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DAILY VARIATIONS OF VARIOUS PARAMETERS OF SEROTONIN METABOLISM IN THE RAT BRAIN. II. CIRCADIAN VARIATIONS IN SERUM AND CEREBRAL TRYPTOPHAN LEVELS: LACK OF CORRELATION WITH 5-HT TURNOVER

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SUMMARY

Rats submitted to regular 12 h cycles of light and darkness for three weeks were sacrificed at various times of the day. 5-HT, 5-HIAA and tryptophan levels were estimated in the fronto-parietal cerebral cortex. Tyrosine and free and total tryptophan levels in serum were estimated in parallel. Significant circadian variations in 5-HT and 5-HIAA levels were found in cerebral tissues. The peaks of 5-HT and 5-HIAA levels were detected during the light and dark periods respectively, the maximal fluctuations being seen between 17.00 h and 21.00 h, two times separating the light off. Important significant circadian variations in free and total serum tryptophan levels were also observed. In both cases, the maximal levels were found during the middle of the dark phase after the peak of 5-HIAA levels. The circadian rhythm of tyrosine levels in serum was in opposite phase with that of tryptophan (free or total). The diurnal changes in tryptophan content in cerebral tissues seemed thus related to those found in serum. Taking in consideration results obtained in previous studies[16,17] carried out in similar experimental conditions, it was concluded that the parallel increase in serum free tryptophan and in tissues 5-HIAA levels seen during the night were not related to a stimulation of 5-HT turnover. Indeed 5-HT synthesis is minimal at this time[16].

INTRODUCTION

During the past few years, we have studied the diurnal variations of serotonin

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(5-HT) metabolism in various structures of the rat brain[16,17]. We were hoping to understand some of the regulatory processes involved in the control of 5-HT synthesis and release in physiological states. Fluctuations in 5-HT metabolism were thus analyzed during two daily successive 6 h periods of light (13.00–19.00 h) and darkness (19.00–01.00 h) in animals housed for at least three weeks in a controlled environment. Thanks to in vivo as well as in vitro studies 5-HT synthesis was shown to be stimulated during the light period; this was associated with the presence of high levels of 5-HT in the brain. The stimulation of [^{3}H]5-HT synthesis was related to an increased initial uptake of [^{3}H]tryptophan in brain slices[18]. By contrast, the transport of [^{3}H]tyrosine, the precursor of catecholamines, was minimal at this time[18]. Curiously, the release and the rate of utilization of 5-HT were accelerated during the dark period when the transmitter synthesis was reduced. The levels of 5-HIAA and the ratio of 5-HIAA levels over those of 5-HT were particularly elevated during the night. These effects precisely studied in the brain stem and the hypothalamus were also detected in other structures such as the cerebral cortex[16].

In various circumstances, the synthesis of central 5-HT depends on the fluctuations of peripheral tryptophan metabolism[25]. Thus, the rise in total[34] or free tryptophan[3,33] levels in serum induced by pharmacological treatments, or stressful situations[20] were associated with parallel changes in the transmitter synthesis. It was thus of interest to examine if the diurnal variations of 5-HT metabolism were related in some ways to those of total or free tryptophan levels in serum. The diurnal fluctuations of these parameters were thus compared with those of tryptophan, 5-HT and 5-HIAA levels in the fronto-parietal cerebral cortex where the circadian rhythm of 5-HT is important[29]. According to Fernstrom and Wurtman[9,10], changes in the levels of neutral amino-acids in serum could influence tryptophan availability for 5-HT synthesis. This led us to estimate also the circadian variations in serum tyrosine levels.

MATERIAL AND METHODS

Male Sprague–Dawley Charles River rats weighing 250–300 g were exposed to alternating periods of 12 h of light (07.00–19.00 h) and 12 h of darkness (19.00–07.00 h) regulated by an automatic light-controlling device; they received food and water ad libitum[16]. Groups of rats, housed for three weeks in this environment, were sacrificed by decapitation at various times of the day, the experiment starting at 09.00 h as indicated in Fig. 1. Their blood was rapidly collected and their brain was dissected to excise the fronto-parietal cerebral cortex.

Estimation of 5-HT, 5-HIAA and tryptophan levels in cerebral cortex

Tissues were homogenized using a polytron apparatus (type PT 10 OD) in 6 ml of an ethanol water solution (74:16 v/v) containing EDTA (0.2%) and ascorbic acid (0.1%) as protectors. Tissues homogenates were treated as described previously for 5-HT, 5-HIAA and tryptophan estimations[16]. Briefly, 5-HT was first retained by ion exchange chromatography on Amberlite CG 50. Tryptophan was then isolated from the Amberlite effluents by ion exchange chromatography on Dowex AG 50

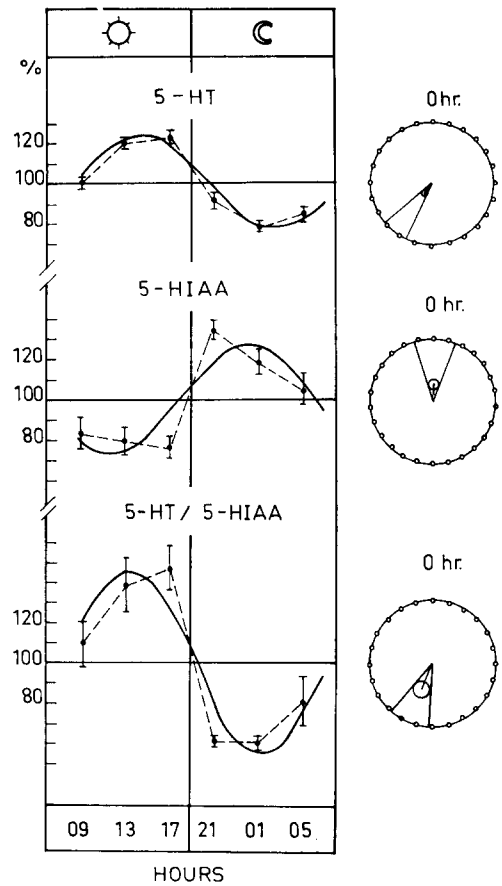

Fig. 1. Daily variations of 5-HT and 5-HIAA levels and of the 5-HT/5-HIAA ratio in the cerebral cortex. 5-HT and 5-HIAA levels were estimated at various times of the light and dark periods in the fronto-parietal cerebral cortex of rats exposed to a regular cycle of 12 h of light (07.00–19.00 h) and 12 h of darkness (19.00–07.00 h). The experimental values joined by dotted lines are the mean ± S.E.M. of data obtained with groups of 4 rats. They are expressed in per cent of the daily mean respective values. The dark lines indicate the fitted theoretical curve determined according to a method described by Kan et al.[19]. The polar representation on the right part of the figure provides statistical information about the characteristics of the circadian rhythms, as described in details elsewhere[19]. Briefly, the length of the inner vector represents the rhythm amplitude (in per cent of the daily mean value); its projection on the hour circle indicates the phase of the rhythm. The inner circle provides the confidence area of the rhythm and thus allows to determine the confidence intervals for the amplitude and the phase.

WX4. Finally 5-HIAA was separated from the effluents of the Dowex column by adsorption on Sephadex G 10. 5-HT, 5-HIAA and tryptophan were then estimated on aliquots of the respective column eluates by the spectrofluorimetric method of Curzon and Green[5] (5-HT, 5-HIAA) and Denckla and Dewey[7] slightly modified by Lin et al.[23] (tryptophan). All data were corrected for respective recoveries.

Estimation of bound and free tryptophan and of tyrosine levels in serum

The blood from the trunk was collected in non-heparinized tubes since heparine

acts on tryptophan binding on serum albumin[13]. After clotting, the serum was obtained by centrifugation of blood samples at 200 × *g* for 20 min at 4 °C. Ultrafiltration of the serum was performed in Amicon Centriflo CF 50 dyalisis cones. Serum samples (0.5 ml) were introduced in cones and centrifuged for 30 min at 800 × *g* at room temperature. Tryptophan and tyrosine were isolated from the ultrafiltrate and in the whole serum according to the methods described by Bourgoin et al.[1]. Briefly, serum and ultrafiltrates samples were mixed with an ethanol water solution (74:16 v/v) containing 0.4 *N* perchloric acid. After a 3–4 h storage at −30 °C the solutions were centrifuged and supernatants were diluted with water (3 ml) and adjusted to pH 2 with potassium hydroxyde. Potassium perchlorate was removed by centrifugation and the final supernatants were passed through Dowex AG 50 WX4. Tyrosine and tryptophan were estimated in acetate eluates according to the spectrofluorimetric methods of Waalkes and Udenfriend[35] and Denckla and Dewey[7] modified by Lin et al.[23], respectively. Since tyrosine is completely free in serum, its determination in ultrafiltrate can be used as an index of recovery of the ultrafiltration procedure. This allowed us to estimate the relative proportion of free and bound tryptophan from the estimation of the amino acid concentration in serum and its ultrafiltrate as described previously[1].

Statistical analysis

Experimental data were submitted to a statistical analysis which was done according to a new method extensively described in part I[19]. This allowed to determine the period, amplitude and phase of the various rhythms.

RESULTS

(1) Circadian variations in 5-HT and 5-HIAA levels in the cerebral cortex

As expected, maximal levels of 5-HT were observed during the second part of the light period. The statistical analysis revealed a significant circadian rhythm ($P < 0.05$) characterized by its phase (14.30 ± 0.44 h) and amplitude (23%) (Fig. 1, Table I). In contrast, the levels of 5-HIAA were maximal at the beginning of the dark period. The phase of the significant ($P < 0.05$) circadian rhythm of 5-HIAA was optimalized at 00.06 ± 1.24 h (Fig. 1, Table I); its amplitude being 27%. The circadian changes in the 5-HT/5-HIAA ratio are illustrated in Fig. 1. This ratio decreased abruptly immediately after the light went off (i.e., between 17.00 and 21.00 h).

(2) Circadian variations in tryptophan and tyrosine levels in the serum and in tryptophan levels in the cerebral cortex

The statistical analysis of the fluctuations of the levels of free and total tryptophan in serum and of those of tryptophan in the cerebral cortex revealed in all cases the occurrence of significant circadian rhythms. The maximal levels were always found during the dark period, the phases being respectively 00.54 ± 2.42 h and 04.44 ± 1.24 h for free and total tryptophan in serum and 03.36 ± 1.13 h for tryptophan in cerebral cortex. The amplitude of the fluctuations of free tryptophan

TABLE I

Characteristics of the various circadian rhythms

The statistics were done according to the method described by Kan et al.[19]. All rhythms had a period (T) of 24 h. The data represent the absolute daily mean value ± S.E.M. of the various parameters studied and the amplitude and phase (± S.E.) of the various rhythms.

Rhythm	*Period (h)*	*Daily mean value*	*Amplitude (%)*	*Phase (h)*
5-HT	24	0.39 ± 0.07 μg/g	23.4 ± 4.5	14.30 ± 0.44
5-HIAA	24	0.58 ± 0.03 μg/g	26.7 ± 9.5	00.06 ± 1.24
5-HT/5-HIAA	24	0.75 ± 0.07	45.5 ± 15.2	13.25 ± 1.18
Trp in Tissues	24	5.58 ± 0.24 μg/g	21.1 ± 6.6	03.36 ± 1.13
Trp in Serum	24	24.40 ± 0.70 μg/ml	13.7 ± 4.9	04.44 ± 1.24
Free Trp in Serum	24	1.72 ± 0.07 μg/ml	20.8 ± 13.5	00.54 ± 2.42
Tyr in Serum	24	21.49 ± 0.86 μg/ml	19.5 ± 8.0	11.48 ± 1.37
Free Trp/Tyr	24	0.086 ± 0.009	46.5 ± 13.8	00.25 ± 1.12

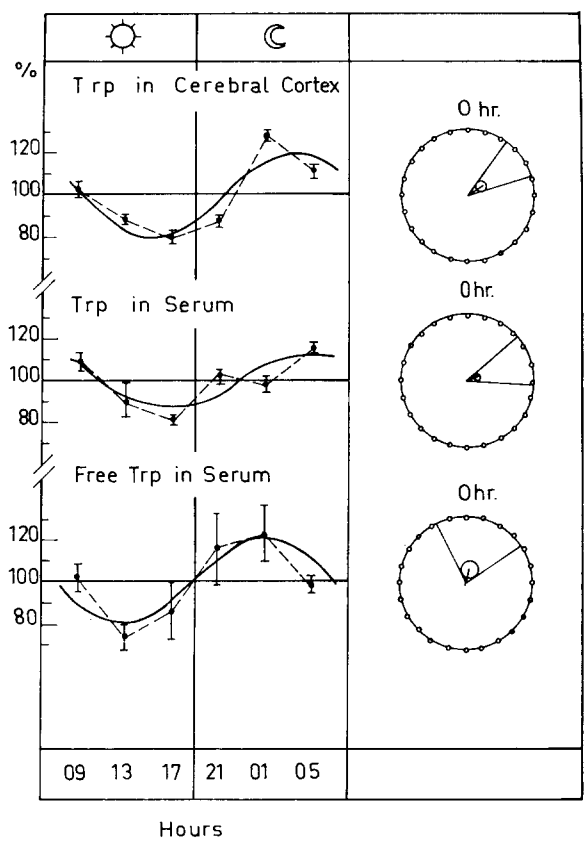

Fig. 2. Daily variations of tryptophan levels in the cerebral cortex and in the serum. In the experiment described in Fig. 1, tryptophan (Trp) levels were estimated in tissues. The levels of the total and free form of the amino-acid were also determined in serum. Data and statistical analysis were represented as described in Fig. 1.

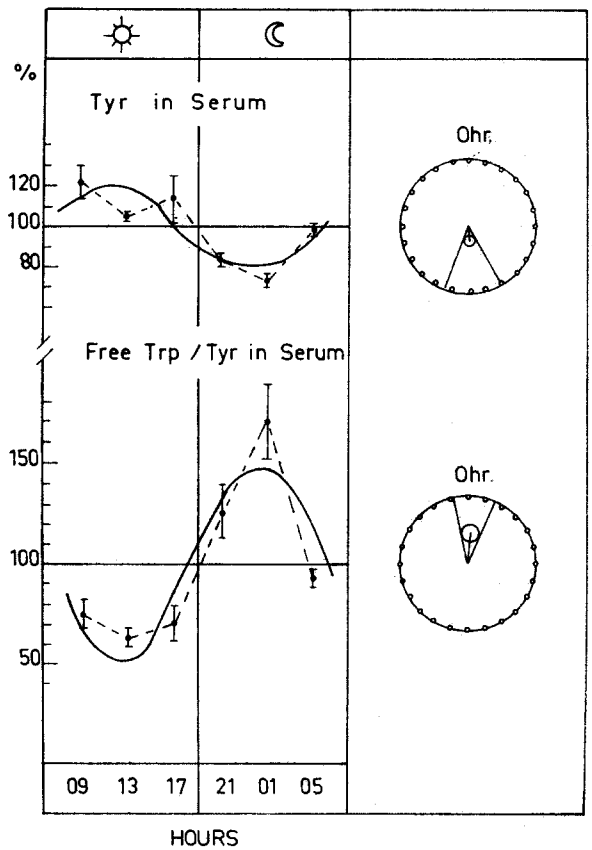

Fig. 3. Daily variations of tyrosine levels and of the free tryptophan/tyrosine ratio in the serum. In the experiment described in Fig. 1, tyrosine (Tyr) levels as well as the free tryptophan/tyrosine (free Trp/Tyr) ratio were estimated in serum. Data and statistical analysis were represented as in Fig. 1.

levels in serum as well as that of tryptophan levels in tissues (21%) were particularly pronounced (Fig. 2, Table I).

In contrast to free or total tryptophan, tyrosine levels in serum were maximal during the light period (phase at 11.48 $\pm$ 1.37 h). The amplitude of the rhythm was similar to that of free tryptophan (20%) (Fig. 3, Table I).

DISCUSSION

Important circadian variations in tryptophan levels occur in cerebral tissues. They seem to be mainly related to the diurnal fluctuations of the content of the free form of this amino-acid in the serum. These variations in the precursor content do not appear to trigger simultaneous and parallel changes in 5-HT metabolism in central serotoninergic neurones. Indeed, the circadian modifications in 5-HT levels[8,26,30,31] or 5-HT synthesis[16] in brain are in opposite phase with those of tryptophan levels in serum (free and total) and in brain.

The free form of tryptophan represents about 7–10% of the total content of the

amino acid in serum which is thus for its large part bound to albumin[2,24]. At its peak level occurring during the dark period, the concentration of free tryptophan in serum was almost twice that found in the middle of the light period (13.00 h). Similar circadian variations in the total serum levels of the amino acid were seen but they were less pronounced. The ratios of free over total tryptophan and of free tryptophan over tyrosine (Fig. 3) levels in the serum were also maximal in the course of the dark period. It is thus not surprising that the circadian variations in tryptophan levels in cerebral tissues were in phase with the serum levels of the free form of the amino-acid. Indeed, in most circumstances, the levels of tryptophan in brain were shown to be dependent on those of the free form of the amino-acid[33] or on the tryptophan/tyrosine (or all neutral amino-acids) ratio in serum[9,10]. Similar parallel circadian variations in serum free tryptophan and in cerebral tryptophan levels were reported by Morgan et al.[27].

The maximal food intake occurs during the night in rats[21,22]. This could partly explain the large quantities of tryptophan found in serum during this period. Free fatty acids are able to displace bound tryptophan[2,4,6]. They could be responsible for the elevation of free tryptophan levels in serum. This does not seem to be the case since in contrast to free tryptophan, free fatty acids levels are elevated during the light period when food consumption is reduced[12,21]. The maximal secretion of insulin, triggered by food intake[32] is observed during the night. This could be responsible in some ways for both the high tryptophan and low tyrosine serum contents detected at this time. Indeed, peripheral injections of insulin induce a rapid elevation in tryptophan levels and a reduction in tyrosine and other neutral amino-acids levels in serum[11]. However, this may not be the only mechanism involved in this phenomenon, since high levels of free and total tryptophan were still found during the dark period in rats fed only during 4 h in the light period[27].

As observed in our previous studies[16,17], marked significant circadian variations in 5-HT and 5-HIAA levels were detected in brain tissues. Their amplitudes were similar (25–26 %) but the peak levels of 5-HT and 5-HIAA were found during the light and dark periods respectively. The maximal changes in 5-HIAA levels and in the 5-HT/5-HIAA ratio occurred between 17.00 and 21.00 h, two times separated by the light off. On the basis of the simultaneous presence of high levels of brain 5-HIAA and serum tryptophan seen during the night period, Morgan et al.[27] have assumed that 5-HT turnover was increased at this time. Our previous studies made in animals submitted to similar experimental conditions as those used in this study, did not support this interpretation. Indeed, on the basis of in vivo and in vitro experiments we reported that the increase in 5-HIAA levels or the reduction in the 5-HT/5-HIAA observed in various brain structures shortly after the beginning of the night were solely related to an enhanced release and utilization of the transmitter[15]. These effects were not associated with a stimulation of 5-HT turnover since 5-HT synthesis was reduced at this time[16]. Therefore, as already discussed elsewhere[3,14,28], the estimations of free tryptophan levels in serum and of 5-HIAA levels in brain tissues do not allow to draw definitive conclusions concerning the rate of 5-HT turnover. A direct evaluation of the rate of 5-HT synthesis should also be made.

The present results also confirm that the diurnal changes in tryptophan and tyrosine transport in cerebral slices[18] are not related to the circadian fluctuations of the amino-acids concentrations in serum. Indeed, tryptophan transport in slices was maximal during the light period whereas the peak level of tryptophan content in serum occurred in the dark period. Similarly the circadian variations in tyrosine transport in slices and in tyrosine levels in serum are in opposite phase[18].

From these various observations as expected, it is doubtful that the fluctuations in tryptophan metabolism occurring in serum are responsible for the circadian variations in brain 5-HT metabolism observed in physiological states. This conclusion is further strengthened by the probable heterogeneity in the circadian fluctuations of the activity of the various serotoninergic pathways. Indeed, the phases and amplitudes of the circadian variations of 5-HT or 5-HIAA levels differ slightly from one structure to another[29,30]. Moreover, in simultaneous studies, pronounced differences in the circadian variations of tryptophan hydroxylase activity were observed between the various groups of serotoninergic cell bodies of the raphe system[19].

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