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Rhythms of Blood Melatonin in Individual Japanese Quail (*Coturnix coturnix japonica*)

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Abstract

We developed a micromodification of the melatonin radioimmunoassay (RIA), reducing sample volumes to 50 μl, and applied it to determine plasma melatonin rhythms in the same population of small animals such as Japanese quail. Plasma samples were measured without any extraction. The validity of the assay was checked by classical techniques, including parallelism, accuracy, sensitivity, precision and reproducibility. The least detectable dose of melatonin of the RIA method was 0.5 to 1 pg/tube. The intra- and inter-assay coefficients of variation for 27 pg melatonin in 50 μl plasma were 4.5 and 4.6%, respectively. Plasma melatonin levels in subjects exposed to 16 hr light and 8 hr darkness (16 L: 8 D) and 8 L: 16 D showed clear diurnal rhythms with high levels occurring in the night-time and low levels during the daytime. However, peak levels and corresponding times were different between the two groups.

Key Words

Melatonin, Daily rhythms, Radioimmunoassay, Photoperiod

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Introduction

Daylength serves as a critical environmental cue for vertebrates in the temperate zone, and restricts their reproduction to a particular period of the year. Most of them appear to measure the daylength using their circadian clocks. It is well established that the major source of melatonin is the pineal gland. The pineal melatonin rhythm, controlled by the circadian oscillator, is reflected accurately in blood, urine and cerebrospinal fluid, with high levels occurring at night. Thus melatonin is considered to be a time-keeping hormone in vertebrates. Melatonin levels in the blood and the pineal gland have been quantified by bioassay[1,2], gas chromatography[3], HPLC[4] and radioimmunoassay, RIA[5,6]. At present, RIA procedures are useful and are easily conducted for the measurement of circulating melatonin levels, however, they have required 500 μl to 1 ml of plasma to detect it[7~9]. These previous techniques, for each individual, do not enable us to examine the phase relationship between melatonin levels and given circadian rhythms such as locomotor activity, because collecting serial samples of blood from small animals is almost impossible.

Thus the present study focused on 1) the improvement of a melatonin RIA that would measure it in a small sample and 2) the application of the RIA to elucidate melatonin changes in the same individual blood of quail under conditions of short days or long days. A portion of this work has been presented in an abstract in the Proceedings of the 10 th Annual Meeting of the Japan Society for Comparative Endocrinology[10].

Materials and Methods

Animals and treatments

Three week-old male Japanese quail (*Coturnix coturnix japonica*) were obtained from a commercial source (Suzukei Company, Toyohashi). They were kept under a short day length of 8 hr light and 16 hr darkness (8 L : 16 D) for 3 weeks before each experiment. Batches of quail were further exposed to one of the following schedules for 1 week ; 16 L : 8 D (N = 5) or 8 L : 16 D (N = 5). All blood samples were obtained at 7 weeks of age. About 100 μl of blood were collected serially at 2 hr intervals from the brachial vein over the next 24 hr with heparinized tubes. During the dark phase, blood collection was made under a dim red light from a bird whose head covered with a light-tight hood. Plasma was separated by centrifugation and stored at −20°C until assay for melatonin.

Melatonin radioimmunoassay

Melatonin concentrations in the plasma were measured directly in 25 to 50 μl samples without any extraction procedures by a double-antibody RIA. Samples, quality controls or standards (0.8 pg to 102 pg) in 50 μl were pipetted into MILLI-3 polypropylene tubes (Lumac, Landgraaf, The Netherlands) and 50 μl of 1% BSA-PBS buffer (0.01 mol/l sodium phosphate, 0.14 mol/l NaCl, 1% NaN_3 and 1% bovine serum albumin, pH 7.5) were added. The rabbit antiserum (supplied by Professor Kawashima, Kyouritsu College of Pharmacy) to melatonin (50 μl ; final dilution 1 : 80000) in 1% NRS EDTA-PBS buffer (0.01 mol/l sodium phosphate, 0.14 mol/l NaCl, 0.05 mol/l EDTA, 1% NaN_3 and 1% normal rabbit serum, pH 7.5) was added into MILLI-3 tubes, mixed and incubated at 4°C overnight. The [^{3}H]-

melatonin (Amersham Japan, Tokyo) in 50 μl (ca. 2000 cpm) was then added, agitated, and incubated further at 4°C overnight. Bound and free melatonin were separated by incubation with anti-rabbit gamma globulins (supplied by Professor Wakabayashi, Gunma University) in EDTA-PBS buffer at 4°C overnight. Following centrifugation at 2000 g for 30 min, the supernatant was aspirated and the precipitate was dissolved in 0.1 N NaOH (100 μl) and mixed with 2 ml of Aqualuma Plus (Lumac) for liquid scintillation counting. The antiserum to melatonin was highly specific for melatonin precursors; cross-reactivity was less than 0.005% with tryptophan, 5-hydroxytryptophan and serotonin, almost 0.005% with N-acetylserotonin. Cross-reactivity with melatonin metabolites and related indoles was less than 0.01%, except for 2.6% with 6-hydroxymelatonin[6].

Results

Melatonin RIA

Parallelism was determined by comparing the displacement curve obtained with serial dilutions of plasma in the night-time with a buffer standard curve (**Fig. 1**). No statistically significant differences were obtained between the slopes of the serially diluted plasma and the relevant portions of melatonin standards. To assess the accuracy of this assay, melatonin (64, 256, 1024 pg/ml; Sigma Chemical Co., St. Louis, MO) was added to pooled plasma collected during the daytime (**Fig. 2**). The correlation coefficient between the amounts added and those assayed was 0.995 and the slope of the regression line was 0.91. Fifty per cent inhibition was produced upon addition of 24 pg melatonin per tube. The least detectable dose of

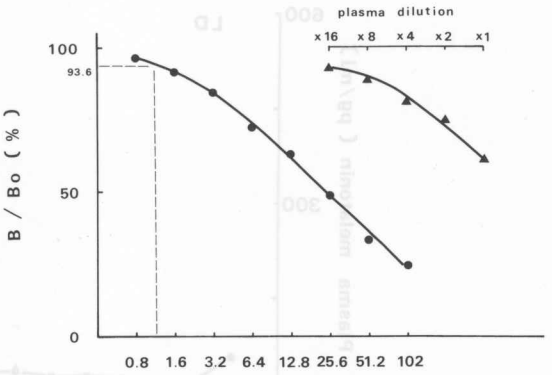

Fig. 1 Inhibition curves obtained with synthetic melatonin and plasma collected from quail during the night-time

Standards or plasma samples, antiserum and tracer solutions in each 50 μl sample were incubated at 4°C and the immunocomplex was precipitated by double antibody method (see details in the text).

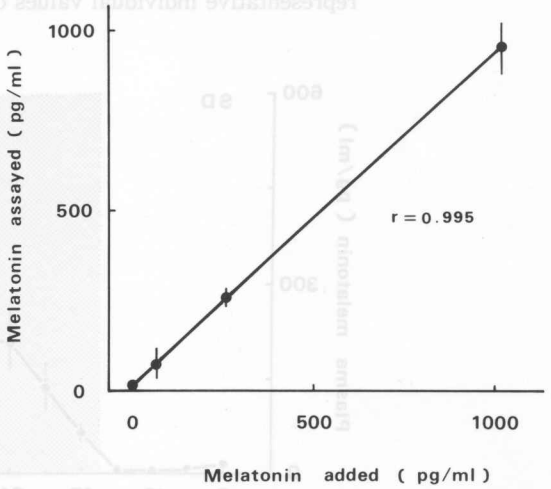

Fig. 2 Accuracy of the RIA method determined by recovery of melatonin added to plasma of quail collected during the daytime

Values represent means ± SEM (n=5).

melatonin (two standard deviations from buffer control tubes) was 0.5 to 1 pg/tube. The intra- and inter-assay coefficients of variation for pooled blood containing 27 pg in 50 μl were 4.5 and 4.6%, respectively. No differences of the displacement curves were noted among three

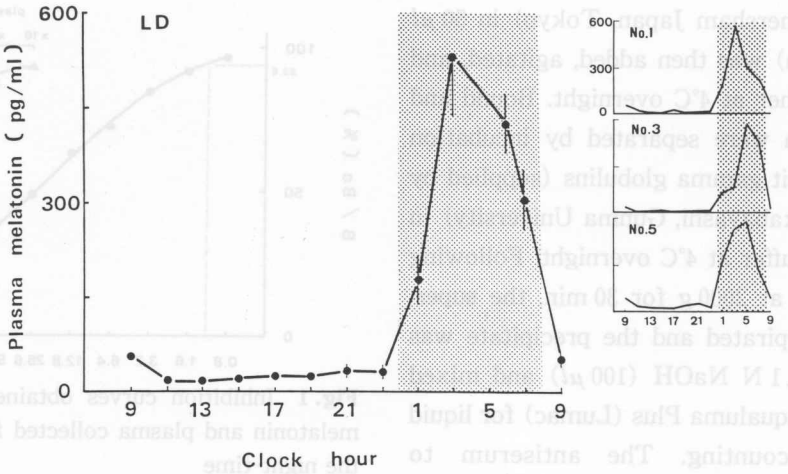

Fig. 3 The diurnal pattern of plasma melatonin in quail exposed to 16 L : 8 D

The points represent the means ± SEM. If no error bar is seen, the SEM is encompassed by the symbol representing the mean. The shaded areas in the figure express the dark period. Right small panels show representative individual values of plasma melatonin.

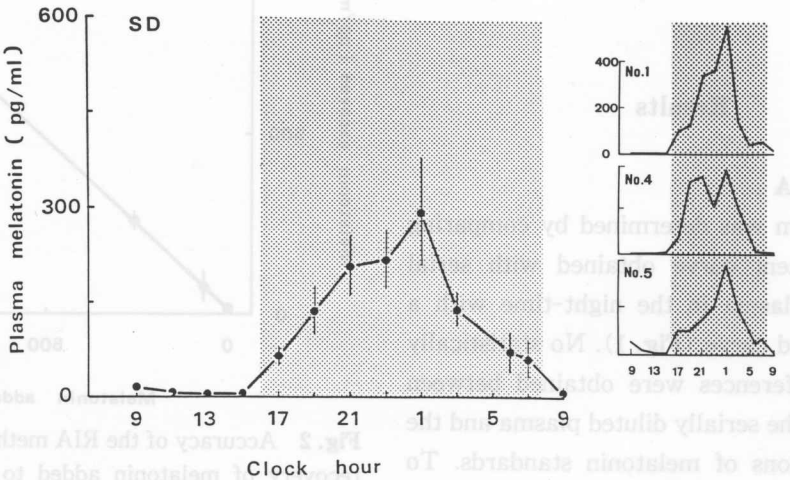

Fig. 4 The diurnal pattern of plasma melatonin in quail exposed to 8 L : 16 D

For details, see the legend of Figure 3 and the text.

different incubation procedures ; advanced, simultaneous and delayed additions of labeled antigen (data not shown).

Diurnal rhythms in plasma melatonin

Plasma melatonin concentrations in quail trained to 16 L : 8 D showed clear rhythms as shown in **Fig. 3**, and to 8 : 16 D in **Fig. 4**. The mean peak levels of melatonin (530 pg/m*l*) in the night-time under 16 L : 8 D appeared to be about 2 times higher than the levels seen under

8 L : 16 D. There was a more than 20 times maximum increase in the night-time blood melatonin levels than the lowest daytime levels in both LD cycle conditions. The peak time for plasma melatonin under 16 L : 8 D was 03.00 to 06.00 hr, and that of 8 L : 16 D was 21.00 to 01.00 hr. Their peaks appeared at about the middle of each dark period.

Discussion

Heretofore it has not been possible to estimate melatonin concentrations in serially collected blood samples from small animals, such as quail, for the study of circulating melatonin rhythms. In the present study we characterized the rhythms of blood melatonin in each individual quail using the techniques of both RIA micromodification and frequent blood samplings. Melatonin concentrations in the blood were found to be high in the night-time and low during the daytime in the same bird. The present results obtained from individual animals confirmed previous reports in changes of plasma melatonin concentrations, based on blood collections from different animal populations by decapitation[8].

Thus, the present study focused on the rhythms of blood melatonin under different photoperiods. Plasma melatonin concentrations in quail exposed 16 L : 8 D and 8 L : 16 D showed clear LD rhythms. The pineal body and its hormone, melatonin, have been implicated in mediating several light-cued physiological processes, including (1) the entrainment and organization of circadian rhythms and (2) the photoperiodic regulation of reproduction in several species. For circadian rhythms, pinealectomy has profound effects on the activity rhythms in some birds ranging from changes in free-running periods for starlings[11] to arrhythmicity for sparrows[12,13]. Daily melatonin injections can entrain the activity rhythms of starlings[14]. Silastic implants which continuously release melatonin can affect the period of the activity rhythms of sparrows[15]. However, it appears that pinealectomy has little or no effects upon the free-running activity rhythms of quail[16] and pigeon[17]. Thus the general concept that the pineal gland is the most important in circadian organization within the Aves is still far from being validated. These results, together with the observations that pinealectomized birds so far studied are still able to entrain to light and dark cycles[18~20] and that the origin of melatonin is not restricted solely to the pineal gland[7,21~23] suggest that the circadian rhythms are controlled by more than one oscillator. It is probable that they are normally coordinated by a hormonal output, possibly melatonin.

While some influences of the pineal gland on reproduction have been reported in quail[24], Indian weaver finch[25] and turkey[26], other attempts have met with no or little success[27,28]. As of this date, the role of melatonin in reproduction has not been clearly defined in birds. In our previous experiments in quail[29,30], quite a brief light pulse (30 min a day) triggers LH secretion and testicular growth when given 12.5~15 hr after dawn, but is totally ineffective or much less effective at other times of the night irrespective of the length of the main photoperiods. These results suggest the existence of a photoinducible phase for gonadotropin secretion which is based on daily rhythms. To examine whether melatonin in the blood is related to the initiation of gonadotropin secretion in Japanese quail, additional studies are necessary.

Acknowledgments

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抄　録

日本ウズラにおける血中メラトニン濃度の日内変化

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小動物の同一個体より連続的に血中メラトニン濃度を測定するには，RIA のマイクロ化が必須である。今回抽出操作を行わず，RIA を改良することにより，試料を 50 μl まで減じることに成功した。標準曲線と血漿試料との平行性，アッセイの感度や正確度，さらにアッセイ内およびアッセイ間変動を測定し，RIA のマイクロ化の有効性を検討した。16 時間照明 8 時間暗黒または 8 時間照明 16 時間暗黒下で飼育した日本ウズラを用い，同一個体より 2 時間毎に採血し，メラトニン濃度の変化を調べた。血中メラトニン濃度はどの個体も夜間に高く，昼間に低値を示し，明らかな日周変化を示した。また，両飼育群間においてメラトニン濃度のピーク時およびピークの値に明らかな差が認められた。

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