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Evolution of synaptic body numbers in the pineal gland of the cat over a 24-hour period in spring

A descriptive morphometric and statistical analysis

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SUMMARY

Eighteen cross-bred male cats (2650±750 g) were kept for three weeks with environmental lighting and under the same feeding (*ad libitum*) and temperature conditions (18-22°C). The animals were sacrificed in the spring in groups of three at 4 h intervals with the following GMT time sequence: 06:00, 10:00, 14:00, 18:00, 22:00, and 02:00 hours. During the circadian cycle, significant variations were observed in the number of synaptic ribbons and synaptic spherules in the pineal glands of the animals, with maximum and minimum values at 02:00 and 14:00 hours, and at 10:00 and 02:00 hours, respectively. Analysis of variance (ANOVA) showed these differences to be statistically significant (F-ratio: 43.513; $p<0.0001$ for synaptic ribbons, and F-ratio: 6.0; $p<0.005$ for synaptic spherules). The number of synaptic ribbons was found to be significantly higher ($p<0.0001$) at night (67.3%) than in the daytime (32.6%), while the number of synaptic spherules was 59.1% and 40.8% in the daytime and at night, respectively. The polynomial correlation between the evolution of the synaptic ribbons and the time point was very positive (R = 0.8). The circadian variations observed in the intermediate synaptic bodys (ISBS) population were not significant, either for triangular synaptic bodies (TSB) ($p<0.07$) or for rectangular synaptic bodies (RSB) ($p<0.2$). Finally, the total number of synaptic ribbons observed in the photophase merged to form "fields" of three or more elements. These fields were more commonly observed in pinealocyte prolongations than in areas close to the nucleus.

Key Words: Pineal body – Synaptic ribbons – Synaptic bodies – Circadian rhythm – Cat

INTRODUCTION

Synaptic ribbons (SR) are ultrastructural organelles found in the prolongations and areas close to the nucleus of pinealocytes in all mammal species investigated to date (Vollrath, 1973; Kurumado and Mori, 1977; Matshusima et al., 1983; Martínez-Soriano et al., 1984; Struwe and Vollrath, 1990). The function of these structures is not known, although under natural conditions their number varies as a function of different light- dark phases (Lues, 1971; Vollrath, 1973; Cimas et al., 1987). In this sense, the lowest values are observed in the daytime while the highest number are found at night. The number of synaptic ribbons also varies according to the experimental conditions (Vollrath and Howe, 1976; Karasek, 1976; Kurumado and Mori, 1980; King and Dougherty, 1982). These organelles are related to the adrenergic innervation of the pineal gland (Romijn, 1975; King and Dougherty, 1982; Karasek et al., 1983; González and Alvarez-Urìa, 1986) and to its indol and amine regulation mechanisms (Mc Nulty and Fox, 1992).

Spherical bodies (SS), with a dense matrix and surrounded by clear vesicles, have also been detected in a number of species, although their pattern of circadian variation is reported to vary considerably from one spècies to another (Vollrath, 1981; Martínez-Soriano et al., 1984, 1999; Khaledpour and Vollrath, 1987; Karasek and Vollrath, 1989).

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Other forms, known as intermediate structures (ISB), are ovoid (OSB), rectangular (RSB) and, trapezoidal or kidney-shaped, and have been described in the hamster (Matshusima et al., 1983), rabbit (Martínez-Soriano et al., 1984, 1999), guinea-pig (Khaledpour and Vollrath, 1987), cow, sheep and pig (Struwe and Vollrath, 1990). They are present in fewer numbers than the synaptic ribbons, but exhibit a similar circadian evolution.

To date, we are unaware of any study in which the characteristics and circadian evolution of synaptic bodies has been investigated in the pineal gland of the cat. The aim of the present study was to examine the circadian evolution of the number of synaptic bodies in this animal model, contrasting the observed analogies and differences with respect to other species.

Materials and Methods

Eighteen cross-bred male cats weighing 2650 ± 650 g were used. The animals were kept for three weeks under environmental lighting and under the same feeding (standard artificial diet and tap water *ad libitum*) and temperature conditions (18-22°C). The animals were sacrificed between May 5 and 6, by intraperitoneal injection of sodium thiopental (40 mg/kg) in groups of three at 4-hour intervals with the following GMT time sequence: 06:00, 10:00, 14:00, 18:00, 22:00 and 02:00 hours.

All animals were subjected to intracardiac 5% glutaraldehyde perfusion after saline cleansing. Once removed, the pineal glands were post-fixed in osmium tetra- oxide and dehydrated in a graded acetone series. The pieces were then contrasted with uranyl acetate and embedded in Epon. Samples were placed on 65 x 65 Fm grids and contrasted with lead citrate according to Reynold's (1963) method.

The number of synaptic bodies was calculated by counting those observed in 8 grid squares (33,800 μm^2). This procedure was carried out once for each animal under 12,000x magnification. The results are given as total numbers and mean per 20,000 μm^2.

Statistical analysis of the data involved a prior descriptive study. Comparative analysis of the variables was performed by contrast and significance testing, accepting an error of (<0.05. Since the variables were continuous and quantitative, paired comparisons were made with a measurements comparison test. For this, the Student t-test was chosen in view of its reliability. Analysis of variance (ANOVA) was used to compare more than two variables simultaneously. The relationships between certain variables were studied by applying classical correlation techniques. Determination of the true degree of relationship between the variables selected was performed with the Pearson correlation test. Calculations were made with the StatWorks program, supported by the Cricket Graph II package, on an Apple Macintosh SIIi microcomputer.

Results

The presence of synaptic-like bodies in the cat was similar to that described for other species, at least in spring, and was represented by ribbons, spheres and intermediate bodies. Regarding the latter, however, we only observed scarce populations with a triangular and rectangular shape. The whole of this heterogeneous population was observed as isolated bodies (Figs. 1, 2 and 3), bodies in pairs (Fig. 4), or bodies grouped as "fields" of more than two elements (Fig. 5) The ribbon forms were always the most abundant presentation and the intermediate forms the most limited.

The bodies were located either deep inside the cytoplasm or, mostly, in zones close to the membrane.

A total of 1052 synaptic bodies (SB) was counted, of which 80.6% (848) corresponded to synaptic ribbon, (SR) 13.9% (147) to spheres,(SS) and only 5.4% (57) to intermediate forms. In turn, of these 2.2% (24) were triangular (TSB) and 3.1% (33) rectangular (RSB) (Table 1).

Of the 848 synaptic ribbons observed along the 24-hour cycle, 571 (67.3%) were visualized in the hours corresponding to darkness (06:00, 22:00, 02:00) and the remaining 277 (32.6%) in the daytime hours (10:00, 14:00, 18:00).

The circadian evolution of the density of synaptic bodies (SB) at each time-point, is shown in Figure 6 and Table 2. The evolution of the SR clearly tended to increase between 14:00 (minimum value) and 02:00 hours (maximum, or peak value). The number of SR was significantly

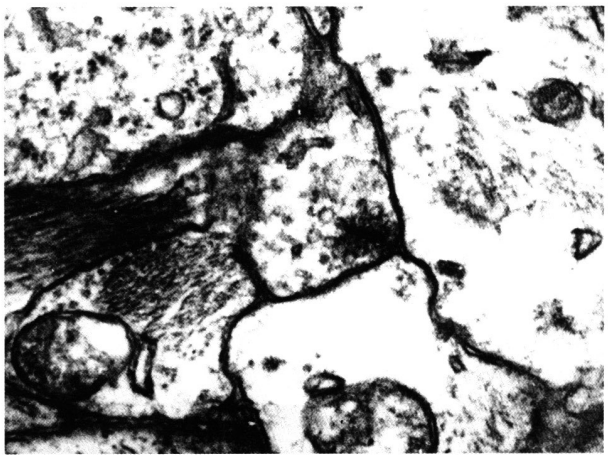

Fig. 1.- "Synaptic" ribbon (SR) appended to membrane. x24,000.

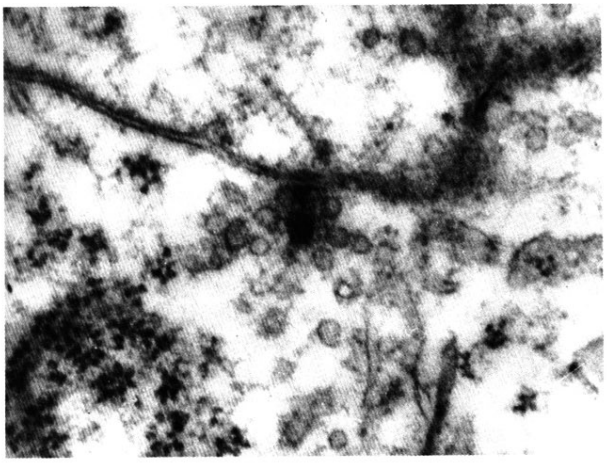

Fig. 2.- Cuadrangular "synaptic" body (CSB) near to membrane. x24,000.

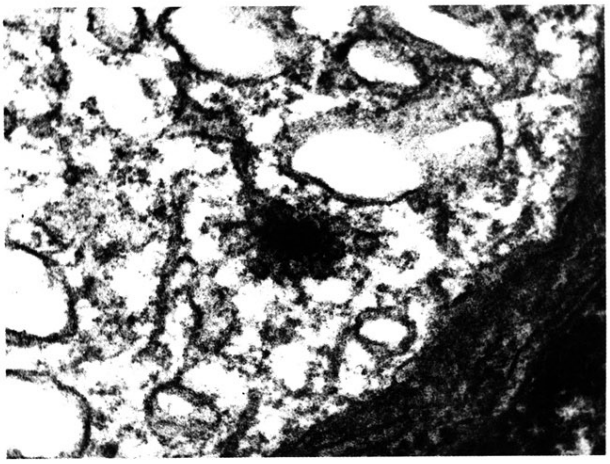

Fig. 3.- "Synaptic" spherule (SS). x24,000.

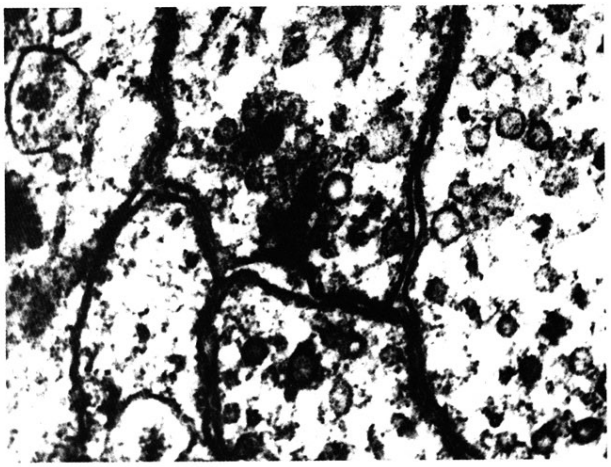

Fig. 4.- Two "synaptic" (SR and SS) near to membrane in pinealocyte prolongation. x24,000.

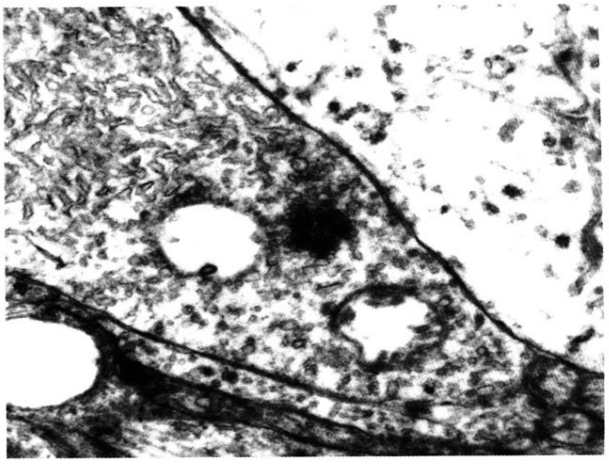

Fig. 5.- Cluster of "synaptic" spherules inside pinealocyte prolongation. x24,000.

larger ($p<0.0001$) during the hours of darkness than in the daytime (Fig. 8, Table 3). These observations reflect a clear circadian evolution, with high and low, statistically significant, fluctuations over 24 h. (ANOVA; F-ratio: 43.513, $p<0.0001$).

The regression and correlation studies between the variables studied, i.e., the number of ribbons and time points (regarding the latter as the independent variable), revealed a marked positive correlation between the two variables ($R = 0.8$).

Interestingly, in the daytime hours (10:00 to 18:00 hours) the number of grouped ribbons (i.e., ribbon fields) was considerably greater than the number of isolated or paired ribbons (180 (65%) and 97 (35%), respectively), although the number of fields was found to be greater between 22:00 and 02:00 hours - no doubt due to the increased presence of bodies in the dark period.

Of the 277 ribbons counted during the daytime hours, 221 (80%) were observed between 10:00 and 14:00 hours, i.e., the large majority of the synaptic ribbons found between the morning and mid-day were grouped. Moreover, these "fields" or groupings were observed in discretely greater (55.6%) numbers in the pinealocyte prolongations than in the perikarya.

In the hours of darkness, (22:00 to 06:00 hours) the grouped forms were observed in fewer numbers than the isolated or paired presentations (137 -23.9%- and 434 -76%-, respectively).

The evolution of the ribbon "fields" along 24 h, as compared with that of SR, is shown in Figure 7. Analysis of variance of the fluctuations seen in the field proved to be significant (F-ratio: 59.385, $p<0.0001$).

The spheres, fewer in number than the ribbons, also followed a statistically significant (F-ratio: 6.2, $p<0.005$) circadian evolution, although

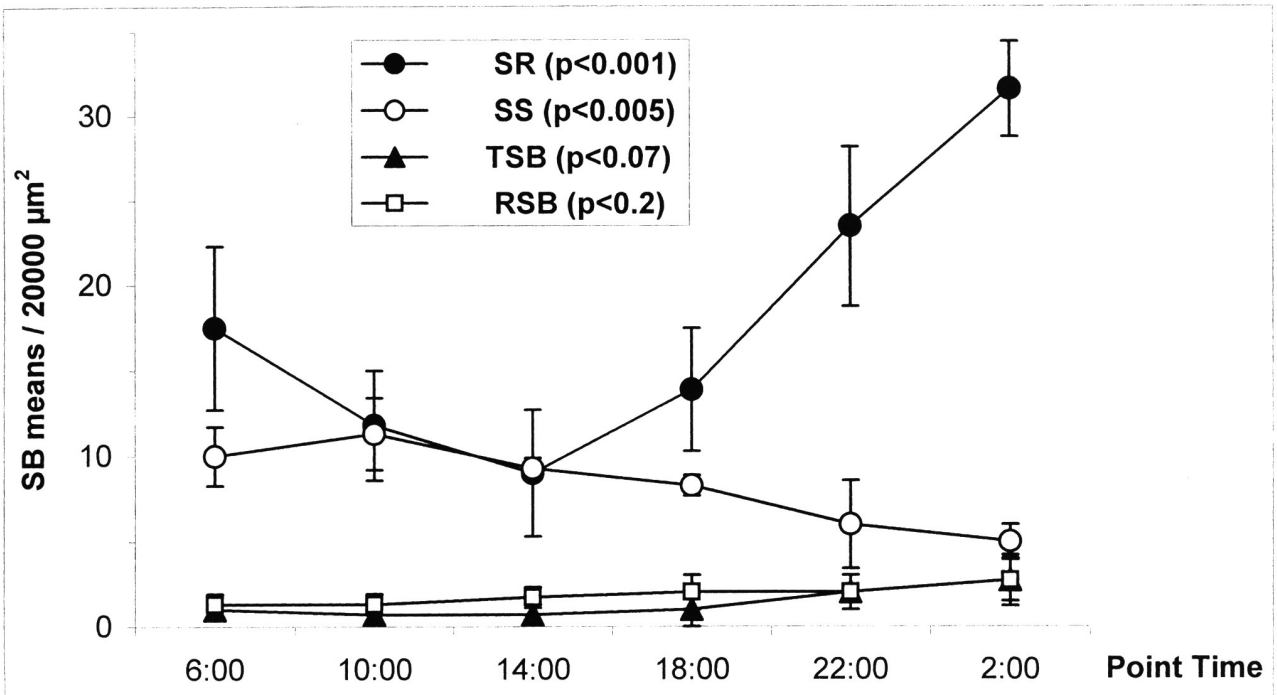

Figure 6.- Circadian evolution of synaptic bodies (SB)

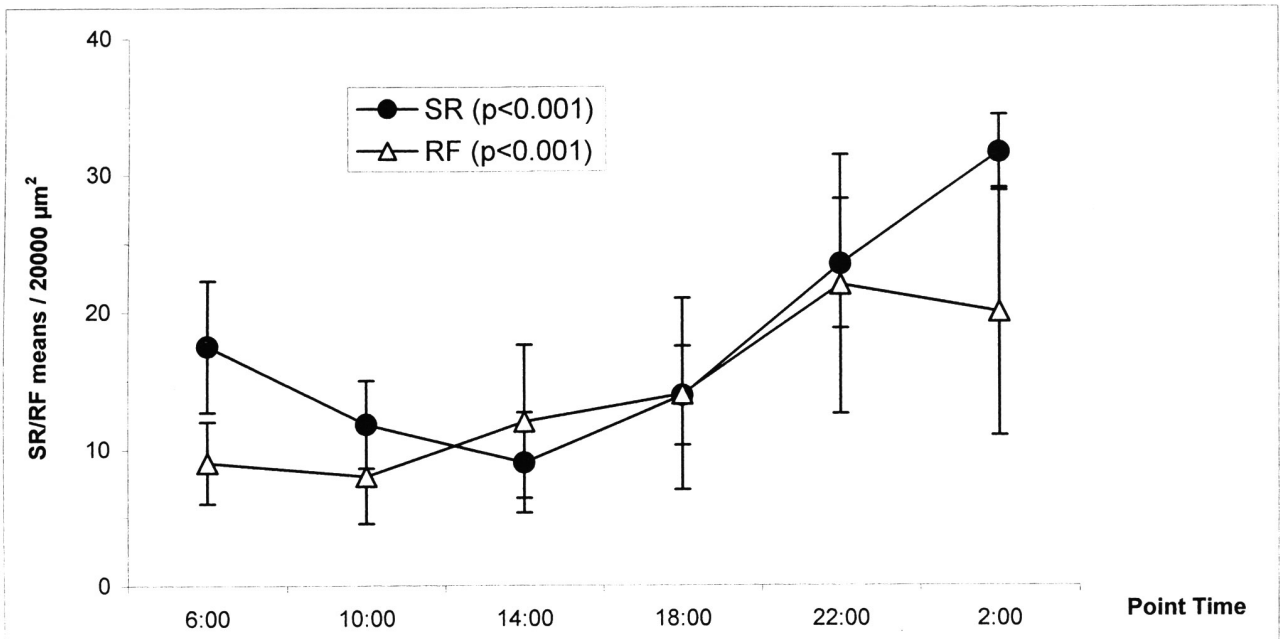

Figure 7.- Circadian evolution of synaptic ribbons (SB) and ribbon fields (RF).

curiously this was inverted with respect to that of the ribbons; thus, a higher peak was observed at 10:00 hours, with a second, lower peak at 02:00 hours (Fig. 6). Between 10:00 and 14:00 hours both two populations (SR and SS) had similar mean values.

The spheres were slightly more abundant (87) in the daytime hours (59.1%), ($p<0.005$) and many of them (54.3%) were found bound to the membrane, particularly in the daytime, when their presence in the form of pairs was also frequent (22.3%).

The intermediate forms (ISB) followed a different trend along the 24 h study period although their rectangular or triangular shape continued to be characteristic.

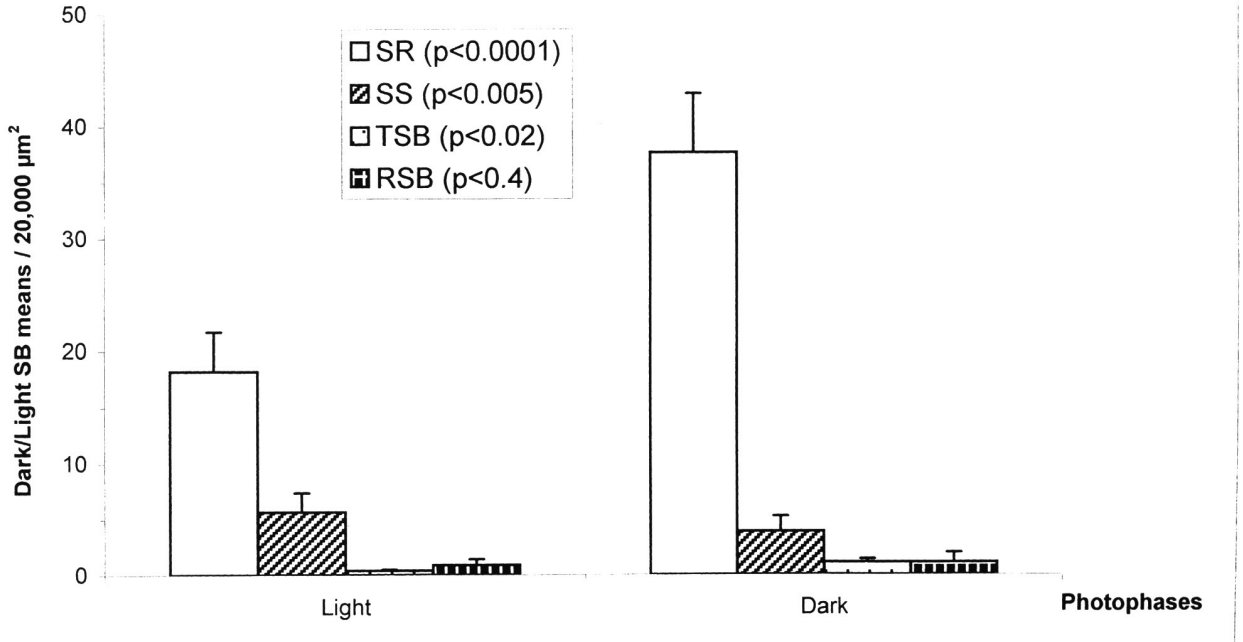

Figure 8.- Dark/Light differences between the different synaptic bodies populations.

The rectangular forms (RSB) tended to increase in number from a minimum presence at 10:00 hours to a maximum abundance at 02:00 hours (Fig. 6), in this sense reflecting the evolution of the ribbons, as described above. However, these variations along the course of the day were not significant (ANOVA; F-ratio: 3.1, $p<0.3$). Their number at night was in turn higher than during the day, but again the differences lacked statistical significance (t-test, $p<0.4$) (Fig. 8, Table 3).

Table 1.- Percentage and distribution of "synaptic bodies" (SB) in the pineal gland of the cat during the spring.

Number and distribution	
Total	1052
Synaptic ribbons	848 (80.6%)
Dark phase	571 (67.3%)
Light phase	277 (32.6%)
Synaptic spherules	147 (13.9%)
Dark phase	60 (40.8%)
Light phase	87 (59.1%)
Intermediate forms:	57 (5.4%)
Dark phase	35 (61.4%)
Light phase	22 (38.5%)
Triangular bodies	24 (2.2%)
Dark phase	17 (75.0%)
Light phase	7 (25.0%)
Rectangular bodies	33 (3.1%)
Dark phase	18 (54.5%)
Light phase	15 (45.4%)

Finally, the evolution of the triangular forms (TSB) was found to be more irregular, with peaks at 18:00 and 22:00 hours. These differences along the course of the day were not significant (ANOVA; F-ratio: 2.9, $p<0.07$). In contrast, the greater increase in these structures in the dark period was statistically significant (t-test, $p<0.02$) (Fig. 8, Table 3).

Discussion

The results obtained in the present study reflect the 24-hour circadian evolution of a heterogeneous population of synaptic bodies in the pineal gland of the cat. In this sense, the synaptic bodies observed were classified as synaptic ribbons (SR), synaptic spherules (SS), and triangular (TSB) or rectangular (RSB) synaptic bodies.

Regarding the evolution of the SR over the 24-hour solar cycle, the results reported in the literature coincide for all species investigated to date, i.e., the guinea-pig (Vollrath, 1973), rat (Kurumado and Mori, 1977; Cimas et al., 1987), baboon (Theron et al., 1979), chipmunk and ground squirrel (Karasek et al., 1983), and rabbit (Martínez-Soriano et al., 1984, 1999). In this sense, SR have been found to exhibit a circadian rhythm, with a greater abundance of elements in the dark hours, and a minimum in the daytime. Our own observations in the cat agree with these descriptions.

The evolution curve and the results of the analysis of variance indicate a significant circadian variation in the number of synaptic ribbons along 24 h (Fig. 5). Likewise, the values obtained

Table 2.- Means and standard deviation of the different populations of "synaptic bodies".

Point-time	SR	SS	TSB	RSB
06:00	17.5 ± 4.8	10.0 ± 1.7	1.0 ± 0.0	1.3 ± 0.6
10:00	11.8 ± 3.2	11.3 ± 2.1	0.7 ± 0.6	1.3 ± 0.6
14:00	9.0 ± 3.7	9.3 ± 0.6	0.7 ± 0.6	1.7 ± 0.6
18:00	13.9 ± 3.6	8.3 ± 0.6	1.0 ± 1.0	2.0 ± 1.0
22:00	23.5 ± 4.7	6.0 ± 2.6	2.0 ± 1.0	2.0 ± 1.0
02:00	31.6 ± 2.8	5.0 ± 1.0	2.7 ± 1.5	2.7 ± 1.2

Table 3.- Dark / light SB means and standard deviation / 20,000 μm^2.

Light	**SR**	18.2 ± 3.5	**SS**	5.7 ± 1.6	**TSB**	0.4 ± 0.2	**RSB**	0.9 ± 0.5
Dark	**SR**	37.6 ± 5.5	**SS**	3.9 ± 1.4	**TSB**	1.1 ± 0.3	**RSB**	1.1 ± 0.9

point to a marked correlation between the number of ribbons and the hourly variations (R = 0.895). Thus, 89.5% of the variations can be attributed to the hourly rhythm -particularly as regards the presence or absence of light- while the remaining 10.5% would be due to unknown factors. This agrees with our earlier results in the rat (Martínez-Soriano et al., 1992).

The functional significance of these structures is not known. In mammals, they have been related to adrenergic innervation, an inverse correlation having been demonstrated between the number of synaptic bodies and the concentration of adrenergic endings in the rat (Karasek et al., 1983).

Similar observations have been made in the cat (González and Alvarez-Urìa, 1986), in which postganglionic electrical stimulation of the cervical sympathetic system was found to induce a decrease in the number of "dense cores" within the adrenergic endings concomitant with an increase in the number of synaptic ribbons.

Studies have also described the correlations between the increase in the number of synaptic ribbons and the embryonic development of innervation in chicks (Robertson et al., 1990). However, the fact that in most species sympathetic denervation or continuous illumination causes an increase in the number of ribbons (Lues, 1971; Vollrath and Maitra, 1986; Roux et al., 1977), together with the observation that the addition to cultured pineal tissue of beta-receptor agonists and antagonists induces no changes in the number of synaptic ribbons in the rat (Seidel et al., 1990) or in the response of the number of synaptic ribbons to isoproterenol (Vollrath et al., 1995), raise reasonable doubts as to the true interpretation of these findings and suggests the existence of adrenergic signal-modulating factors.

Of the factors possibly involved, special mention should be made of environmental illumination in the sense that it could modulate the photophase production of inducible cAMP early regulator (ICER), through which transcription is regulated for cAMP synthesis (Stehle et al., 1993), this being the secondary messenger of fundamental importance in the formation of synaptic ribbons.

Another factor to be considered is neuropeptide Y, which regulates noradrenergic transmission in the rat pineal body at both pre- and postsynaptic level (Simmoneaux et al., 1994) through a clear circadian rhythm of secretion (Shinohara and Inouye, 1994).

The present findings regarding the spherules clearly point to the existence of a difference in the rhythmic course of the both the ribbons and the synaptic spherules, with a maximum peak at 10:00 in the morning, and a minimum at 02:00 hours. Curiously, however, this evolution of the spherules was found to be precisely the inverse of the evolution observed for the synaptic ribbons.

Differences in the evolution and behaviour of the spherules have already been discussed by other authors in different species and under different conditions (Lues, 1971; Romijn, 1975; Karasek et al., 1983; Kosaras et al., 1983; Martínez-Soriano et al., 1984; Vollrath et al., 1985). In the guinea-pig (Khaledpour and Vollrath, 1987), a clear day/night inversion has been described in the number of spherules with respect to the rods, as was also found in the present study. Similar situations seem to occur circanually in the proximal and intermediate portions of the rabbit pineal gland (Martinez-Soriano et al., 1999).

The functional significance of these structures has been much debated, although no consistent

hypothesis has yet been advanced. However, it does seem clear that their evolution depends on factors other than those that regulate the number of synaptic ribbons. The results of the present study and of those commented above (Khaledpour and Vollrath, 1987; Martínez-Soriano et al.,. 1999) point in this direction and even allow us to speculate about the possible existence within the pineal gland of some species with at least one system involving two coupled oscillators — one regulating the pineal function in the daytime, and another that would function at night— as already suggested by Pittendrigh and Daan (1976) and Illnerova and Vanececk (1982) in relation to the regulation of melatonin production. However, recently (Adly et al., 1999) evolutionary ultrastructural changes in the morphology of synaptic ribbons in the retina of the Balb/c mice have been described. After exposure to light in the morning (07:00-08:00 h.) the synaptic ribbons form distal swellings, giving rise to club-shaped profiles and they also decrease in length. The swellings appear to bud off, thus forming spherical synaptic bodies. It could explain the contact —points observed between SR and SS curves at 10:00 and 14:00 point-time described in this work (Figure 6).

Pineal intermediate synaptic bodies (ISB) are scarce in the pineal gland of the cat. Here, we only observed two different types of this type of structure: rectangular and triangular. They were found to be more abundant at night than in the daytime, although the differences were only statistically significant in the case of the triangular bodies. Similar differences have been documented in the pineal gland of the rabbit in the spring. Both types of intermediate synaptic bodies have also been reported in the pineal glands of the hamster (Matshusima et al., 1983), rabbit (Martínez-Soriano et al., 1984, 1999), guinea-pig (Khaledpour and Vollrath, 1987) and in artiodactyles (Struwe and Vollrath, 1990). In this sense, numerical and photophase fluctuations similar to those observed in the cat have been described, with the exception of the artiodactyles, in which the SIB were found to predominate over both the spherules and ribbons.

These results suggest a degree of uniformity regarding the numerical and photophase fluctuations of the intermediate body populations in rodents —a trend apparently similar to that found in the cat- although further research is required to confirm this. In contrast, the number of synaptic intermediate bodies in species such as the pig, birds, and cattle seems to be completely different, and in view of the lack of studies in these species, little can be said of their photophase fluctuations. What does seem to be apparent from the descriptions made to date on the pineal glands of rabbit and artiodactyles is the existence of seasonal photophase differences in the number of these structures. In this sense, it may be speculated that seasonal changes would modulate the fluctuations in the number of bodies or that interspecies variations might be due to the length of the gland, its degree of innervation and the number of nerve fibers involved. The most recent studies suggest that pineal innervation is not only adrenergic but also parasympathetic (Moller, 1992; Panshuwan-Fujito et al., 1991) and of central origin (Mikkelsen and Moller, 1990; Mikkelsen et al., 1991). This widens the possibility that each fiber might release different neuropeptides serving to modulate different functional aspects, the effective structural reflection of which could be the different types of synaptic bodies observed.

It is quite another question whether the different types of SB are separate entities or not.

Recent studies point to the notion that SR profiles are cross sections through thin plate- or lamella-like organelles (McNulty et al., 1986; Jastrow et al., 1997). However, without systematic reconstructions based on serial sectioning the true three dimensional structure of the SIB is difficult to assess. In any case, the still insufficient information available about these pineal structures prevents us from establishing any coherent hypothesis regarding their true functional significance.

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