

## **Influence of light/dark, seasonal and lunar cycles on the nuclear size of the pinealocytes of the rat**

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**Summary.** Morphological and physiological studies suggest a possible division of the pineal parenchyma into an external or "cortical" and another central or "medullar" layer. We have studied the possible influence of the light/dark, seasonal and lunar cycles on the nuclear size of the pinealocytes of the rat in both the hypothetical "cortical" and "medullar" layers. Forty male Wistar rats were used. Experiment was carried out in two seasons, winter and spring, two lunar phases, full moon and new moon, and the two circadian phases, photophase and scotophase. The nuclear volume of the pinealocytes, calculated from the Jacoby's formula, was the karyometric parameter used as measurement of the nuclear size. Main results showed that nuclear volume of the cortical pinealocytes was greater than that of the medullar pinealocytes only during the photophases of winter new-moon days and spring full moon days, whereas in all the remaining situations, the greater nuclear sizes were found in the pinealocytes of the medullar layer. These results support the existence of independent morphological variations of the pinealocyte in the central and peripheral zones of the pineal gland.

**Key words:** Pineal gland, Seasonal periods, Lunar cycles, Photophases, Cortical layer, Medullar layer, Biological rhythms

### **Introduction**

The pineal gland of mammals is the main organ in transducing the neural signal, generated by the endogenous circadian pacemaker, the suprachiasmatic nucleus, into a hormonal signal by the circadian synthesis and secretion of the hormone melatonin. It is well documented that this circadian rhythm in melatonin production is coupled to the external light-dark cycle, being influenced not only by the 24-hour changes in environmental light, but also by the lengthening or shortening of the daylength during the different seasons

(Vollrath, 1981; McNulty and Prechel, 1992).

It is, however, lesser-known if the circadian rhythms in which the pineal gland is involved might also be altered by the lunar cycles. Although the main biological rhythm synchroniser is the environmental light and the moon influence on geomagnetism is weak, effects of artificial magnetic fields on the pineal gland have been previously reported (Wilson et al., 1981; Semm, 1983; Cremer-Bartels et al., 1983, 1984; Reuss and Olcese, 1986; de la Guardia et al., 1988; Giménez et al., 1991). Since the pineal gland is functionally dependent on the sympathetic system, and the sympathetic adrenal system is, in turn, sensitive to magnetic stimuli, the effect of the latter on the pineal may, therefore, be indirect (Reuss and Olcese, 1986; Martínez-Soriano et al., 1992).

On the other hand, morphological and physiological studies suggest a possible division of the pineal parenchyma into an external or "cortical" and another central or "medullar" layer (Quay and Renzoni, 1966; Romijn, 1975; Diehl, 1981; Semm, 1983; Becker and Vollrath, 1983; Diehl et al., 1984; Karasek et al., 1990) and even many more (Hira et al., 1998). Determinations of the size of the pinealocytes in the peripheral and central regions at various time points during a 24-hour period and different seasons in rats, have been carried out by different authors regarding regional and day-night differences in pinealocyte size (Jung and Vollrath, 1982; Cimas et al., 1992).

On the basis of this premise, we have studied the possible variations that the photophasic, seasonal and lunar cycles can exert on the nuclear size of the pinealocyte of the rat in the cortical and in the medullar layers.

### **Materials and methods**

Forty male Wistar rats (mean body weight  $240 \pm 37$ g) subjected to the same nutritional and environmental conditions (temperature, 18-20 °C, natural light) were studied. The rats were divided into 8 groups of 5 animals each. Experiment was carried out in winter (four groups) and in spring (the other four groups). Half of the groups of each season (two groups) were sacrificed during the new-moon days, one group in the photophase (between

10:00 h and 12:00 h) and the other in the scotophase (between 00:00 h and 02:00 h), whereas the other half were during the full moon days at the same two time-points.

The animals were fixed by perfusion with Karnovsky's (1965) solution. The pineal glands were removed and post-fixed in osmium tetroxide for 90 minutes, dehydrated in graded series of acetone, stained with 5% uranyl acetate and 1% phosphotungstic acid in 70% acetone, and finally embedded in Epon resin.

Nuclear size was determined from semithin sections (1µm) obtained with an ultramicrotome and stained with toluidin blue. Measurements from 100 cortical and 100 medullar pinealocyte nuclei have been reported to be sufficiently representative of each animal. The 200 nuclei measured per rat came from four semithin sections (50 nuclei each section and 25 nuclei each pineal region) taken from the pars distalis of the pineal gland. Each one of the four selected sections was at least 15 µm away from the preceding one, in order to avoid including the same pinealocyte nucleus in more than one section. Only clearly visible pinealocyte nuclei were considered. The nuclear volume (V) was calculated using Jacoby's (1935) formula from the following karyometric indexes: greater diameter (A), lesser diameter (B) and a constant (k).

$$V = \frac{1}{6} \cdot A \cdot B^2 \cdot k$$

The statistical evaluation of the data was made after a descriptive study. Comparative analysis of the variables was carried out by contrast and significance test. P-values smaller than 0.05 were considered statistically significant. When comparing the means of more than two variables, we used analysis of variance (ANOVA).

## Results

### Global description

Nuclei of the pinealocytes, considered without distinguishing between cortical and medullar zones, reached a bigger volume during the dark phases than during the light phases of the winter season, regardless of the lunar cycle, and the spring full-moon days. Likewise, nuclei reached a bigger volume during full moon than during new moon in winter and in the spring scotophases. Finally, when we analysed the evolution between both seasons, bigger nuclear volumes were always found in winter, except for the photophase of the new-moon days where highest measurements corresponded to spring (Fig. 2).

Each one of the environmental or external rhythms considered (light/dark, seasonal and lunar cycles) exerted, for themselves, a statistically significant individual influence. When we considered the seasonal factor, the differences were relevant and highly significant (F-test: 21.738;  $p < 0.0001$ ), like the differences between the lunar phases considered (F-test: 27.573;  $p < 0.0001$ ) and between the day and night periods (F-test: 34.612;  $p < 0.0001$ ). If we considered the joint interaction of the three external factors, we could see a statistically significant influence on the variations of the nuclear sizes (F-test: 13.557;  $p < 0.0002$ ) (Table 1).

The influence of the interactions between the main external factors on the nuclear volume variations was also studied. The analysis of the interaction between the seasonal rhythm and the lunar phases showed an important volumetric decrease when the seasonal rhythm combined with the full moon level and the inverse effect when it combined with the new moon. The same inverse interaction was observed between the seasonal and

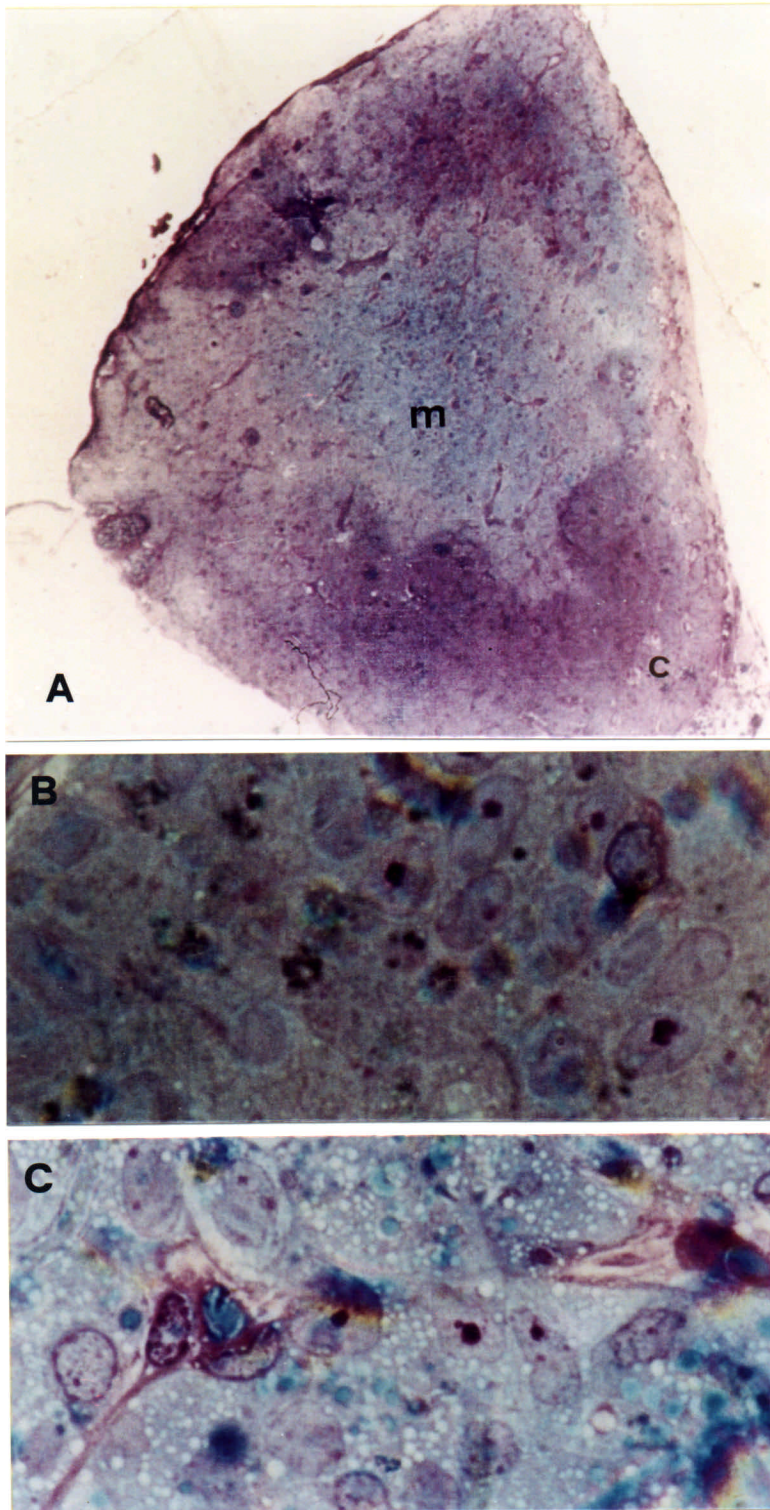
**Table 1.** Multiple analysis of the variance. Four-way analysis. Global analysis.

SOURCE	df	SUM OS SQUARES	MEAN SQUARES	F-TEST	P VALUE
Season (A)	1	1056521.479	1056521.479	21.738	.0001
Lunary Phase (B)	1	1340113.665	1340113.665	27.573	.0001
AB	1	2997313.067	2997313.067	61.671	.0001
Photophase (C)	1	1682194.48	1682194.48	34.612	.0001
AC	1	2351977.998	2351977.998	48.393	.0027
BC	1	5121.712	5121.712	0.105	.7455
ABC	1	658876.727	658876.727	13.557	.0002
Location (D)	1	4376406.446	4376406.446	90.046	.0001
AD	1	1585731.061	1585731.061	32.627	.0001
BD	1	24971.365	24971.365	0.514	.4735
ABD	1	2756868.058	2756868.058	56.723	.0001
CD	1	439166.46	439166.46	9.036	.0027
ACD	1	395878.446	395878.446	8.145	.0043
BCD	1	2069602.267	2069602.267	42.583	.0001
ABCD	1	5593316.77	5593316.77	115.084	.0001
Error	7984	388038433.743	48602.008		

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photophasic rhythms. However, a lack of interaction was appreciated between the lunar cycles and the light-dark phases. A parallelism of the variation of the nuclear

volumes in each lunar cycle could be appreciated related to the light-dark phases. As a consequence, there was an increase of the nuclear size due to the effect of both the



**Fig. 1.** A. Low-power photomicrograph of a coronal section of the pineal gland showing the cortical (c) and medullary (m) layers. High-power photomicrograph of the peripheral (B) and central layers (C).

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**Table 2.** Multiple analysis of the variance. Three-way analysis. Analysis of the external factor influence on the variations of the nuclear volumes of the cortical area.

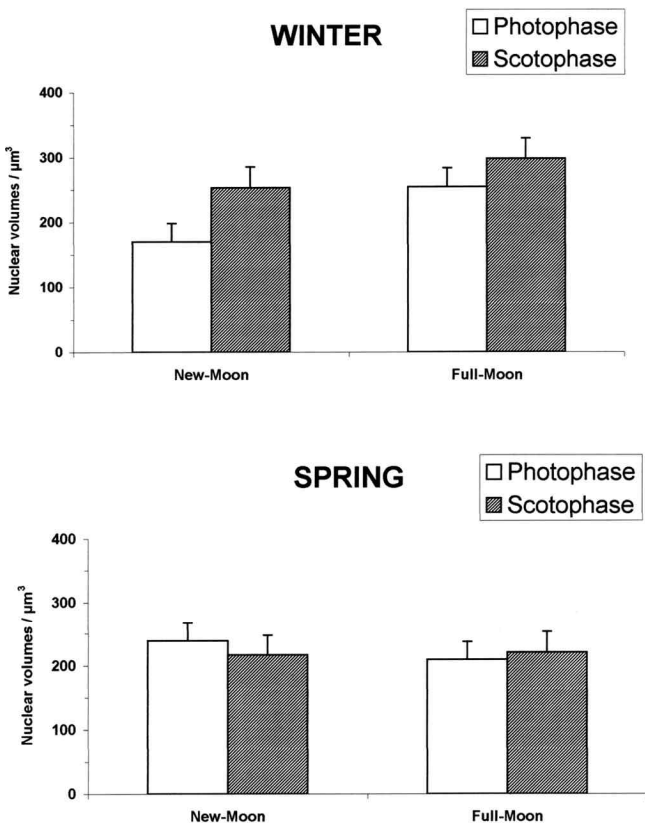
SOURCE	df	SUM OF SQUARES	MEAN SQUARE	F-TEST	P VALUE
Season (A)	1	26769.702	26769.702	0.588	.4432
Lunary Phase (B)	1	865475.472	865475.472	19.014	.0001
AB	1	2512.915	2512.915	0.055	.8143
Photophase (C)	1	201167.006	201167.006	4.42	.0356
AC	1	408994.341	408994.341	8.985	.0027
BC	1	934406.139	934406.139	20.528	.0001
ABC	1	1206381.602	1206381.602	26.504	.0001
Error	3992	181707111.463	45517.813		

night and the full moon (Fig. 3).

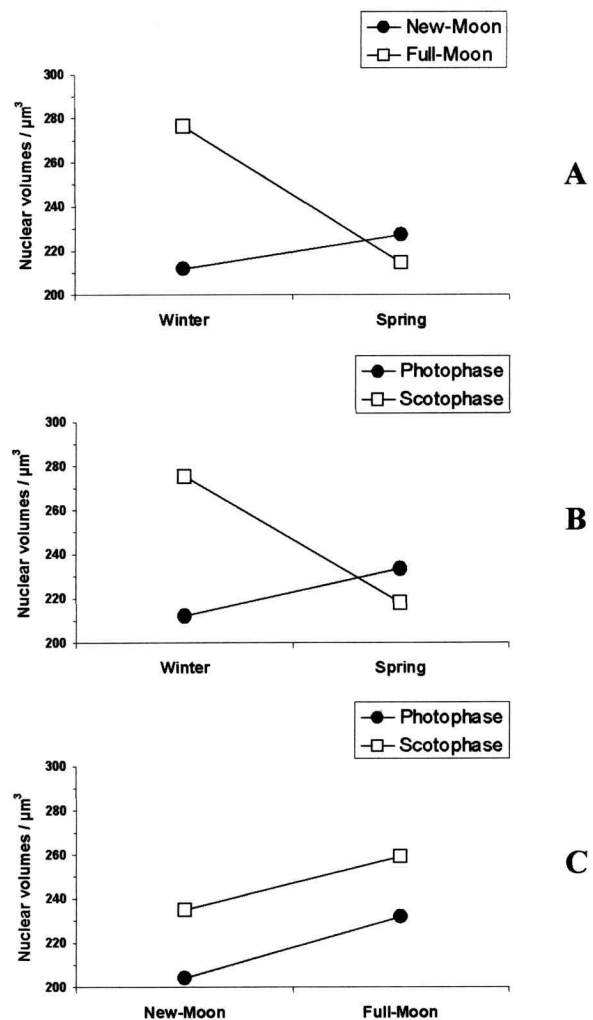
Local description

When the location of the pinealocyte into the cortical or into the medullar layers was considered the nuclear sizes of the medullar pinealocytes were always bigger than those of the cortical ones, except for the photophases of both the winter new-moon days and the spring full-moon days when cortical values were higher, and for the scotophase of the spring full-moon days when nuclear size of the pinealocytes in both layers was similar (Fig. 4).

The analysis of the external factors influence on the variations of the nuclear volumes of pinealocytes of the cortical area showed a statistically significant difference between the light-dark phases (F-test: 4.42;  $p < 0.0356$ ). The existing difference between both lunar phases is also



**Fig. 2.** Histogram showing the nuclear volume variations of the pinealocytes. Global description.



**Fig. 3.** Influence of the interactions between external factors on the variations of the nuclear volume of the pinealocytes. **A.** Seasonal rhythms/lunar phases. **B.** Seasonal rhythms/photophasic rhythms. **C.** Lunar phases/photophasic rhythms.

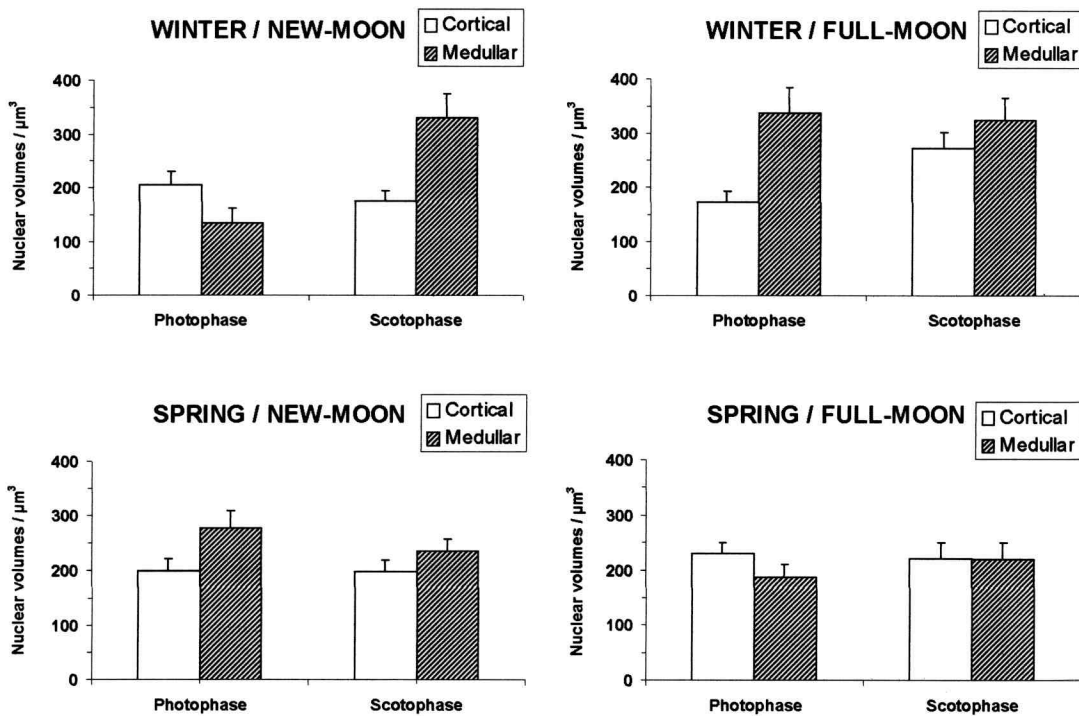
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significant (F-test: 19.014;  $p < 0.0001$ ), but not that between the seasons (F-test: 0.588;  $p < 0.4432$ ). The joint interaction of the three external factors was strongly

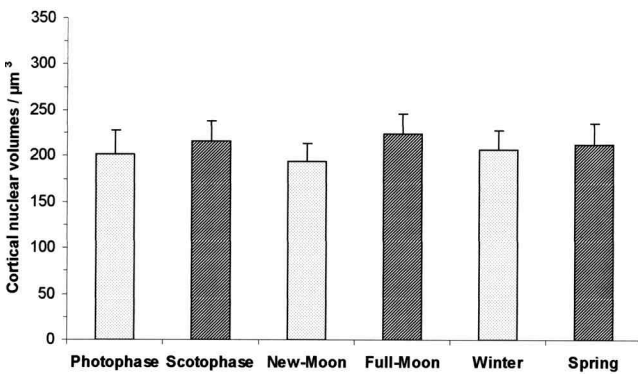
significant (F-test: 26.504;  $p < 0.0001$ ) (Table 2). These differences in the nuclear volumes of the cortical pinealocytes according to the influence of each external

**Table 3.** Multiple analysis of the variance. Three-way analysis. Analysis of the external factor influence on the variations of the nuclear volumes of the medullar area.

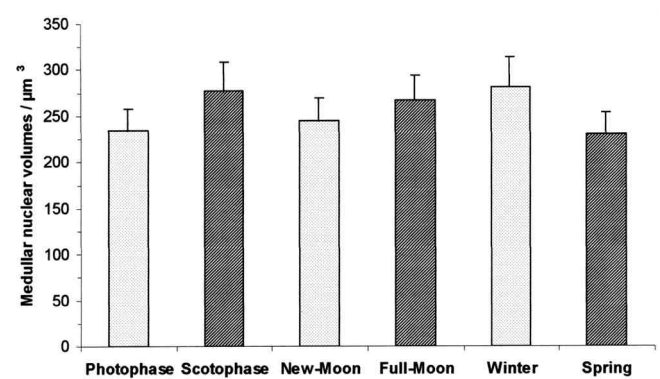
SOURCE	df	SUM OF SQUARES	MEAN SQUARE	F-TEST	P VALUE
Season (A)	1	2615482.838	2615482.838	50.603	.4432
Lunary Phase (B)	1	499609.557	499609.557	9.666	.0001
AB	1	5751668.211	5751668.211	111.281	.8143
Photophase (C)	1	1920193.934	1920193.934	37.151	.0356
AC	1	2338862.103	2338862.103	45.251	.0027
BC	1	1140317.839	1140317.839	22.062	.0001
ABC	1	5045811.895	5045811.895	97.624	.0001
Error	3992	206331322.28	51686.203		



**Fig. 4.** Histogram of the comparison between the seasonal, photophasic and lunar cycles and the peripheral/central karyometric index.



**Fig. 5.** Histogram showing the external factor influence on the variations of the nuclear volume of the cortical area.



**Fig. 6.** Histogram showing the external factor influence on the variations of the nuclear volume of the medullar area.

factor were graphically represented (Fig. 5).

In the case of the medullar layer, the analysis of the external factors influence on the variations of the nuclear volumes showed that all the external rhythms exerted a statistically significant influence (Table 3). The variations of the nuclear volumes of the medullar pinealocytes in each situation could also be graphically represented (Fig. 6).

Finally, we analyzed the influence of the location factor, cortical or medullar, on the nuclear variations of the pinealocyte. Individually considered the location factor exerted a statistically significant influence (F-test: 90.046;  $p < 0.0001$ ). Likewise, the joint influence of all the factors considered in the study, the external and the location factors, was statistically significant (F-test: 115.084;  $p < 0.0001$ ) (Table 1).

In general, the nuclear volume of the pinealocytes was higher in the central area of the pineal gland than in the peripheral one (Fig. 7).

## Discussion

The existence in the pineal gland of a peripheral layer and a central layer functionally different goes back to the sixties with the works of Renzoni and Quay (1964) and Quay and Renzoni (1966). They were the first ones to point out possible differences in the size of the pinealocyte nucleus and nucleolus. Subsequently, Romijn (1975) provoked again the controversy suggesting the existence of two zones with a different dye craving for the toluidin blue in the rabbit pineal.

Since then, the results of the works developed in this sense have been contradictory: in some cases the existence of such differences has been denied (Welsh et al., 1979) and in other instances it has been accepted.

In this last case, we have to highlight the fact that some researchers conditioned the existence of two zones to the studied pineal region (Becker and Vollrath, 1983; Diehl et al., 1984; Matshushima et al., 1990; Hira et al., 1998), to the seasonal phase when the assessment is made (Popova et al., 1975), and to sex (López-Iglesias et

al., 1987), age (Hira et al., 1989), the time point of the day (Matshushima et al., 1983, 1989), or the combined seasonal and circadian phases (Cimas et al., 1992).

These morphological differences supported what had also been suggested from the electrophysiological point of view. Semm et al. (1981) indicated the existence of pinealocytes groups with a different response capacity to the stimulus according to their location in the pineal parenchyma. They were the first neurobiologists that pointed out the existence of cellular "islets" with independence of response inside the pineal.

The results of our work clearly showed that there were fluctuations in the size of the nuclear volumes according to the peripheral or central location of the pinealocyte and in dependence, for importance of influence, of the seasonal phase and the lunar cycle, although their effect would be executed in a clear interaction with each other.

These results also supported the importance, previously reported, of the season, photophase and topography in the study of the morphofunctionality of the pineal gland (Popova et al., 1975; Matshushima et al., 1983, 1989, 1990; Cimas et al., 1992; Sakai et al., 1996; Hira et al., 1998). There were, however, little differences regarding the absolute value of the nuclear values of the pinealocytes that, according to these authors, were higher in the peripheral zone. These differences could have their bases in the different methodology used by them and us, but in any case the objective of our work was not to obtain absolute values but relative ones in a study of a dynamic nature, oriented to analyze the variability of the pinealocyte before the influence of some determined natural factors, a variability that can also be an explanation of the differences between the values found in both of them.

We share the opinion of authors like Quay and Renzoni (1966), de la Guardia et al. (1988), Giménez et al. (1991) and Guillot et al. (1995), who postulated that the medullar layer was more susceptible of modifications before natural stimulus (light and gravity changes) and experimental ones (magnetic fields induction, irradiation with coherent light).

Effectively, from our results we could derive that external factors (like light-dark, seasonal and lunar cycles) were influent, both in isolation and associated to each other, on the pinealocytes nucleus. This influence could vary according to the peripheral and central location of the nucleus, being the central or medullar layer which showed the biggest fluctuations under the action of these external factors.

This evidence, joined to the opinions of the authors mentioned above and to the existence of a latitude factor verified a long time ago (Quay, 1963a,b; Cuello and Tramezzani, 1969), showed one of the possible reasons for discrepancy or for the occasional coincidences in the results of the different papers published about these aspects.

Finally, the recent work of Hira et al. (1998), that revealed the existence of a gradient of variation of the

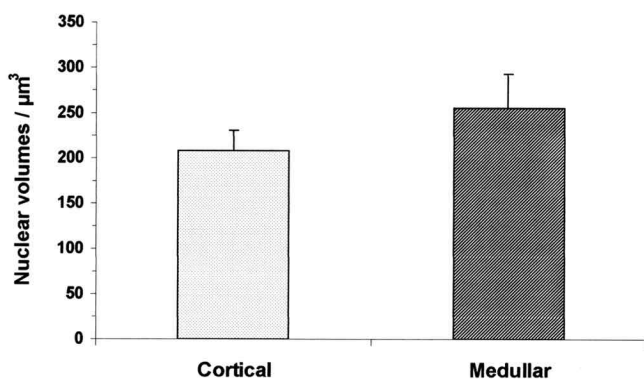


Fig. 7. Histogram of the comparison between cortical and medullar nuclear volume mean values.

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nuclear size from the periphery to the central zone and from proximal to distal, in relation with the distribution of vessels and nerves, could question, together with the existing electrophysiological data, if the pineal gland is really a multifunctional organ arranged in “stratus” or “islets” that regulate different endocrine-metabolic rhythms and that, of course, requires a strict methodology regarding the circadian, synodic, seasonal, sexual and topographical conditions to be considered when making a work about it.

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