Circadian Rhythm in Leu-β-Naphthylamide Hydrolysing Activity in Selected Photoneuroendocrine Areas of Adult Male Rats*

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Soluble Leu-β-naphthylamide hydrolysing activity (arylamidase activity) was assayed in retina, superior cervical ganglia, several brain areas and serum of adult male rats, at different time points of a 12:12h light:dark cycle (from 7h to 19h light). The results demonstrated a left or right biased asymmetrical distribution of this activity depending on the time point studied: While the levels of activity were significantly higher in the left retina than in the right one at 10h of the light period, these were predominant in the right side, at 01h of the dark period. In addition, this activity was higher in the left hypothalamus at 13h of the light period. No asymmetries were disclosed in the rest of time points and structures studied. On the other hand, this activity demonstrated a diurnal variation in the left anterior hypothalamus and total posterior hypothalamus but no periodic variation was evidenced in right anterior hypothalamus or the rest of zones studied. These results may reflect the role of this activity regulating the functional status of its susceptible endogenous substrates.

Key-words: Circadian rhythm, Arylamidase activity, Asymmetry, Rat brain.

The diurnal rhythm that several neuropeptides display within the brain is probably regulated by te suprachiasmatic nucleus (SCN) of the anterior hypothalamus (19). This nucleus is integrated in a photoneuroendocrine circuit related to the melatonin rhythm generating system, which includes: Retina, SCN, paraventric-

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ular nuclei, intermediolateral nucleus of the spinal cord, superior cervical ganglia (SCG) and pineal gland (16, 25). In addition, the existence of a pineal influence on the hypothalamic pituitary axis (24) and other selected brain regions (18) has been suggested.

Neuropeptides (11, 20, 23) as well as their receptors (10, 22, 26), have been demonstrated in virtually all structures of this circuit; nevertheless, their role in the biological functions of this pathway is still unknown. On the other hand, the major proposed mechanism of inactivation of neuropeptides is their enzymatic degradation by peptidases (7). Therefore, the study of the regulation of these enzymes may provide insights into the nature of their role within the mechanisms that control neuropeptide activity.

Leucine-β-naphthylamide hydrolysing activity (LeuHA) (Leu-arylamidase activity) has been involved in the inactivation of several neuropeptides including dynorphins (8), substance F (13), enkephalins (14) and angiotensin II (1). Although much of the current work is being performed to investigate the circadian variation of neuropeptides, no attention has been devoted to their putative rhythmic control by peptidases. Therefore, in order to analyze a cyclic pattern of arylamidase activity, fluorometrically LeuHA has been determined using Leu-β-naphthylamide (LeuNNap) as substrate, at different time points of a 12:12 h light:dark cycle, in several rat brain areas (some of them included in the above circuit) and serum. In addition, since an asymmetrical distribution of brain LeuHA has been previously reported (2), a bilateral study was performed as well.

Materials and Methods

Male Sprague-Dawley rats weighing 200-250 g, housed under controlled temperature (25 °C) and lighting (light on 07.00 h, off 19.00 h) conditions, with food and water ad libitum, were employed in this study. Their brains were perfused with saline through left cardiac ventricle under equithensin anesthesia (2 ml/kg body wt), quickly removed and cooled in dry ice, at 10 h (n = 10), 13 h (n = 6), 16 h (n = 6), 22 h (n = 7), 1 h (n = 8) and 4 h (n = 8). During the dark period, the animals were perfused under a dim red light and additional light was used only after removal of the eyes. Before removing the brain, both eyes were taken out and their corresponding retinas (Re) quickly dissected and cooled in dry ice. The pineal gland (Pi), left and right superior cervical ganglia (SCG) and pituitary gland, separated into anterior (APt) and intermediate-posterior (IPPt) lobes, were also dissected and cooled in dry ice. Left and right anterior (AHt), posterior hypothalamus (PHt) and the most caudal area of the cortex: occipital cortex (OC), were dissected in agreement with the stereotaxic atlas of KÖNIG and KLIPPEL (17). The anterior hypothalamic area was considered between the stereotaxic planes A6360 μ and A5150 μ, anterior to the interauricular line, and the posterior one between A5150 μ and A3430 μ. Blood samples were obtained before perfusion from the left cardiac ventricle. Tissue samples were homogenized in 10 volumes of 10 mM HCl-Tris buffer (pH 7.4) and ultracentrifuged to obtain the soluble fraction (100,000 g, 30 min, 4 °C). The resulting supernatants were used to detect enzymatic activity and proteins content, assayed per triplicate.

Soluble LeuHA was measured in a fluorometric assay employing LeuNNap as substrate according to the method of GREENBERG (12) modified by ALBA *et al.* (3), briefly: 10 μl of each supernatant were incubated during 30 min, at 25 °C with 1 ml of the substrate solution (1 mg/100 ml LeuNNap, 10 mg/100 ml BSA and 10 mg/100 ml dithiothreitol in

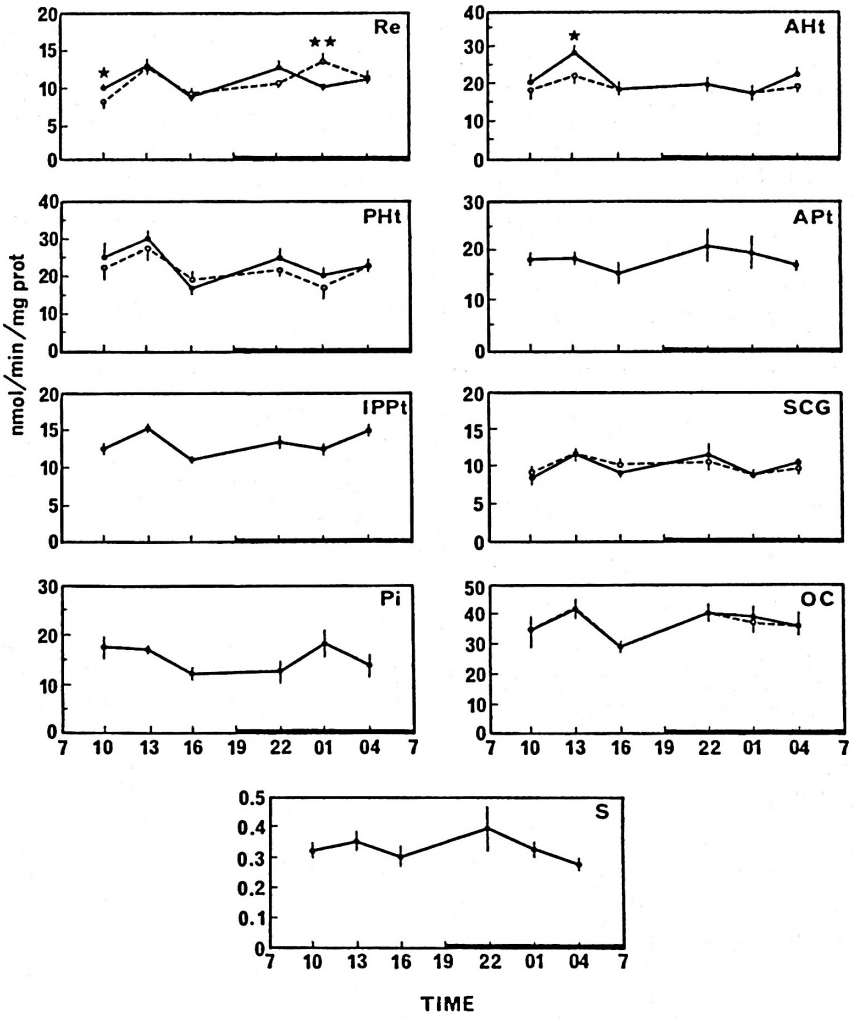

Fig. 1. *Specific soluble LeuHA in a 12:12 h light:dark cycle (from 7 h to 19 h light) in retina (Re), anterior hypothalamus (AHt), posterior hypothalamus (PHt), anterior pituitary (APt), intermediate-posterior pituitary (IPPt), superior cervical ganglia (SCG), pineal gland (Pi), occipital cortex (OC) and serum (S).* The rats were sacrificed at 10 h (n = 10), 13 h (n = 6) and 16 h (n = 6) of the light period and at 22 h (n = 7), 1 h (n = 8) and 4 h (n = 8) of the dark one. Values represent mean ± SEM levels expressed as nmol of LeuNNap hydrolyzed per min per mg of protein. (—) left (---) right, * $p < 0.05$, ** $p < 0.01$.

10 mM phosphate buffer pH 7.4). After incubation, the enzymatic reaction was stopped by adding 1 ml 0.1 M acetate buffer (pH 4.2). The quantity of β-naphthylamine released as a result of the enzymatic activity was determined fluorometrically at 412 nm of emision wavelength with an excitation wavelength of 345 nm. Proteins were quantified by the method of BRADFORD (9). Specific soluble LeuHA was expressed as nmol of Leu-NNap hydrolyzed per min per mg of protein.

To analyze differences in activity between left and right sides in Re, OC, SCG and AHt, the paired Student's *t* test was applied. For PHt, the distribution of the differences made the *t* test inappropriate and the Wilcoxon signed rank test was used instead. The circadian variation was modeled by assuming that the same average value M is seen 10, 16, 22 and 4 h, while 13 h has a higher average, M+A, and 1 h has the corresponding lower value, M-A. An F test of the significance of the parameter A was then performed.

Results

The levels of specific soluble LeuHA analyzed in brain areas, retina, superior cervical ganglia and serum are shown in figure 1. The comparisons between the left and right enzymatic activity of Re, OC, SCG, AHt and PHt, demonstrated an asymmetrical distribution in Re and AHt depending on the time point studied: While the activity in left Re was higher than in the right Re ($p < 0.05$) at 10 h of the light period, LeuHA was predominant in the right side ($p < 0.01$) at 1 h of the dark period. The left anterior hypothalamic activity also was superior than the right one ($p < 0.05$) at 13 h of the light period. Since Re and AHt displayed an asymmetrical distribution at selective time points, the left and right sides of these structures were treated as individual areas when analyzing the circadian variation. No differences between left and right activities of OC, SCG or PHt, at any time point, were evidenced. Therefore, we used the average of left and right values for the analysis of a potential diurnal rhythm.

A diurnal variation in LeuHA was demonstrated in left AHt ($p = 0.0005$) and PHt ($p = 0.004$). However, no periodic variation was evidenced in right AHt or the rest of zones studied.

Discussion

In neuropeptide research, including the study of proteolytic enzymes, several asymmetries have been evidenced (2, 5), suggesting that the functions in which those peptides are involved, could be lateralized in the same way. Our results demonstrated that selectively, at two time points of the light period (10 h for Re and 13 h for AHt), LeuHA was higher in the left side than in the right one. However, at 1 h of the dark period, the activity was predominant in the right Re. In this sense, an asymmetrical distribution of LeuHA has been reported in hypothalamus (2); however, no reason for this specific distribution was adduced. The findings here reported establish a direct connection between a neurochemical asymmetry and a biological rhythm. In a similar way are the results of BAKALKIN *et al.* (6) wich also demonstrated a brain lateralization conditioned by the time of the day: The luliberin content is asymmetrically distributed in hypothalamus and seems to shift within the day, being maximal in the morning and minimal in the evening. Nevertheless, the functional meaning of these results is still unknown.

Although it has not yet been determined whether this rhythm is endogenous or is entrained by the external light/dark cycle, it could be hypothesized that, since the retina and anterior hypothalamus are

functionally connected by the retinohypothalamic tract, an asymmetrical distribution of arylamidase activity in these locations may suggest an environmental lighting influence which results in a lateralized processing of susceptible substrates at certain time points.

The putative endogenous substrates of LeuHA Met-enkephalin (4), substance P (15) and dynorphin (21) display a circadian rhythmicity in hypothalamus showing the highest levels in the dark hours when the lowest levels in the same location are found. Therefore, since a role for LeuHA in controlling these neuropeptides has been proposed, their high hypothalamic levels in the dark hours could be due to a diminished LeuHA activity.

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Resumen

Se estudia la actividad enzimática soluble responsable de la hidrólisis de la leucina-β-naftilamida (actividad arilamidasa) en retina, ganglio superior cervical, diversas áreas cerebrales y suero de ratas machos adultas, a diferentes horas de un ciclo de luz/oscuridad de 12 h/12 h (luz de 7 h a 19 h). Dependiendo de la hora estudiada, la actividad muestra una distribución asimétrica de predominio izquierdo o derecho; mientras que a las 10 h del período de luz los niveles son significativamente mayores en la retina izquierda, a las 1 h del período de oscuridad predominan en la derecha. Además, a las 13 h del período de luz, la actividad hidrolítica es mayor en el hipotálamo anterior izquierdo. No se observan asimetrías en el resto de horas y estructuras estudiadas. Por otro lado, los niveles de actividad muestran una variación diurna en el hipotálamo anterior izquierdo y en el hipotálamo posterior, considerado como un conjunto, pero no se observa variación periódica ni en el hipotálamo anterior derecho, ni en el resto de zonas estudiadas. Estos resultados pueden reflejar el papel que representa esta actividad arilamilasa, regulando el estado funcional de sus sustratos endógenos.

Palabras clave: Ritmo circadiano, Actividad arilamidasa, Asimetría, Cerebro de rata.

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