Melatonin induces Cry1 expression in the pars tuberalis of the rat

Hugues Dardente*, Jérôme S. Menet, Vincent-Joseph Poirel, Dominique Streicher, François Gauer, Berthe Vivien-Roels, Paul Klosen, Paul Pévet, Mireille Masson-Pévet

Neurobiologie des Rhythmes, UMR 7518 CNRS/ULP, IFR 37, 12, rue de l’université, 67000 Strasbourg, France

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Abstract

In mammals, interacting transcriptional/post-translational feedback loops involving ‘clock genes’ and their protein products control circadian organisation. These genes are not only expressed in the master circadian clock of the suprachiasmatic nuclei (SCN) but also in many peripheral tissues where they exhibit similar but not identical dynamic to that seen in the SCN. Among these peripheral tissues, the pars tuberalis (PT) of the pituitary expresses clock genes. We show here that the PT of the rat, like that of other rodents, rhythmically expresses Per1. We also report rhythmic expression of another clock gene, Cry1. The peak of Cry1 mRNA expression occurs during the night concomitantly with rising blood plasma melatonin concentrations. Using an acute injection paradigm, we demonstrate that Cry1 expression is directly induced by melatonin in the PT. Melatonin injection at the end of the subjective day also affects Per1 expression, leading to diminished mRNA levels. These data support the existence of a time-measurement model in the PT based on direct opposite actions of melatonin on Per1 and Cry1 expression.

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1. Introduction

Melatonin is secreted during the night by the pineal gland and provides the organism with both a daily and a seasonal message. High levels of high affinity melatonin receptors are expressed in the pars tuberalis (PT) of mammals pituitary (for review, see Ref. [32]). The PT plays an important role in the mediation of the seasonal message carried by melatonin [13,19,28]. The melatonin rhythm depends on the presence of the suprachiasmatic nuclei (SCN; Ref. [21] and references therein) which are the site of the master circadian pacemaker. The outlines of the molecular clock mechanism have become clearer with the identification of numerous putative clock genes and their protein products as well as characterization of their complex and intricate relationships (reviewed in Ref. [25]). Unexpectedly, clock genes have been found not only in the SCN but also in many other brain regions, peripheral tissues and isolated cells, apparently interacting through a common model of transcriptional/posttranslational feedback loops [1,3,29]. In this study, we analyzed whether Per1 and another clock gene, Cry1, whose proteins are known to interact in the molecular clock [8,10,12], are expressed in a circadian manner in the PT of the rat as recently shown in the sheep [14]. In rodents, only data concerning Per1 are available. Per1 is rhythmically expressed in the PT of the mouse, the golden and the Siberian hamsters [16,17,26]. Experiments with pinealectomized hamsters, as well as melatonin injections, suggest that the expression of Per1 in the PT is controlled by melatonin, unlike what is observed in the suprachiasmatic nuclei [17,18,23,30]. A time-measurement model, based on different phase relationships of the Per and Cry mRNA peaks under long and short photoperiods, was recently proposed in sheep [14].

To determine whether Cry1 and Per1 are directly regulated by melatonin in the PT of the rat, and clarify whether Cry1 expression is directly linked to melatonin
onset, we applied a single acute melatonin injection either at the end of the subjective day (CT11.5) or during the subjective day (CT6). Altogether, the results provided here clearly demonstrate that melatonin directly and transiently induces Cry1 and give further support to the existence of a time-measurement mechanism that would be driven by melatonin through direct opposite actions on Per1 and Cry1.

2. Material and methods

Adult male Wistar rats (200–250 g, Dépré, St Doulchard, France) were given food and water ad libitum. They were kept on a 12-h light, 12-h dark (LD) cycle with lights on (200 lx) at 8:00 a.m. with a constant dim red light. Room temperature was maintained at 20±1 °C. The experiments were conducted under constant darkness (DD) rather than under LD cycle, as described below, to avoid any possible effect of light (i.e. masking).

We studied the effect of an acute melatonin injection on the expression of Per1 and Cry1 in the PT of the rat at two different circadian times: CT11.5 (Experiment 1) and CT6 (Experiment 2).

2.1. Experiment 1

After 2 weeks of adaptation, two groups of animals were transferred into DD for 2 days. At the end of the second subjective day (CT11.5), one group of rats was subcutaneously injected with melatonin (1 mg/kg). Melatonin (Sigma, France) was dissolved in ethanol and diluted with 0.9% saline to make a 5% ethanol in saline solution. Vehicle-treated rats received ethanolic saline vehicle only. The animals (n=4 for each time point and group) were sacrificed by decapitation every 30 min from CT12 to CT13.5 and then every 2 h from CT15 until CT11 the subsequent subjective day.

2.2. Experiment 2

After 2 weeks of adaptation, two groups of animals were transferred into DD for 2 days. During the subjective day (CT6), one group was subcutaneously injected with melatonin (1 mg/kg) and the other was injected with vehicle exactly as described for Experiment 1. Rats (n=5 for each circadian time and group) were then decapitated at CT6.5, CT7.5, CT8.5, CT12.5 and CT19. Trunk blood was collected in heparinized tubes and plasma melatonin concentrations were measured in duplicate by radioimmunoassay (RIA) using 2[125I]melatonin and a rabbit antiserum (R19540, INRA Nouzilly, France) at a final dilution of 1:250 000. The minimum detection levels for the assay were between 5 and 10 pg/ml plasma. Validation of the RIA assay has been reported previously [15].

For both experiments, brains were dissected out and frozen in cold isopentane. Serial coronal sections (20 μm) were cut in a cryostat and thaw-mounted onto gelatin-coated slides. Sections were kept at −80 °C until processed for in situ hybridization. All procedures used in animal experimentation complied with the French law, implementing the European Communities Council Directive 86/609/EEC.

Rat Per1 and mouse Cry1 clones were kindly donated by Professor H. Okamura (Department of Anatomy and Brain Science, Kobe University School of Medicine, Japan). Antisense and sense probes were transcribed from the corresponding linearized plasmids using the appropriate polymerase in the presence of α[35S]UTP (1250 Ci/mmol, NEN-Dupont, Zaventem, Belgium) according to the manufacturer’s protocol (MAXIscript, Ambion, USA). Hybridization was carried out as described previously [6]. Briefly, sections were postfixed in 4% formaldehyde for 10 min, acetylated twice in 0.5% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, incubated in 0.1 M Tris (pH 7.0) with 0.1 M glycine for 30 min, rinsed and dehydrated in a graded ethanol series (70, 90, 95 and 100%, 1 min each), and finally, air-dried. Hybridization was carried out with either sense or antisense radiolabeled probe (450 pM for each probe) in a solution containing 50% deionized formamide, 10% dextran sulfate, 50 mM dithiothreitol, 1× Denhardt’s solution, 2× SSC, 1 mg/ml salmon sperm DNA, 1 mg/ml yeast RNA, at 54 °C for 16 h. After hybridization, the sections were rinsed twice for 10 min in 2× SSC before being treated with Ribonuclease A for 30 min at 37 °C (Sigma, 2 μg/ml). The slides were then rinsed twice and stringency washes were carried out (15 min in 0.5× SSC at 53 °C and then 30 min in 0.2× SSC at 62 °C). Finally, the sections were dehydrated in a graded ethanol series (70, 90, 95 and 100%, 1 min each) and air-dried. The slides were exposed to an autoradiographic film (BioMax, Kodak, France) for 5–15 days. Quantitative analysis of the autoradiograms was carried out using a computerized analysis system (Biocom-program RAG 200). Three independent measures in one PT were taken together with a measure in the subparaventricular zone where no specific signal is expected. Specific signal was then defined as the value resulting from subtraction of the latter measure from the measure in the PT. This was repeated on three sections per animal, thereby leading to nine measures per animal that finally gave an animal mean. Data are depicted as relative optical density (OD) mean±S.E.M. No specific signal was observed when slides were hybridized with corresponding sense probes (data not shown). Differences between the melatonin-treated (melatonin) and the vehicle-treated groups (saline) were assessed by two-way ANOVA and post-hoc Tukey test.
3. Results

3.1. Experiment 1, melatonin injection at day/night transition (CT11.5)

Data are shown in Fig. 1 and representative autoradiograms at specific time points are presented in Fig. 2. In the control group, *Per1* mRNA exhibits peak levels at CT1 (time effect \( P < 0.001 \)). A similar time effect was also observed in the melatonin-treated group. No interaction between group and time was observed, excluding further pairwise comparisons. A trend toward lower levels could however be noticed in the melatonin-treated group (peak level at CT1 cut by roughly 50%).

There is a significant effect of time (\( P < 0.001 \)) on *Cry1* mRNA in the control group with a peak at CT19. Melatonin greatly affects the expression of *Cry1* (time effect \( P < 0.001 \)). Indeed, the hormone induces a strong, rapid and transient expression of *Cry1* mRNA (two-way ANOVA interaction group \( \times \) time, \( P < 0.001 \)). The peak of *Cry1* mRNA is maximal 2 h after the injection and basal levels are reached again 6 h later.

3.2. Experiment 2, melatonin injection during the subjective day (CT6)

Data are shown in Fig. 3. Melatonin assay in the plasma of both groups (Fig. 3A) revealed that: (1) its concentration is highest at CT19 in the control group (around 90 pg/ml, time effect, \( P < 0.001 \)) and (2) it reaches very high values (around 50 ng/ml) half an hour after injection and thereafter stays at higher levels than in the controls.

Fig. 2. Representative autoradiograms of sections hybridized for *Per1* and *Cry1*. Panel A shows the amplitude of *Per1* rhythm (with low levels at CT17 and high levels at CT1) in the saline-treated group. Panel B illustrates the effect of melatonin injection on the expression of *Cry1* at CT13.5. Scale bar=1 mm.
Expression of Per1 mRNA in the controls was found to be very low at all time points consistent with experiment 1. Melatonin treatment had no effect on Per1 mRNA expression (data not shown).

Fig. 3B depicts mRNA levels for Cry1 at the different time-points examined. In the saline-treated group, Cry1 mRNA levels were higher at CT19 than any other time point (time effect, \( P < 0.001 \)). Melatonin injection at CT6 greatly affected Cry1 expression (two-way ANOVA, interaction group×time, \( P < 0.001 \)). Melatonin injection advanced the peak of Cry1 to CT8.5 (time effect, \( P < 0.001 \)) with values at this point being similar to those seen in the saline group at CT19. Cry1 levels then declined, so that by CT12.5 they were back to basal values. Pairwise comparisons gave highly significant differences between saline and melatonin-treated groups only at CT8.5 and CT19 (\( P < 0.001 \)).

4. Discussion

In this study, we report that two components of the molecular clock (for review, see Ref. [25]), namely Per1 and Cry1, are rhythmically expressed in the PT of the rat. Furthermore, other components of the clock, like Timeless [31], Clock and Per2 (personal observation) are also expressed in the PT of rodents, raising the possibility that the PT of rodents might contain a complete set of clock genes, as demonstrated in the PT of sheep [14], as well as
in numerous other peripheral tissues and isolated cells [3]. However, the clock gene expression in the PT differs from what is observed in other peripheral tissue as it may be driven by melatonin, as initially suggested by Sun et al. [26]. This hypothesis is supported by the following observations: Per1 is undetectable in melatonin-deficient strains of mice [26], pinealectomy in the golden hamster abolishes Per1 expression [18], a single melatonin injection before lights-on diminishes the peak level of Per1 [17] and finally, Per1 mRNA and protein levels are greatly diminished in animals exposed to short photoperiods [17,20] and absent in MT1 melatonin receptor knock-out mice [33].

Our observation that Cry1 is rapidly and very strongly induced by melatonin, together with the observation that in non-treated animals, a peak of expression occurs during the dark phase (i.e. at a time when melatonin is present in the bloodstream) suggested that melatonin may gate the expression of Cry1 in the PT. Barassin et al. [4] previously showed by microdialysis that the onset of melatonin secretion in the Dépré Wistar rat breeding colony used in the present study occurs late in the night (i.e. CT17–18), that is just before the peak of Cry1 mRNA observed in control animals of experiment 1. These data then suggested that the Cry1 endogenous peak of expression (observed at CT19) in the PT may be directly and acutely driven by melatonin. This hypothesis was also in agreement with data describing an advanced peak of Cry1 mRNA in sheep under short photoperiod compared to long photoperiod, coherent with the blood plasma melatonin patterns under both photoperiods [14].

However, melatonin injected at the end of the day is known to affect functioning of the clock [2,22]. Therefore, it could not be ruled out that the induction of Cry1 mRNA in the PT of rats injected with melatonin at CT11.5 results from an indirect action of the hormone on the SCN, leading to an advanced phase of the circadian clock and, consecutively, to an advanced phase in the PT. This alternative hypothesis was tested by melatonin injection at CT6, when it does not affect the circadian clock. Here too, we found that Cry1 was induced, with kinetics and amplitude roughly similar to those observed in the first experiment. This result, together with the facts that: (1) melatonin injected at CT11.5 had no effect on clock gene expression in the SCN [23] and (2) that melatonin did not affect clock gene expression in the Japanese quail SCN [30], strongly argue against the possibility that Cry1 induction in the PT may occur indirectly, through changes induced by the hormone at the level of the SCN. Plasma melatonin concentration assessed by radioimmunoassay (Fig. 3A) together with data obtained by Barassin et al. [4] demonstrate that, in this rat strain, the nocturnal rise in plasma melatonin levels is delayed until the later part of the night. Cry1 mRNA levels in the saline-treated groups of both experiments also peak by the second half of the subjective night (CT19). Thus, these data strongly support a model in which melatonin may be directly responsible for the expression of Cry1 in the PT, in vivo. The length of the Cry1 peak, which is shorter than the elevated levels of melatonin both in controls [4] and in melatonin-injected animals (Fig. 3), suggests that the mechanism leading to this gene induction is rapidly desensitized. The absence of the endogenous peak of Cry1 in the melatonin-treated group of experiment1 (Fig. 1) supports this conclusion. We therefore propose that Cry1 may act as a melatonin onset sensor rather than a marker for the duration of the melatonin signal.

The molecular events leading to the induction of Cry1 are unknown but may involve the transcription factor Rev-erb α (also known as NR1D1). REV-ERB α has been recently implicated as a repressor of Bmal1 expression in the molecular clock [24,27], thereby playing a role similar to that described for VRILLE in the Drosophila molecular circadian clock [5,9]. Additionally, Etchegaray et al. [7] demonstrated that REV-ERB α might be responsible for the delayed peak of Cry1 expression when compared to Per1 and Per2. Thus, Rev-erb α and other related orphan nuclear receptors such as RORβ [11] are good candidates for future studies investigating Cry1 induction in the PT.

As demonstrated in other rodent species, Per1 mRNA in the PT of the rat peaks early in the day, when blood plasma melatonin levels are back to low levels. The acute injection of melatonin at the end of the subjective day has the tendency to diminish Per1 peak level by roughly 50% but, importantly, has no effect on the phase of its rhythm.

Thus, on one hand, Cry1 expression appears to be anchored to the onset of melatonin secretion (thereby acting as a melatonin onset sensor) whereas, Per1 expression appears to be linked to the offset of melatonin secretion. This dual effect of melatonin, together with its photoperiod-dependent pattern in plasma levels may provide the basis of a time-measurement mechanism in the PT of rodents. A modified phase relationship between Per1 and Cry1 peaks may lead to perturbations in the occurrence of PER/CRY dimers [8,10,12] thereby modifying their nuclear translocation and repression of the transcriptional activity of CLOCK/BMAL1 dimer as already suggested in sheep by Lincoln and co-workers [14]. This would eventually lead to different seasonal output signals from the PT. This model may help to understand how the PT is involved in the seasonal control of prolactin secretion by the pars distalis [13,19]. The validation of such a model will, however, require further experiments.

In conclusion, we report that acute melatonin injection either at the end or in the middle of the subjective day causes a premature advance in the induction of Cry1 expression in the PT of the rat. This effect occurs without any change in the phase of the peak of Per1. We propose that Cry1 and Per1 may be important markers of photoperiod length in the PT and that opposite roles of melatonin on their transcription may constitute the basis of a time-measurement model.
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