. By knowing the number of cycles where this line passes (C_T), ΔC_T was calculated as the difference between C_T for each gene of interest and for 18S RNA. Next, the values for each well were subtracted from the average of wells where expression was lower (calibrator) in DD, thereby obtaining $\Delta\Delta C_T$. This value was placed as the negative power in base 2 ($2^{-\Delta\Delta C_T}$), thus obtaining the number of times that the gene was expressed at the various time points, as compared to the minimal expression. The log values were averaged and expressed as percentage of the minimal value in DD.

Means \pm SEM of 6 to 10 cultures were calculated. Eventual temporal differences in each experimental condition (DD, 14L:10D, or DD in the presence of melatonin) were analyzed by ANOVA followed by the Tukey test. The various treatments were compared by two-way ANOVA followed by the Bonferroni test. Differences were considered significant when $P \leq 0.05$.

Results

Under DD, significantly high values of the expression of *Opn4x* were observed at 0 and 12 h, with a gradual and significant reduction between 3-9 and 15-21 h. In 14L:10D, there was a statistically significant increase in *Opn4* mRNA values at 6 h. Compared to DD, mRNA levels were remarkably lower under 14L:10D conditions (Figure 1). When cells were subjected to DD and treated with 1.0 nM melatonin

Table 1. Specific real-time PCR primers for *Xenopus laevis* genes.

Selectivity	Primer	Sequence
18S	Forward	5'-CGGCTACCACATCCAAGGAA-3'
	Backward	5'-GCTGGAATTACCGCGGCT-3'
<i>Opn4x</i>	Forward	5'-TCTTGAATTAAGGAGGAAAAGTG-3'
	Backward	5'-TGTCGCTGCTGATTATGTGA-3'
<i>Clock</i>	Forward	5'-TCCCCTTTCCCTGTGCAA-3'
	Backward	5'-GTGTCAGTCCTGTGATGGGATAAC-3'
<i>Per1</i>	Forward	5'-TGAAGGCCCTTAAAGAGCTAAAGA-3'
	Backward	5'-TTGCCAGTGTGCCAGACTTG-3'
<i>Per2</i>	Forward	5'-AAGGAAGTGGGAGAGACTCATACT-3'
	Backward	5'-AAACACAGTCTGTCACTTCCGTTACT-3'
<i>Bmal1</i>	Forward	5'-TCCCACGTGCAACGCC-3'
	Backward	5'-CGGGCCATGCGCAG-3'

for 1 h, 24 h before starting mRNA extraction, significantly lower values in mRNA relative expression were noted at 12 h. Compared to DD, there was a noticeable flattening of the expression with hormone treatment (Figure 1).

In DD, significantly high values of *Clock* expression were observed at 0 and 12-15 h. During the 14L:10D photoperiod, there was a statistically significant increase in mRNA values at 12 h. Compared to DD, it could be noted that there was an increase in mRNA values at 12 h under 14L:10D conditions (Figure 2). When cells were subjected to DD and treated with 1.0 nM melatonin for 1 h, 24 h before

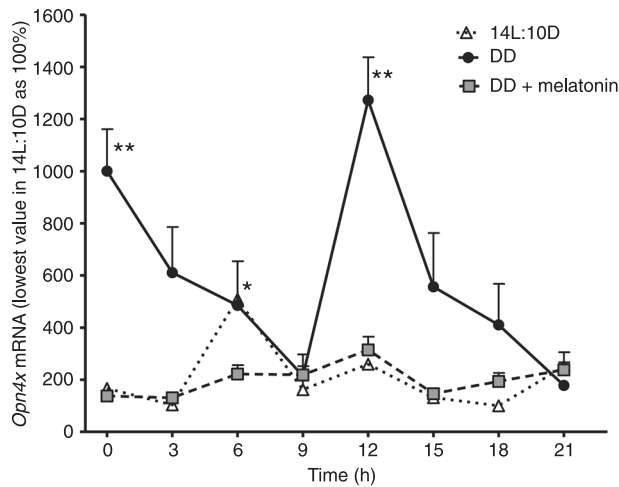


Figure 1. Relative *Opn4x* expression under a 14-h light:10-h dark (14L:10D) regime, constant darkness (DD) or DD plus 1.0 nM melatonin. Each point is the mean ± SEM for 6-10 cultures. * and **P < 0.05 for time course differences in 14L:10D and DD, respectively (ANOVA followed by the Tukey test).

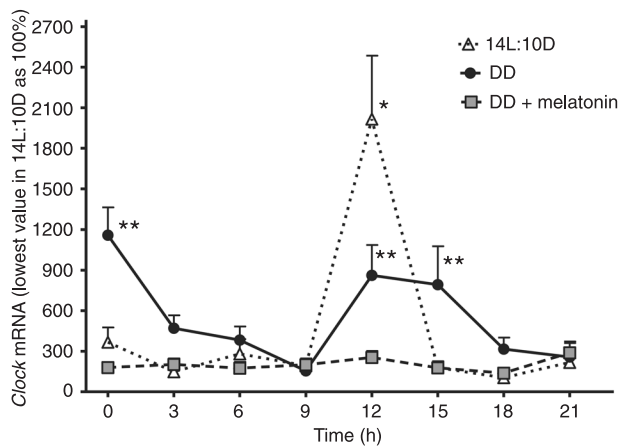


Figure 2. Relative *Clock* expression under a 14-h light:10-h dark (14L:10D) regime, constant darkness (DD) or DD plus 1.0 nM melatonin. Each point is the mean ± SEM for 6-10 cultures. * and **P < 0.05 for time course differences in 14L:10D and DD, respectively (ANOVA followed by the Tukey test).

starting mRNA extraction, the mRNA values were reduced compared to DD (Figure 2).

In DD, *Bmal1* expression was low and constant, except at 9 and 18 h, where a small but significant increase was observed. Under 14L:10D conditions, there was a statistically significant decrease in *Bmal1* values between 3-9 h, as compared to the other times. Comparing 14L:10D to DD, it can be noted that, as a rule, the values of *Bmal1* mRNA were elevated during the dark phase of the L:D cycle (Figure 3). When the cells were subjected to DD, and treated with 1.0 nM melatonin for 1 h, 24 h prior to starting mRNA extraction, no change in the time course of mRNA relative values could be observed. When compared to DD, one can note that hormone treatment did not change these levels (Figure 3).

In DD, similar to *Bmal1*, *Per1* expression was also low and constant. Under the 14L:10D photoperiod, there was a statistically significant increase in the time course of mRNA values during the light phase of the cycle. When the DD condition was compared to the 14L:10D cycle, mRNA values were significantly higher between 3 and 12 h (Figure 4). When the cells remained in DD and were treated with 1.0 nM melatonin for 1 h, 24 h before starting mRNA extraction, there was a significant increase in *Per1* mRNA values between 0 and 3 h in hormone-treated cells (Figure 4).

In DD, significantly high values of *Per2* expression were observed at 3 and 12 h. Under the 14L:10D cycle, *Per2* mRNA was low and constant between 6 and 12 h, with a gradual increase towards 12 h. When DD was compared to the 14L:10D photoperiod, significantly lower mRNA values were observed at 3 and 21 h in the L:D cycle (Figure 5). When the cells in DD were treated with 1.0 nM melatonin for 1 h, 24 h before starting mRNA extraction, significantly

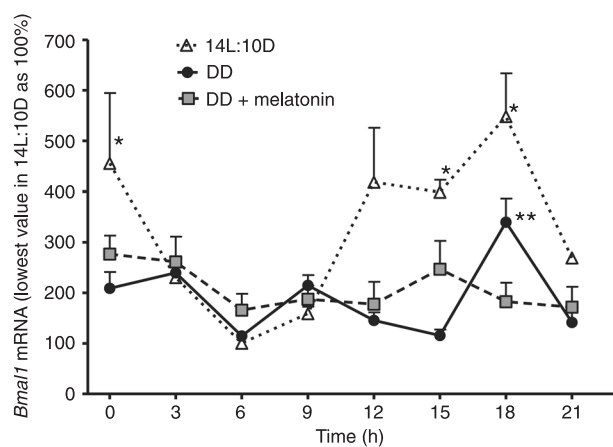


Figure 3. Relative *Bmal1* expression under a 14-h light:10-h dark (14L:10D) regime, constant darkness (DD) or DD plus 1.0 nM melatonin. Each point is the mean ± SEM for 6-10 cultures. * and **P < 0.05 for time course differences in 14L:10D and DD, respectively (ANOVA followed by the Tukey test).

high values of mRNA were observed at 12 and 21 h. Compared to DD, melatonin did not affect *Per2* levels, with the exception of the 3-h point, where the values were lower in the presence of the hormone (Figure 5).

Discussion

Biological clocks have been identified in various tissues in the most diverse species. However, the clocks in the various types of cells may present different operational characteristics, and constitute cell and/or self-sufficient molecular oscillators adjusted by environmental cycles, especially light-dark. In *Drosophila*, for example, in which circadian clocks were encountered in all the tissues studied, the main pacemaker maintains oscillations for a long time even under conditions of constant darkness, with little or no rhythm damping. Nevertheless, the differences between the central pacemaker and the machinery of the clock itself, and in the remaining vertebrate and invertebrate systems, have not yet been entirely clarified (34).

The dermal melanophores of *X. laevis* respond to light by dispersing melanosomes, a characteristic of embryonic melanophores of this amphibian (33). The ability of amphibian melanophores to react directly to light, although frequent, is not always present. Two primary chromatic responses are perceived, the most common being the dispersion of pigment granules throughout the cytoplasm in light-exposed melanophores (33).

In previous studies, it was observed that *Opn4*, the already cited photopigment discovered in *X. laevis* dermal melanophores, is expressed in the retina of all classes of vertebrates examined to date from teleosts to mammals, including humans (14,18). In the present study, we were able to confirm the function of photopigments, whose presence was confirmed by the Western blot technique (data not shown), in a cell-line that had been preserved in our laboratory for about 5 years. It was also possible to suggest that the melanophores of this amphibian respond to light in a way similar to that described for other cell types (17).

Besides *Opn4*, the embryonic dermal melanophores of *X. laevis* also express the clock genes *Clock*, *Per1*, *Per2*, and *Bmal1*. According to data from the present study on this cell line, when the cells were exposed to a light:dark cycle, *Per1*, *Per2*, and *Bmal1* genes showed an expression pattern apparently dependent on time of day. However, in constant darkness there is no variation of time-dependent expression, except maybe for *Bmal1*. Carr and Whitmore (17) observed that in DD each cell of a population continues to oscillate, but with fluctuations in free-running period, and these oscillations are not in phase. As we collect data of cell populations, the expression of clock genes may also be attenuated due to anti-phase oscillations of individual cells.

In our experiments, ZT 0 (0 h) corresponded to the time when the light was switched on (9 am). Furthermore, all

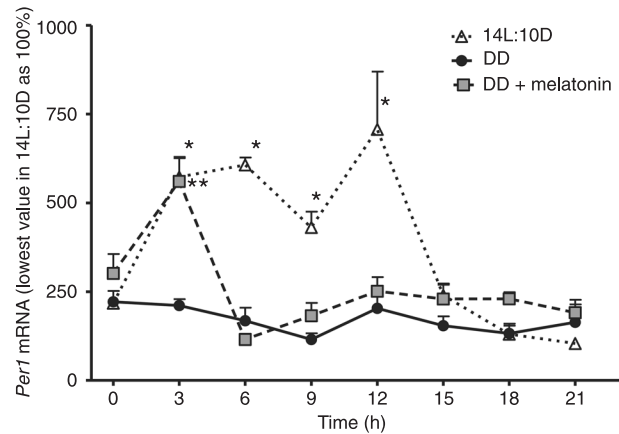


Figure 4. Relative *Per1* expression under a 14-h light:10-h dark (14L:10D) regime, constant darkness (DD) or DD plus 1.0 nM melatonin. Each point is the mean \pm SEM for 6-10 cultures. * and **P < 0.05 for time course differences in 14L:10D and DD + melatonin, respectively (ANOVA followed by the Tukey test).

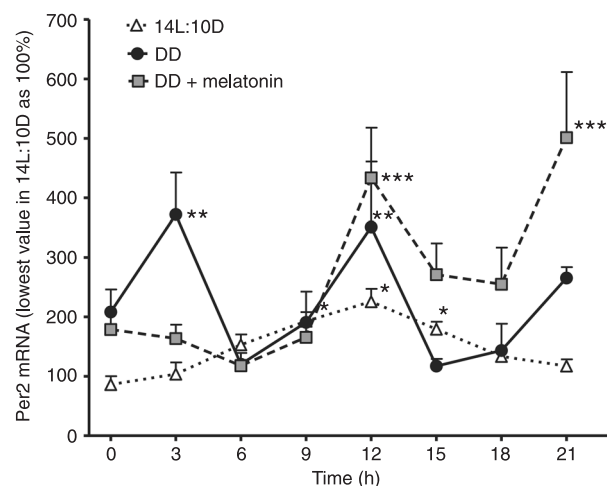


Figure 5. Relative *Per2* expression under a 14-h light:10-h dark (14L:10D) regime, constant darkness (DD) or DD plus 1.0 nM melatonin. Each point is the mean \pm SEM for 6-10 cultures. *, ** and ***P < 0.05 for time course differences in 14L:10D, DD and DD + melatonin, respectively (ANOVA followed by the Tukey test).

experiments were set up at the same time of day (3 pm), the culture medium being changed simultaneously when necessary. This procedure was required since Yoshikawa et al. (35) showed that the rhythmicity of cell cultures, obtained from organs containing central or peripheral circadian oscillators, are influenced by the time when the cultures were obtained and the medium was changed.

In the mammalian circadian system, *Per1* and *Per2* gene expression is light-entrained (9); in *Xenopus* retina, under a 12L:12D regime, *Per2* rhythm is light-entrained

with lower expression just before the beginning of the light phase, and peaking during the day. On the other hand, in this amphibian, *Per1* retina expression is rhythmic under light-dark and DD conditions (36). In *X. laevis* melanophores under a 14L:10D photoperiod regime, there is an increase in *Per2* expression during the light period, similar to what occurs in the retina. Furthermore, *Per1* expression is higher in the light phase, thereby implying that its expression is dependent on the time of day, similar to what is observed in mammals.

As to the *Bmal1* gene, results indicate that, in a 14L:10D photoperiod regime, there is a decrease during the 3- to 9-h period, contrary to what is observed for *Per1* and, to a lesser extent, for *Per2*, in the light phase. Furthermore, there is a visible increase in expression during the 12- to 15-h period (end of the light and beginning of the dark phases), which is maintained at a high level until 18 h. Overall, these results are the opposite of those observed in *Per1* and *Per2* expression.

Similar results were observed by Durgan et al. (37) in a mammalian model (rat cardiomyocytes) in which they analyzed oscillation patterns over a period of 24 h, with sample collection every 3 h, using the same protocol as ours. Under a 12L:12D photoperiod, the authors reported a decrease in *Bmal1* expression in the light phase and an increase in the dark, the opposite occurring with *Per2* expression. Farhat et al. (27) showed that under the 12L:12D condition, there was robust daily expression of *Per1* and *Cry1b* clock genes, whereas *Opn4x* and *Clock* expression seemed to vary in an ultradian pattern. For both *Per1* and *Cry1b* genes, expression was higher during the light phase, whereas for the *Clock* gene there was an increase in expression coincident with the dark phase during the subjective night.

Generally speaking, a circadian cycle begins in the early morning hours with activation of *Per* and *Cry* transcription by CLOCK/BMAL1. Furthermore, according to Albrecht and Eichele (38), transcription levels reach their apex around midday and, in fact, our data indicate an increase in expression over the 12-15 ZT period for both *Per* genes,

this corresponding to the end of the light phase and the beginning of the dark one. *Bmal1* expression remains high throughout the dark phase. According to the proposed model for mammals, this high expression would facilitate synthesis of the BMAL1 protein, and the consequent dimerization with CLOCK, thereby leading to an increase in *Per* expression. Our data with these transcripts corroborate this model (6).

Melatonin, the hormone secreted by the pineal gland, is directly related to biological clock machinery. In all species, it is secreted at night and is associated with sleep, the maintenance of body temperature, immunological response and other nocturnal physiological events. There is evidence that the exogenous administration of melatonin can adjust these rhythms to both endogenous cortisol secretion and to that of melatonin itself (39). Under certain circumstances, melatonin can synchronize the circadian rhythm (24 h) in free-running individuals, i.e., it is able to rescue the endogenous period of their internal biological clocks (40). In *X. laevis* dermal melanophores, our data indicate that melatonin, after a pulse of 1 h, practically does not affect melanopsin or clock gene expression, but, in general, causes a flattening of the temporal curve.

The results obtained here for *X. laevis* dermal melanophores, although less complex than the mammalian system, may contribute to a better understanding of the inherent molecular mechanisms present in most organisms, which could be confirmed by investigation from a phylogenetic perspective.

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