Brain Research, 123 (1977) 125–136

DAILY VARIATIONS OF VARIOUS PARAMETERS OF SEROTONIN METABOLISM IN THE RAT BRAIN. I. CIRCADIAN VARIATIONS OF TRYPTOPHAN-5-HYDROXYLASE IN THE RAPHE NUCLEI AND THE STRIATUM*

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SUMMARY

Daily changes in tryptophan-5-hydroxylase (TrH) activity have been studied in six different 5-HT-containing cell groups (B_3, B_4, B_5, B_6, B_7, B_8) in the brain stem of rats maintained in a regular cycle of 12 h of light and 12 h of darkness. Significant circadian alternations of TrH activity were observed in most of the raphe nuclei tested, although the changes were not identical from one structure to another. The striatum showed daily variations in TrH activity in opposite phase to the nucleus raphe dorsalis which projects specific terminals into this area. Monoamine oxidase (MAO) activity was simultaneously estimated in these nuclei but did not exhibit any significant rhythm, suggesting a specific regulation of TrH. Total protein levels were also subject to daily changes.

INTRODUCTION

Histochemical studies have recently demonstrated heterogenous projections from the various serotoninergic raphe nuclei. In particular, nucleus raphe dorsalis and nucleus raphe centralis have been shown to innervate specific rostral structures (striatum, hippocampus, hypothalamus and cortex)[2,3,5,8]. A similar distribution of 5-hydroxytryptamine (5-HT, serotonin) levels and its anabolic enzyme, tryptophane-

* Some of the results described in this paper have been previously published as a short communication[14].

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5-hydroxylase (EC 1.4.16.3, TrH) has also been demonstrated[4,14,19,21]. It has been shown that the intraneuronal 5-HT level and its biosynthesis[1,11,20] are subject to nycthemeral rhythms. These observations suggest that the activity of TrH, at the level of 5-HT groups[6] including individual raphe nuclei (B3, B4, B5, B6, B7 and B8) may be subject to similar variations. The activity of monoamine oxidase (EC 1.4.3.4., MAO) was simultaneously evaluated in each of the same groups in order to compare the variations of anabolic and catabolic enzymes. To differentiate between terminals and cell bodies, TrH activity was also measured in the striatum[9], which is a major projection area of 5-HT neurons from nucleus raphe dorsalis[8].

MATERIAL AND METHODS

(I) Preparation of animals

Twenty-four male albino rats of the OFA strain (IFFA Credo, France), weighing approximately 250 g each, were used. All animals, caged in fours, were housed in a room at a constant temperature (24 ± 1 °C) on an automatic fixed dark–light (Mazda Fluor, daylight, 200 lux) schedule (light on at 07.00 h, off at 19.00 h). Each day, between 8 and 10 a.m., the cages were cleaned out and food (Extra Labo M25 biscuits) and water given ad libitum.

(II) Preparation of samples and enzymes assays

At various times (17.00, 21.00, 01.00, 05.00, 09.00 and 13.00 h) 4 rats were decapitated. A stereotaxic section was made at the anterior frontal plane of de Groot[7]. The posterior part of the brain, caudal to this section and including the mesencephalic and serotoninergic groups, was removed and promptly frozen on dry ice. Beginning at the caudal extreme of the IVth ventricle, a Leitz 1300 freezing microtome was used to cut 500 μm sections which were preserved (—10 °C) on glass plates. Serotoninergic groups, located with maps described elsewhere[6,7,15,24,25], were removed from the adequate 500 μm thick slices using a stainless steel circular punch (0.9 mm diameter).

The B7 group of the dorsal raphe nucleus was identified in the central gray matter, medial and immediately ventral to the central aqueduct (Fig. 1A). Tissue was punched out of 4 slices (2 mm total) beginning at the caudal extreme of the aqueduct (Fig. 1A). The B8 group, identified as the centralis raphe nucleus, was removed from the same 4 slices, 1 mm below B7 group (Fig. 1A).

The B5 group, including the pontine raphe nucleus, and the B6 group were removed from the 3 caudally adjacent slices (1.5 mm total), including the two A6 groups (locus coeruleus) (see Fig. 1B). The B6 group, located in the midline at the level of the floor of the IVth ventricle, and the B5 group, comprising the middle third of the slice between the floor of the IVth ventricle and the ventral surface of the brain, were punched out (Fig. 1B).

The B4 group was removed from the same area as B6 but 1 mm more caudally (Fig. 1C). The B3 group (including nucleus raphe magnus) was removed 2–2.5 mm caudal to the B5 group. The latter was dissected out about 1.5 mm below the fourth ventricle (Fig. 1C).

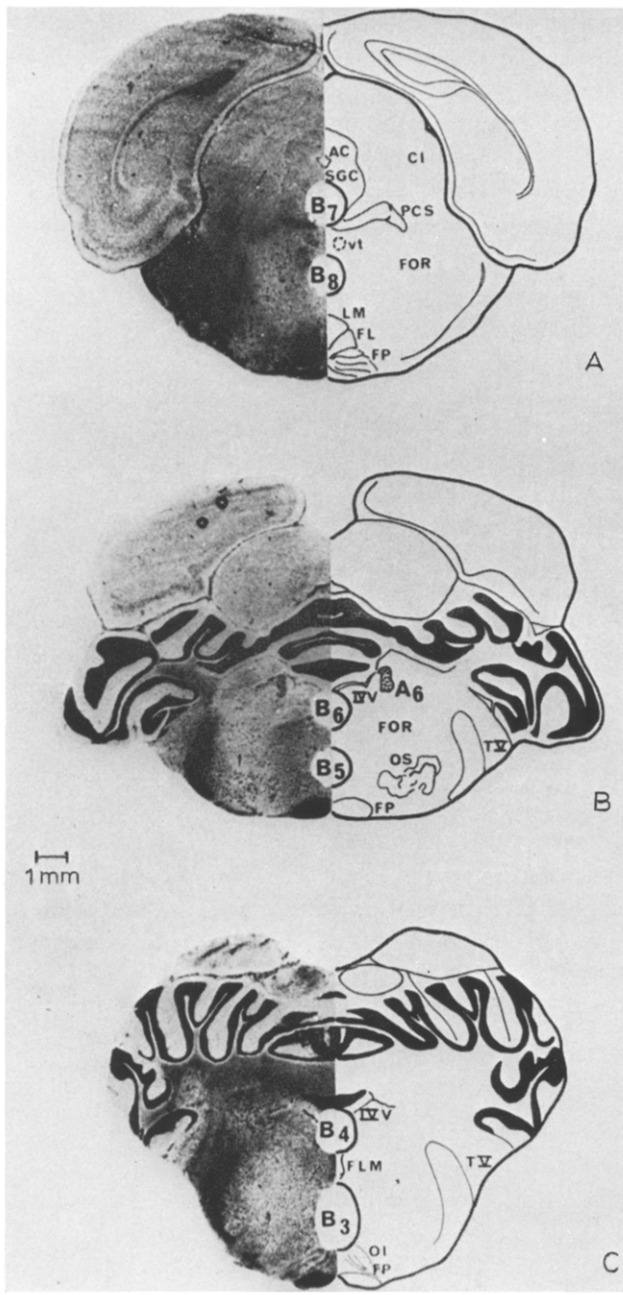

Fig. 1. Localization of raphe system nuclei in the rat brain stem. 5-HT groups were removed from 500 μm thick as decribed in the text. Punches are indicated by circular lines. This figure shows 40 μm thick sections cut from 500 μm slices and stained with cresyl violet. Abbreviations: AC, aqueductus cerebri; CI, colliculus inferior; FL, fasciculus longitudinalis; FLM, fasciculus longitudinalis medialis; FOR, formatio reticularis; FP, fibrae pyramidales; OI, oliva inferior; OS, oliva superior; PCS, pedunculus cerebellaris superior; TV, tractus nervi trigemini; vt, nucleus ventralis tegmenti; IV V, ventriculus quartus.

After punching, histological controls were made on each slice by cutting them again and staining using the classical Nissl technique. Only structures with complete removal of the raphe nuclei were used for the following determination.

After excision the different nuclei were gently sonicated (10 sec, 40 W, 30 kHz) in 25 μl (raphe nuclei) or 100 μl (caudate nuclei) of ice cold phosphate buffer (0.1 *M*, pH 7.60). Aliquots (20 μl) were assayed for TrH as previously described[14]. The use of phosphate buffer allowed the best direct chromatographic separation of [^{14}C]5-HTP and [^{14}C]5-HT. The reaction was verified to be linear for more than 30 min in each structure examined. Another aliquot (5 μl) was assayed for MAO according to the method of Parvez et al.[22], with minor modifications. Briefly: 25 μl of phosphate buffer (0.2 *M*, pH 7.40) were added and then samples were incubated for 20 min at 37 °C with 0.050 μCi (2.4 10^{-5} *M*) of [^{14}C]5-hydroxytryptamine creatinine sulfate (specific activity: 59 mCi/mmole generally labeled; Radiochemical Centre, Amersham) in 5 μl of buffer. The reaction was stopped by 20 μl of HCl 2 *N* and then 300 μl of toluene was added. After agitation (25 min) and centrifugation (9,000 × *g*, 5 min) 200 μl of the upper phase was transferred to counting vials in order to measure radioactivity in 10 ml of scintillating toluen (2.5 diphenyloxazole (PPO), 0.4%; 1,4 bis (2-(5-phenyl-oxyazolyl)) benzene (POPOP), 0.01%). All values were corrected with respect to a buffer blank.

Enzyme activities were expressed in pmoles of 5-HTP produced/h/structure and in pmoles of deaminated 5-HT/h/structure for TrH and MAO respectively. Total proteins levels were estimated by the Folin reagent method[17].

(III) Statistical analysis

A specific method of estimation was developed for this type of data (short chronological series without replications) and based on the following considerations.

The measured enzyme activity (x) is a function of the time of observation (t) according to the formula

$$x(t) = \alpha + \beta \sin\left(\frac{2\pi}{T} t + \varphi\right)$$

$$\theta = \frac{2\pi}{T} t \quad (T = \text{period}),$$

α (mean level), β (amplitude) and φ (phase angle) must be estimated. The measure of x at time t_i for the v^{th} animal can be considered as a random variable

$$X_{iv} = \alpha + \beta \sin(\theta_i + \varphi) + Z_{iv}$$

were Z_{iv} represents the random measure errors, independent and normally distributed with zero mean and variance σ^2. The estimation of β and φ is particularly simplified if:

- the sampling is periodic
- the time of observation covers at least one period.

Estimations a, b, p of α, β, φ are determined by least-squares regression techniques[16].

$$a = \frac{1}{r} \sum_{i=1}^{r} x_{i.} \qquad x_{i.} = \frac{1}{n} \sum_{\nu=1}^{n} x_{i\nu}$$

$$b = \frac{2}{r} \left[\left(\sum_{i=1}^{r} x_{i.} \sin \theta_i \right)^2 + \left(\sum_{i} x_{i.} \cos \theta_i \right)^2 \right]^{\frac{1}{2}}$$

$$\operatorname{tg} p = \frac{\sum_i x_{i.} \cos \theta_i}{\sum_i x_{i.} \sin \theta_i}$$

where r is the number of sampling times and n the number of sacrificed animals for each sampling time.

Because of the orthogonal properties of sine – cosine functions, the polar representation of sinusoidal variation, derived from Cosinor techniques[10], is located inside a circle of confidence with center at point (b cos p, b sin p) and radius

$$R = 2\,s \sqrt{\frac{F[2, r(n-1)]}{r.n}}$$

for a given confidence level (0.95), where

$$S^2 = \frac{1}{r(n-1)} \sum_i \sum_\nu (x_{i\nu} - x_{i.})^2$$

is an estimator of error variance σ^2. The associated confidence intervals are therefore $b \pm R$ for amplitude and are determined by the tangents for phase p (Fig. 2). If the circle overlaps the origin, there is no significant sinusoidal variation of period T.

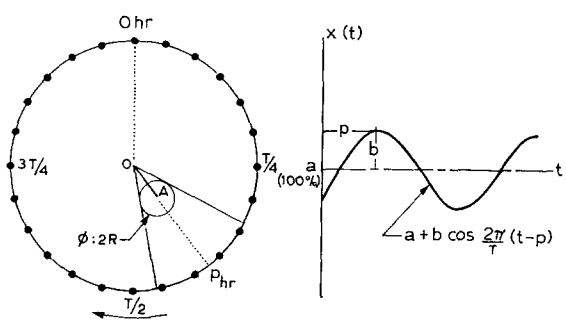

Fig. 2. Polar representation (left) of a sinusoidal rhythm of period T (hr). $\vec{OA}$ is a rotativy vector (counter-clockwise) with a constant angular velocity of $2\pi/T$ radians p/h. Its length represents amplitude b, expressed in percentage relatively to the daily mean level a (100%). The plot versus time t of the projection of $\vec{OA}$ on a vertical axis gives a sine function (right). Conventionally, we have chosen to represent the position of $\vec{OA}$ at local midnight time in order to read clockwise (bottom arrow), directly the value of phase angle p on the polar plot. With this representation and according to the trigonometric conventions, p (hr) represents the temporal position of maximum of a + b cos $2\pi/T$ (t — p), equivalent to x(t). The inner circle (radius R) is the confidence area of the rhythm and gives the confidence intervals for b and p. For combined rhythms (fundamental plus first harmonic), there will be a confidence area for each term. In this case, the outer circle, divided in 24 equal parts, will be equivalent to 24 h in considering the fundamental and 12 h for the first harmonic.

For temporal variations which are not necessarily sinusoidal, it is possible to fit them by a linear combination of harmonic sines (period T, T/2, T/3 etc) by applying the preceding method to each term of the combination. Conventionally, a variation will be called a "sinusoidal rhythm" in the case of only one significant term, and "combined rhythm" in the other cases, without any functional implication of this terminology.

In order to test the hypothesis of homogeneity of error variance between each sampling time, Cochran's test[26] was applied on the raw data. If S^2_r represents the estimator of σ^2 at time t_i, this test uses the statistic $F_{max} = S^2 \text{ largest} / \sum_{i=1} S_i^2$ to compare with F_{max} (r, n–1) theoretical values.

For the following, all the numerical values of amplitudes (b) will be expressed as a percentage relative to the daily mean level (a = 100 %), and the phase angle p expressed in h, relative to local midnight. (2π radians equivalent to 24 h for fundamental and 12 h for the first harmonic.)

RESULTS

All the enzyme activities were expressed by structure (see Discussion). The wet weight of each structure was determined and we did not observed any significant daily variations of this weight. Table I gives results obtained from 4 rats sacrificed at 09.00 h.

Except for MAO activities in B5 (including nucleus raphe pontis), all the data satisfied to the criterion of homogeneity of variance.

(A) Tryptophan hydroxylase activity

B_3 group (nucleus raphe magnus) – B_4 group (Fig. 3). Significant sinusoidal variations were observed in both of these groups, but with different periods: T = 24 h for B4 with maximum activity at the end of the light period (18.30 h) and T = 12 h for B3 with two maxima by nycthemer (01.40 and 13.40 h). The daily mean activity (pmoles/h/st ± S.E.M.) in B3 (204 ± 13) was 4 times greater than in B4 (48 ± 4) even though the relative amplitudes were in the same range (16.3 % and 20.7 % respectively).

TABLE I

Dissection of 5-HT groups of the rat brain stem

Two to four microdisks (900 μm diameter, 500 μm thickness) of fresh tissue were pooled for each 5-HT group as described in the methods. The mean weight values ± S.E.M. were obtained from 4 animals sacrificed at 09.00 h.

5-HT groups	*B8*	*B7*	*B6*	*B5*	*B4*	*B3*
Number of microdisks per structure	4	4	3	3	2	4
Mean weight ± S.E.M. (mg of fresh tissue)	2.15 ± 0.03	2.12 ± 0.02	1.42 ± 0.02	1.55 ± 0.03	1.02 ± 0.05	2.12 ± 0.10

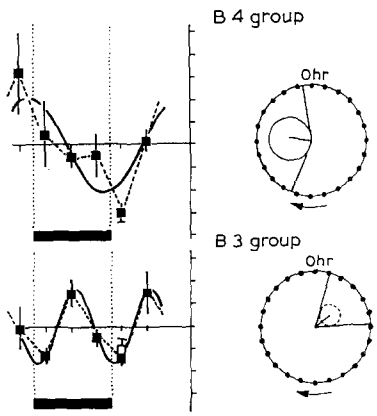

Fig. 3. Daily variations of TrH activity in the B4 and B3 serotoninergic group[6]. (a) *The orthogonal plots* (left) show the tissue course (■- - - -■) of the mean enzyme activity expressed per structure as a percentage (± S.E.M. as vertical bars ■) of the daily mean level (100%). The dark line (■∿■) indicated the fitted theoritical curve. The mean TrH activity (± S.E.M. as vertical bars ⊟) measured in five additional experiments at 09.00 h (see results) is also represented. The period of darkness between 19.00 and 07.00 h is delimited by vertical dotted lines. Scale: ordinates, 10%; abscissae, 4 h. (b) The respective *polar plots* (right) indicate the amplitude b (%), the phase p (hours) and their confidence interval (0.95) (see statistical analysis and Fig. 1). The radius of outer circle is equivalent to 50% of the daily mean activity. Solid inner circles: fundamental period (T = 24 h); dashed inner circles: first harmonic (T = 12 h). (c) For B_3: T = 12 h, b = 16.3% ± 9.5% p = 01.40 h ± 01.15 h. For B_4: T = 24 h, b = 20.7% ± 19.8% p = 18.30 h ± 05.00 h.

B_5 group (nucleus raphe pontis) – B_6 group (Fig. 4). Enzyme activity in B5 group exhibited a significant ($P < 0.05$) reduction (—50%) after the onset of the light period (09.00 h). However the variability of the measurements did not permit the detection of any smoothed rhythm by the present method of estimation. B6 group

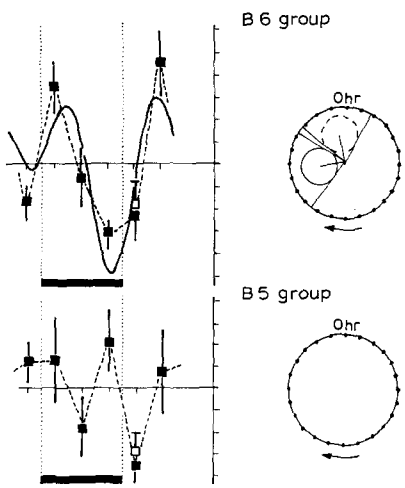

Fig. 4. Daily variations of TrH activity in B_5 and B_6 groups[6]. Same representation as in Fig. 2. For B_5: no significant variations; for B_6: "combined" rhythm: T = 24 h, b = 23.3% ± 16.7% p = 17.30 h ± 02.30 h; T = 12 h, b = 26.1% ± 16.7% p = 11.30 h ± 01.15 h.

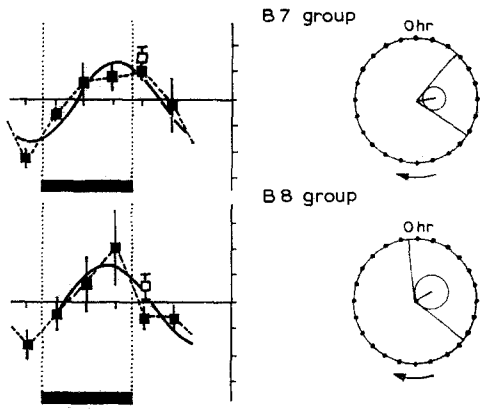

Fig. 5. Daily variations of TrH activity in B_7 and B_8 groups[6]. Same representation as in Fig. 2. For B_7: T = 24 h, b = 14.5% ± 9.7%, p = 05.30 h ± 02.30 h. For B_8: T = 24 h, b = 14.8% ± 11.3%, p = 04.00 h ± 04.30 h.

possessed a pronounced "combined rhythm" with a minimum activity before the onset of the light period (05.30 h). Furthermore, B6 group exhibited a daily mean activity double that observed in B5 group (75.3 ± 9 and 44 ± 5 pmoles/h/st ± S.E.M., respectively).

B_7 group (nucleus raphe dorsalis) – B_8 group (nucleus raphe centralis) (Fig. 5). TrH activity in both of these groups exhibited a significant "sinusoidal rhythm". In spite of different daily mean values (694 ± 35 and 363 ± 26 pmoles/h/st ± S.E.M., respectively for B7 and B8), the TrH activity fluctuates in a comparable manner in both groups: identical amplitudes (14.5% and 14.8%, respectively) and maximum at the end of the dark period (5.30 h and 4.00 h respectively).

The TrH activity was also measured in each group at 09.00 h in 5 additional experiments (4 rats each) with the same conditioning of animals, in order to assure the day-to-day reliability of the measurements and to confirm the estimated sine variations. At this time and in each structure, we never found significant differences between the two series of measurements (see Figs. 3–6).

Striatum (Fig. 6). In this area, TrH activity showed a large circadian variation (b = 52%) around the daily mean level (4 ± 0.6 pmoles/h/st ± S.E.M.) with a

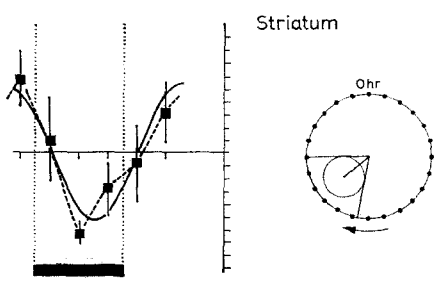

Fig. 6. Daily variations of TrH activity in the striatum. Same representation as in Fig. 2 with a different scale: orthogonal plot (left), 10% vs. 04 h; polar plot (right), radius of outer circle equivalent to 100% of the daily mean activity. T = 24 h, b = 52% ± 34%. p = 15.20 h ± 02.20 h.

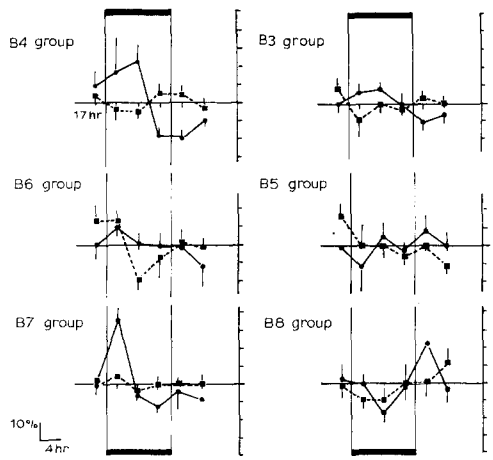

Fig. 7. Daily variations of MAO activity (black squares ± S.E.M.) and total protein levels (black circles ± S.E.M.) in six 5-HT groups of the rat brain[6]. Results are expressed as follow: daily mean level (a) in pmoles of deaminated 5-HT/h/st and mg/st for MAO activity and total protein levels (± S.E.M.) respectively, amplitude (b) in % and phase (p) in hours ± confidence interval (0.95) for each significant sine term. NSR: no significant rhythm. B_3 group: MAO, a = 0.380 ± 0.009, NSR; protein, a = 0.325 ± 0.006, T = 24 h, b = 7.90 ± 7.00, p = 23.10 ± 04.12. B_4 group: MAO, a = 0.316 ± 0.006, NSR; protein, a = 0.252 ± 0.010, T = 24 h, b = 21.43 ± 15.31, p = 21.22 ± 3.02. B_5 group: MAO, a = 0.258 ± 0.008, NSR; protein, a = 0.203 ± 0.006, NSR. B_6 group: MAO, a = 0.256 ± 0.08, NSR; protein, a = 0.211 ± 0.006, NSR. B_7 group: MAO, a = 0.453 ± 0.005, NSR; proteins, a = 0.283 ± 0.038, T = 24 h, b = 15.19 ± 8.30, p = 20.19 ± 02.42 and T = 12 h, b = 15.36 ± 8.30, p = 8.59 ± 02.36. B_8 group: MAO, a = 0.400 ± 0.005, NSR; protein, a = 0.270 ± 0.006, T = 12 h, b = 12.00 ± 9.00, p = 08.01 ± 04.01.

maximum in the middle of the light period (15.20 h), at the moment where TrH activity in the related perikarya (B7) is minimum (Fig. 5).

(B) Monoamine oxidase activity (Fig. 7)

With our experimental conditions and the present method of statistical estimation, the MAO activity did not show any significant circadian variations in any of the groups. Nevertheless, B5 and B6 groups exhibited significant ($P < 0.05$) sharp variations (in contrast with sinusoidal or smoothed rhythms) of this enzyme activity (+30% between 13.00 and 17.00 h for B5 and —30% between 21.00 and 01.00 h for B6).

(C) Total protein levels (Fig. 7)

Except for B5 and B6 groups, all the groups exhibited significant variations of total protein levels: sine rhythms (T = 24 h) for B3 and B4 groups and "combined" rhythms for B7 and B8 groups.

DISCUSSION

A biological rhythm can be considered as a recurrent temporal variation. Any analysis should begin by the estimation of its period of recurrence. Because of the presence of an external synchroniser (L12–D12) the period estimation was not con-

sidered in this work, as it was assumed that any variation would be synchronized on the same period (fundamental T = 24 h) or on a multiple period (T = 12 h, 8 h . . .). Furthermore, because a sampling interval of 4 h was used (6 × n measurements by 24 h), it was hazardous to estimate components of a period less than or equal to 8 h. Thus, only the fundamental (T = 24 h) and the first harmonic (T = 12 h) were used for a statistical estimation.

Another point is the signification of what we called "combined rhythm" and the mode of its statistical estimation. For a pure sine variation and from the analytical expression of the confidence area in a polar representation, it is easy to understand that the detection is effective only if the amplitude of the rhythm (b) is greater (with a scale factor) than the standard deviation of observations (s), that is, if the circle of confidence does not overlap the pole (which symbolizes the daily mean level). From Fourier's theorem, any periodic function can be expressed by a linear combination of successive harmonic sines. This combination does not necessarily imply an action of several harmonic oscillators for the biological generation of the recurrent variation. This Fourier expansion permits only a better "explanation" of the temporal variation than with a pure sine. In the same way, the 12 h-harmonic will be significant, from a best fitting point of view, only if its amplitude is greater than the standard deviation. The formulae of estimations remains unchanged for the 12 h-harmonic only if the number of sampling times over 24 h is even, thus insuring the two conditions necessary to build an exact test (periodic sampling covering at least one period).

Tryptophan hydroxylase is specifically localized in the 5-HT cells and all the cells were punched out together with surrounding non-5-HT tissue as was demonstrated by the subsequent histological controls. Considering this fact and the observation that the wet weight of the punches did not exhibit any significant daily variations, the expression of enzyme activities per structure is more descriptive of the absolute value of this enzyme in 5-HT tissue, which is independant of the diameter of the punch (and therefore of the non-5-HT tissue punched around raphe nuclei).

Furthermore, significant circadian variations of total protein levels were observed in some of the groups. The complex mechanisms of such variations remain unknown but could be related to some previous studies on daily variations in amino-acid incorporation into brain proteins[23]. Consequently, this confirms that we are able to express the results of enzyme activities (TrH and MAO) with respect to each structure and not to total protein levels. Expression of TrH activity by mg of proteins will overestimate the rhythm of enzyme activity expressed by the structure in the case of out of phase enzyme activity and total protein level rhythms (B7 for example, Fig. 5 and 7). On the other hand, for phase-locked variations of TrH activity and protein levels by the structure (B4, Fig. 3 and 7), the expression of TrH activity by mg of total proteins will tend to underestimate the enzyme rhythm expressed by structure.

Except for B5 group, characteristic daily variations of TrH activity were observed in each structure studied. These rhythms were clearly different from one serotoninergic group to another. In fact the results indicate that the median (B6 and B5) and caudal (B4 and B3) 5-HT groups showed "combined rhythms" compared to the rostral 5-HT groups (B7 and B8). This heterogeneity is particularly marked between B6-B5 groups

and B7-B8 groups. The latter groups exhibited circadian rhythms of low amplitude which were in synchronism in the two nuclei (Fig. 4 and 5). A different rhythm was also observed in B4 group in which TrH activity had a circadian cycle which was 180 degrees out of phase with B7 and B8. In other words, it would seem that the rostral 5-HT nuclei possess non-combined, in-phase rhythms of the activity of the specific enzyme of 5-HT biosynthesis.

On the other hand, these variations in TrH activity, although different in each structure seem specific for this enzyme. In fact MAO, which was assayed simultaneously in the same regions, did not seem to exhibit any significant rhythms. Excepting sharp variations observed in B5 and B6, this enzyme activity showed only very small daily variations. In any case, there is no apparent synchronisation between the TrH and MAO variations. The double neuroanatomical and biochemical specificity of TrH appears to be an important element in the regulation of biosynthesis of the serotonin. The differences observed between the various 5-HT containing areas suggest that these regions perform different physiological roles. This functional implication is well supported by selective lesions of the raphe nuclei, which show that these different areas act differently on the sleep–waking cycle[13].

These nycthemeral alternations of TrH activity could be explain by two distinct mechanisms: (1) variations of tryptophan levels at the sites of hydroxylation[12], (2) variations of the efficiency of TrH or/and the synthesis of this enzyme. Without further kinetic studies for each cosubstrate (tryptophan or 6-MPH4) it is difficult to emphasize one of these hypotheses. But the low levels of endogenous tryptophan in the samples (about 0.06 nmoles for a tissue concentration of 2.10^{-5} M) compared with the quantity of exogenously added substrate (8 nmoles) makes the first hypothesis unlikely. It is more likely that the rhythms were produced by alternations in the efficiency or quantity of the enzyme. Moreover the existence of a 12-h phase difference between raphe dorsalis and striatum which contains numerous terminals from this nucleus, could be explained by the delay required by molecules of the enzyme, newly synthesized in the cell bodies, to be transported in the striatal area, since Meek et al. have shown axonal transport of TrH proceeds at a rate of 5–7 mm/24 h[18]. Such a process of control would not exclude the existence of other regulatory factors at the level of terminals, and this may explain the discrepancy between the amplitude of the rhythms at these two sites.

ACKNOWLEDGEMENTS

This research was supported by DRME (grants 74/013 and 74/025), INSERM (U. 52) and CNRS (LA 162).

We are very grateful to Professor L. Martel for his statistical advices and to P. Keane and A. McRae for their skillful assistance in the preparation of this manuscript.

REFERENCES

1 Bobillier, P. and Mouret, J., The alternation of diurnal variations of brain tryptophan, biogenic amines and 5-hydroxyindolacetic acid in the rat under limited time feeding, *Int. J. Neurosci.*, 2 (1971) 271–282.

2 Bobillier, P., Petitjean, F., Salvert, D., Ligier, M. and Seguin, S., Differential projections of the nucleus raphe dorsalis and nucleus raphe centralis as revealed by autoradiography, *Brain Research*, 85 (1975) 205–210.

3 Bobillier, P., Seguin, S., Petitjean, F., Salvert, D., Touret, M. and Jouvet, M., The raphe nuclei of the cat brain stem: a topographical atlas of their efferent projections as revealed by autoradiography, *Brain Research*, 113 (1976) 449–486.

4 Brownstein, M. J., Palkovits, M., Saavedra, J. M. and Kizer, J. S., Tryptophan hydroxylase in the rat brain, *Brain Research*, 97 (1975) 163–166.

5 Conrad, L. C. A., Leonard, C. M. and Pfaff, D. W., Connections of the median and dorsal raphe nuclei in the rat: an autoradiographic and degeneration study, *J. comp. Neurol.*, 156 (1974) 179–206.

6 Dahlstrom, A. and Fuxe, K., Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons, *Acta physiol. scand.*, 62, Suppl. 232 (1964) 1–55.

7 De Groot, J., *The Rat Forebrain in Stereotaxic Coordinates*, North Holland Publ., Amsterdam, 1959, 40 pp.

8 Fuxe, K. and Jonsson, G., Further mapping of central 5-hydroxytryptamine neurons: studies with the neurotoxic dihydroxytryptamines. In E. Costa, G. L. Gessa and M. Sandler (Eds.), *Advances in Biochemical Psychopharmacology, Vol. 10*, Raven Press, New York, 1974, pp. 1–12.

9 Glowinski, J. and Iversen, L. L., Regional studies of catecholamines in the rat brain. I – The disposition of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]dopa in various regions of the brain, *J. Neurochem.*, 13 (1966) 655–669.

10 Halberg, F., Tong, Y. L. and Johnson, E. A., Circadian system phase, an aspect of temporal morphology; procedures and illustrative examples. H. von Mayersbash (Ed.), In *Cellular Aspects of Biorhythms*, Springer, Berlin, 1967. pp. 20–48.

11 Hery, F., Rouer, E. and Glowinski, J., Daily variations of serotonin metabolism in the rat brain, *Brain Research*, 43 (1972) 445–465.

12 Hery, F., Rouer, E., Kan, J. P. and Glowinski, J., The major role of tryptophan active transport in the diurnal variations of 5-hydroxytryptamine synthesis in the rat brain. In *Advances in Biochemical Psychopharmacology, Vol. 11*, Raven Press, New York, 1974, pp. 163–167.

13 Jouvet, M., Régulation biochimique des états de vigilance, *Int. J. Neurol.*, 10 (1975) 141–157.

14 Kan, J. P., Buda, M. and Pujol, J. F., Tryptophan-5-hydroxylase activity in the raphe system of the rat brain stem, *Brain Research*, 93 (1975) 353–357.

15 König, J. R. F., and Klippel, R. A., *The Rat Brain*, Williams and Wilkins, Baltimore, Md., 1963, 162 pp.

16 Linnik, Y. V., *Méthode des Moindres Carrés*, Dunod, Paris, 1963, 356 pp.

17 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., Protein measurement with the Folin phenol reagent, *J. biol. Chem.*, 193 (1951) 265–275.

18 Meek, J. L. and Neff, N. H., Tryptophan 5-hydroxylase: approximation of half-life and rate of axonal transport, *J. Neurochem.*, 19 (1972) 1519–1525.

19 Meek, J. L. and Neckers, L. M., Measurement of tryptophan hydroxylase in single brain nuclei by high pressure liquid chromatography, *Brain Research*, 91 (1975) 336–340.

20 Morgan, W. W., Saldana, J. J., Catherine, A. Y. and Morgan, J. F., Correlation between circadian changes in serum amino acids or brain tryptophan and the contents of serotonin and 5-hydroxyindolacetic acid in regions of the rat brain, *Brain Research*, 84 (1975) 75–86.

21 Palkovits, M., Brownstein, M. J. and Saavedra, J. M., Serotonin content of the brain stem nuclei in the rat, *Brain Research*, 80 (1974) 237–249.

22 Parvez, H. and Parvez, S., Microradioisotopic determination of enzyme catechol-O-methyltransferase, phenylethanolamine-N-methyltransferase and monoamine oxidase in a single concentration of tissue homogenate, *Clin. Chim. Acta*, 46 (1973) 85–90.

23 Richardson, K. and Rose, S. P. R., A diurnal rhythmicity in incorporation of lysine into rat brain regions, *Nature (Lond.)*, 233 (1971) 182–184.

24 Taber, E., Brodal, A. and Walberg, F., The raphe nuclei of the brain stem in the cat. I. Normal topography and cytoarchitecture and general discussion. *J. comp. Neurol.*, 114 (1960) 161–188.

25 Valverde, F., Reticular formation of the albino rat's brain stem. Cytoarchitecture and corticofugal connections, *J. comp. Neurol.*, 119 (1962) 25–53.

26 Winer, B. J., *Statistical Principles in Experimental Design*, McGraw-Hill, New York, 1971, 907 pp.