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Influence of light/dark, seasonal and lunar cycles on serum melatonin levels and synaptic bodies number of the pineal gland of the rat

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Summary. Synaptic bodies (SB) are ultrastructural organelles observed in the pinealocytes of mammals. According to its shape, they have been classified into synaptic ribbons (SR), synaptic spherules (SS), and intermediate synaptic bodies (ISB). They have been related to the melatonin regulation and production mechanisms of the pineal gland. Circadian and circannual fluctuations of both melatonin and SB have been reported. The possibility that other external factors, apart from light-dark or seasonal cycles, might influence pineal function has been suggested. We studied the evolution of the number of SB and serum melatonin levels not only during light-dark and seasonal phases but also during lunar cycles. Forty male wistar rats were used. Experiment was first carried out in winter and repeated identically in spring. Each season, one group of animals was killed during the new-moon days and a second group during the full-moon days: half of both groups in the photophase and the other half in the scotophase. The number of SB was measured at electron microscopic level whereas serum melatonin levels were determined by radioimmunoassay techniques. Main results showed that SR number and serum melatonin levels were higher during scotophases, winter and full-moon days. The SS only showed a light predominance during winter, whereas predominance of the ISB was found only during the scotophases. These results support the influence of the photophasic factors on the SR and ISB variations. In the case of the SS the influence of the lunar cycles is always dependent on the other factors. Finally, the serum level of melatonin is clearly influenced by the photophasic rhythms and the seasonal periods but not by the lunar cycles.

Key words: Pineal gland, Synaptic bodies, Melatonin, Lunar cycles, Seasonal periods, Circadian phases

Introduction

There are a lot of examples of biological rhythms in the physiology and behavior of the mammals. One of the most comprehensively studied are the circadian rhythms, endogenously generated oscillations with periods of approximately 24 hours. These rhythms adapt to the environment, so the endogenous oscillators must be synchronized to the external 24-hour daily light-dark cycle. Light is the main cue responsible for the entrainment of these oscillators to the enviroment. Through a multisynaptic neural pathway involving the retinohypothalamic tract, the suprachiasmatic nucleus and the superior cervical ganglia, the pineal gland is capable to show light-dark cycle changes, transducing the photoperiodic information into a hormonal signal by the circadian secretion of its hormone, melatonin.

Related to the hormone regulation mechanisms of the pineal gland and with similar circadian fluctuations as the melatonin, have also been described, in the pinealocytes of mammals, ultrastructural organelles known as synaptic bodies (SB). According to shape, SB have been classified as synaptic ribbons (SR), the most frequent form (Fig. 1A), synaptic spherules (SS), the second most frequent SB (Fig. 1B) and intermediate synaptic bodies (ISB) the less observed SB, which include the ovoid-, quadrangular- and triangular-shaped SB (Fig. 1C). The SB has been reported in rats (Martínez Soriano et al., 1992a), hamsters (Matshusima et al., 1983), rabbits (Martínez Soriano et al., 1984, 1992b, 1999), cow, sheep and pig (Struwe and Vollrath, 1990).

Not only has it been observed circadian rhythm (Kurumado and Mori, 1977), it has also been observed a circannual behavior in the pineal gland. Seasonal influence on pineal metabolism related to photoperiod have been reported. Duration of the nocturnal melatonin secretion is increased with shortening of the daylength preceding the winter solstice and vice versa (McNulty and Prechel, 1992). A circannual rhythm in frequency of pinealocyte SB has also been described (Karasek et al., 1988).

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However, the observation that secretion products of the pineal gland show circannual rhythms in animals kept under constant laboratory conditions, suggest the possibility that other factors apart from circadian and circannual rhythms might influence pineal function.

There is abundant information about the effect of magnetic fields on lower vertebrates (Rodda, 1984), birds (Semm et al., 1984; Demaine and Semm, 1985; Bardasano et al., 1986) and mammals (Wever, 1973; Semm et al., 1980; Reuss et al., 1983; De la Guardia et al., 1988; Giménez et al., 1991; Lerchl et al., 1991; Martínez Soriano et al., 1992a,b).

Moreover, both melatonin and the SB have shown similar circadian fluctuations depending not only on the luminosity but also on the magnetic fields (Martínez Soriano et al., 1992a,b). Night time exposure of the pineal gland to magnetic fields is known to depress melatonin synthesis (Welker et al., 1983). Likewise, retinal hydroxindole-O-methyl-transferase activity is modified by magnetic fields (Cremer-Bartels et al., 1983).

These considerations and the evident effects of the lunar cycles on the biological natural cycles, led us to study the numerical behaviour of SB and serum melatonin levels during light/dark, seasonal and lunar cycles.

Materials and methods

Forty male Wistar rats (240±37g) subjected to the same nutritional and environmental conditions (18-20 °C, natural light) were studied. The rats were divided into 8 groups of 5 animals each. Animals of four groups were sacrificed in winter: 2 during the new-moon days (one group in the photophase, between 10:00 and 12:00 hour, and the second in the scotophase, between 00:00 and 02:00 hour); and the other two during the full-moon days, in the same above mentioned conditions. The same procedure and conditions was followed for the sacrifice of the four spring groups.

The animals were fixated by perfusion with Karnovsky's (1965) solution. The pineals were removed and post-fixed in osmium tetroxid for 90 minutes, dehydrated in graded series of acetone, block-stained in 0.5% uranyl acetate and 1% phosphotungstic acid in 70% acetone, and finally embedded in Epon resin in such a way that the glands were cut transverselly. The sections were mounted on 300-mesh copper grids and stained with 20% uranyl acetate in 100% methanol for 1 minute followed by lead citrate according to Reynolds (1963) for 5 minutes.

The number of SB was calculated by counting those observed in 8 grid squares each measuring 65x65 µm (total area 33.800 μm^2, magnification x12,000) and expressed in 20,000 μm^2.

Serum melatonin levels were determined by radioimmunoassay (RIA) techniques. Briefly, after extraction of blood, serum was removed by centrifugation and stored at -20 °C until assayed. The melatonin concentration was measured using RIA kit (Eurodiagnostic, Appledown, Netherlands) with variation coefficient of limit detection between 20% and 80%. Results were expressed in pg/ml.

The statistical evaluation of the data was made after a descriptive study.

Results

Synaptic ribbons (SR)

Number of SR during the scotophase was always larger compared to the corresponding photophase, regardless of the season and lunar phase. Likewise, values in winter were higher than in the corresponding spring, except for the photophase of full-moon days. Finally, considering the influence of the lunar cycles, the number of SR was at full-moon days greater than at corresponding new-moon days, except for the winter photophase (Fig. 2).

The difference between both seasons was highly significant (F-test: 51.037; $p<0.0001$), like the one between both lunar phases (F-test: 12.946; $p<0.001$), and between the light-dark phases (F-test: 283.695; $p<0.0001$). The joint interaction of the three external factors studied (light-dark, seasonal and lunar cycles) showed a statistically significant influence on the variations of the SR number (F-test: 34.722; $p<0.0001$) (Table 1).

However, interaction of only two of the three factors considered in the study, seasonal and lunar cycles, seasonal and light-dark cycles, or lunar and light-dark cycles (Fig. 3), did not exert a statistically significant

Table 1. Multiple analysis of the variance. Three-way analysis. Analysis of the external factor influence on the variations of the number of the synaptic ribbons.

SOURCE	df	SUM OF SQUARES	MEAN SQUARE	F-TEST	P VALUE
Seasons (A)	1	469.225	469.225	51.037	.0001
Lunary Phases (B)	1	119.025	119.025	12.946	.0011
AB	1	22.5	22.5	2.447	.1276
Photophases (C)	1	2608.225	2608.225	283.695	.0001
AC	1	28.9	28.9	3.143	.0858
BC	1	44.1	44.1	4.797	.0359
ABC	1	319.225	319.225	34.722	.0001
Error	32	294.2	9.194		

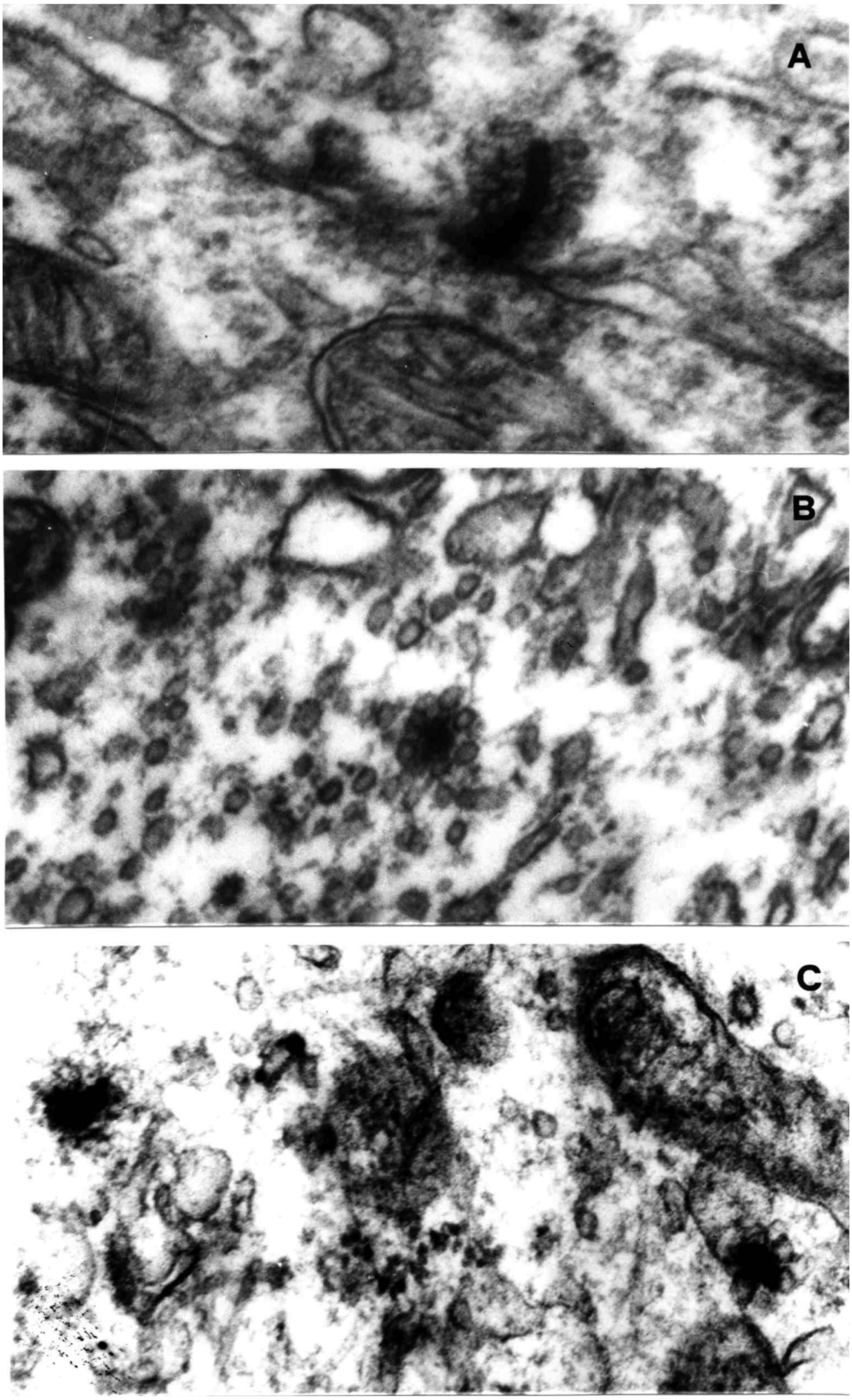

Fig. 1. Electron micrographs showing the different synaptic bodies. **A.** Synaptic ribbons. **B.** Synaptic spherules. **C.** Intermediate synaptic bodies. x 36,000

Table 2. Multiple analysis of the variance. Three-way analysis. Analysis of the external factor influence on the variations of the number of the synaptic spherules.

SOURCE	df	SUM OF SQUARES	MEAN SQUARE	F-TEST	P VALUE
Seasons (A)	1	76.05	76.05	20.462	.0001
Lunary Phases (B)	1	5	5	1.345	.2499
AB	1	24.2	24.2	6.511	.0128
Photophases (C)	1	68.45	68.45	18.417	.0001
AC	1	1.25	1.25	0.336	.5638
BC	1	16.2	16.2	4.359	.0404
ABC	1	156.8	156.8	42.188	.0001
Error	72	267.6	3.717		

influence on the variations of the number of SR (Table 1).

Synaptic spherules (SS)

The SS number was greater during the scotophase of the winter full-moon days and the spring new-moon days whereas in the remainder situations the trend was reversed. The SS predominated in winter only during the photophase of the new-moon days and the scotophase of the full-moon days. Finally, when we considered the lunar phase, larger SR values were obtained at full-moon days in winter scotophase and spring photophase, being this situation completely reversed at the new-moon days (Fig. 4).

With respect to the seasonal factor, the difference of

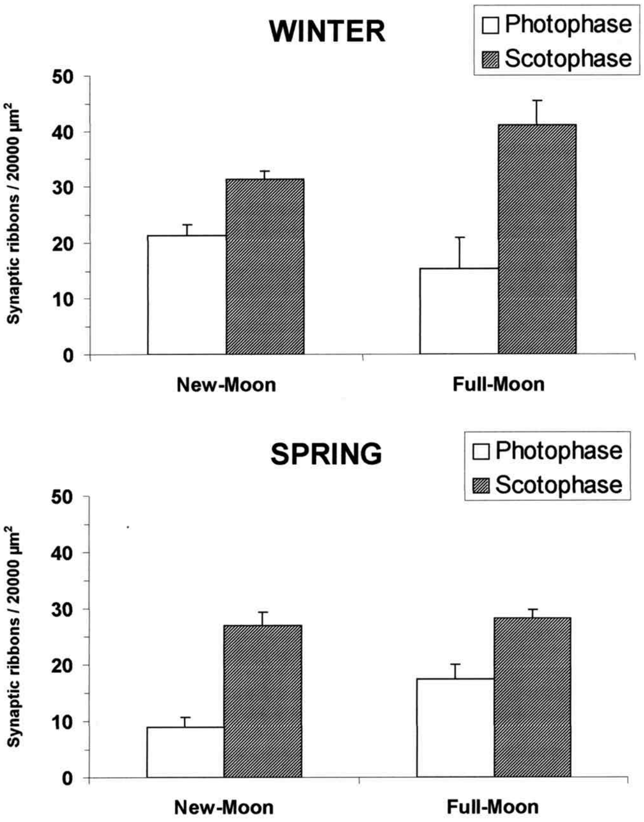

Fig. 2. Histogram of the comparison between the seasonal, photophasic and lunar phases and the number of synaptic ribbons.

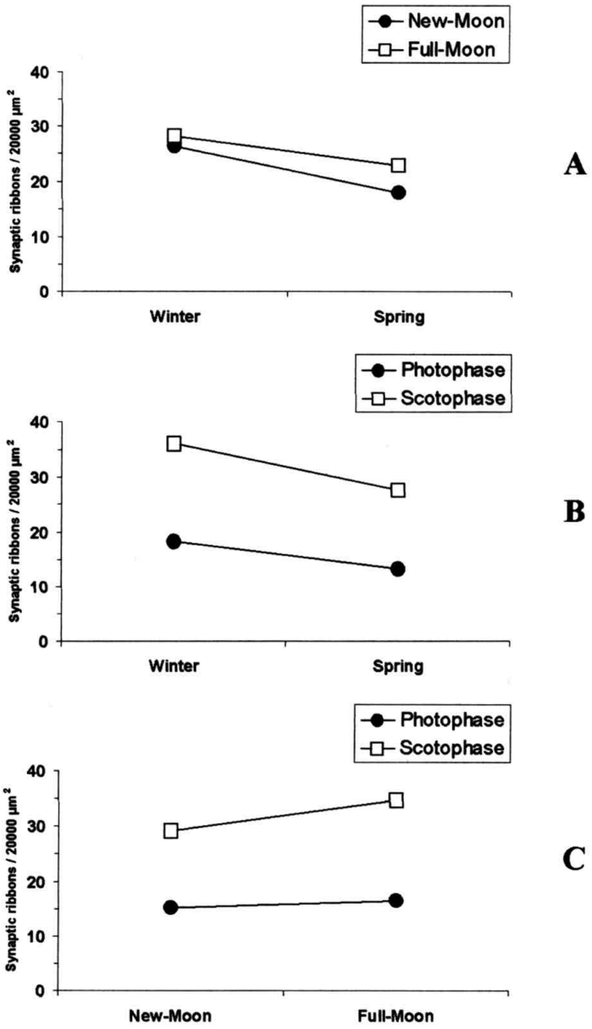

Fig. 3. Influence of the interactions between the external factors on the variations of the number of synaptic ribbons of the pinealocytes. **A.** seasonal rhythms/lunar phases. **B.** Seasonal rhythms/photophasic rhythms. **C.** Lunar phases/photophasic rhythms.

the SS values between both seasons is relevant and highly significant (F-test: 20.462; p<0.0001). The same happens with the light-dark factor (F-test 18.417; p<0.0001). However, difference between both lunar phases was not statistically significant (F-test: 1.345; p<0.249). Considering the joint interaction of the three external factors analyzed, we observed a statistically significant influence on the variations in number of the synaptic spherules (F-test: 42.1888; p<0.0001) (Table 2).

Both interactions, between seasonal and lunar phases and between lunar and light-dark cycles (Fig. 5), exerted a statistically significant influence on the SS variations (F-test: 6.511; p<0.012 and F-test: 4.359; p<0.04, respectively). However, interaction between seasonal and light-dark cycles (Fig. 5) did not exert a statistically significant influence on the number of SS (F-test: 0.336; p< 0.563) (Table 2).

Intermediate synaptic bodies (ISB)

There was a predominance of the ISB during the scotophase, with the only exception of the winter new-moon days, when the amount found was similar to that of the scotophase. As in the SS, the number of ISB was larger in winter only during the photophase of the new-moon days and the scotophase of the full-moon days. Likewise, when we considered the lunar phase, the number of ISB was greater during the full-moon days in winter scotophase and spring photophase (Fig. 6).

Analysing these main effects, we observed that the difference of values of the ISB between seasonal phases was relevant and highly significant (F-test: 11.945; p<0.0009), like that related to the lunar cycles (F-test: 5.309; p<0.0241), whereas light-dark phases differences were not statistically significant (F-test: 0.747; p<0.3904). Considering the joint interaction of the three factors, we observed that there was not a significant influence on the ISB number variations (F-test: 2.074; p<0.1542) (Table 3).

The analysis of the interaction between seasonal and lunar cycles, seasonal and light-dark cycles, or lunar and light-dark cycles (Fig. 7) resulted not statistically

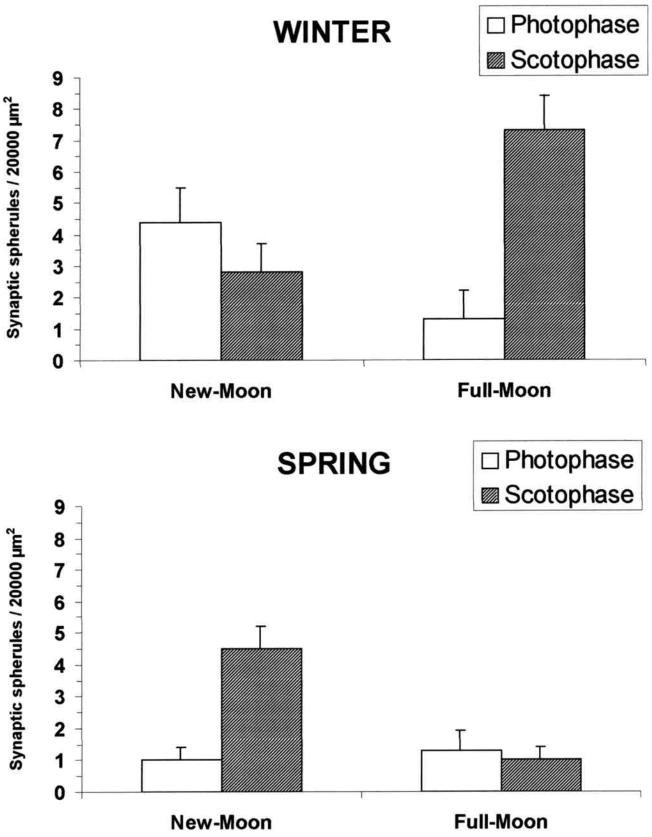

Fig. 4. Histogram of the comparison between the seasonal, photophasic and lunar phases and the number of synaptic spherules.

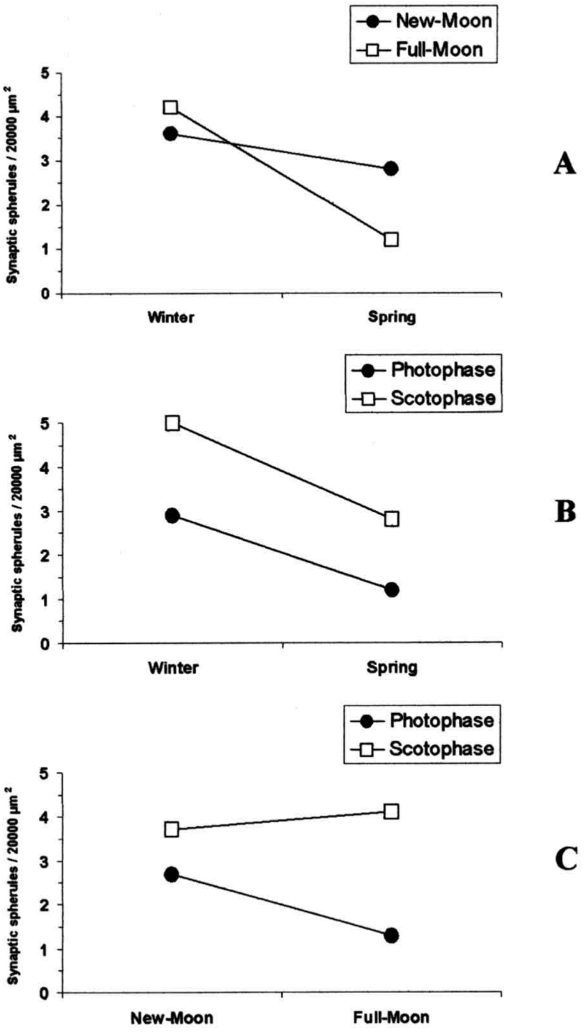

Fig. 5. Influence of the interactions between the external factors on the variations of the number of synaptic spherules of the pinealocytes. **A.** seasonal rhythms/lunar phases. **B.** Seasonal rhythms/photophasic rhythms. **C.** Lunar phases/photophasic rhythms.

Table 3. Multiple analysis of the variance. Three-way analysis. Analysis of the external factor influence on the variations of the number of the intermediate synaptic bodies.

SOURCE	df	SUM OF SQUARES	MEAN SQUARE	F-TEST	P VALUE
Seasons (A)	1	7.2	7.2	11.945	.0009
Lunary Phases (B)	1	3.2	3.2	5.309	.0241
AB	1	0.05	0.05	0.083	.7742
Photophases (C)	1	0.45	0.45	0.747	.3904
AC	1	0.2	0.2	0.332	.5664
BC	1	0.2	0.2	0.332	.5664
ABC	1	1.25	1.25	2.074	.1542
Error	72	43.4	0.603		

significant (F-test: 0.083; p<0.7742; F-test: 0.332; p<0.5664; F-test: 0.332; p<0.5664, respectively) (Table 3).

Melatonin

Serum melatonin levels were higher during the scotophase than during the corresponding photophase. They were also more elevated in winter than in spring. Likewise, values of melatonin in serum were higher at the full-moon days, except during the winter scotophase (Fig. 8).

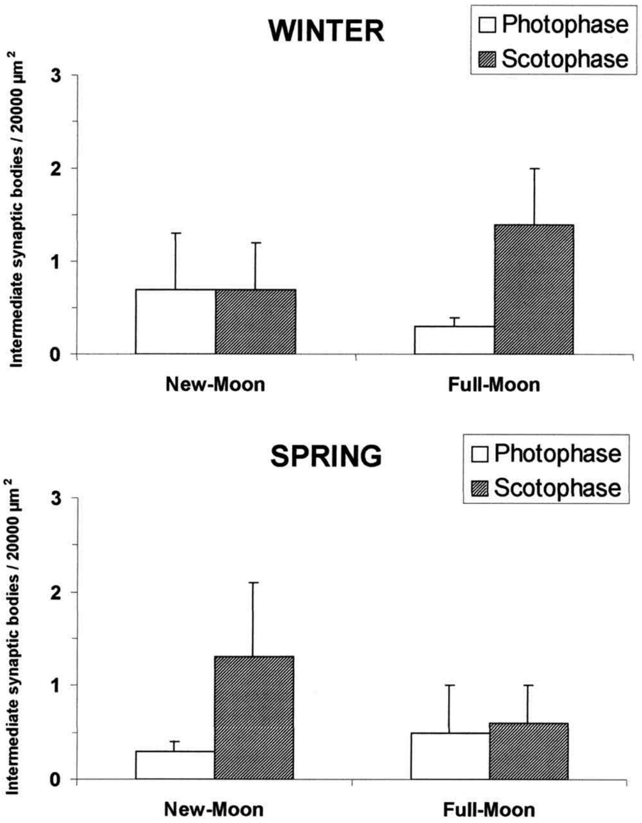

Fig. 6. Histogram of the comparison between the seasonal, photophasic and lunar phases and the number of intermediate synaptic bodies.

The effect of the season factor (F-test: 5.391; p<0.0268) and the light-dark cycle factor (F-test: 12.452; p<0.001) was statistically significant, whereas the lunar

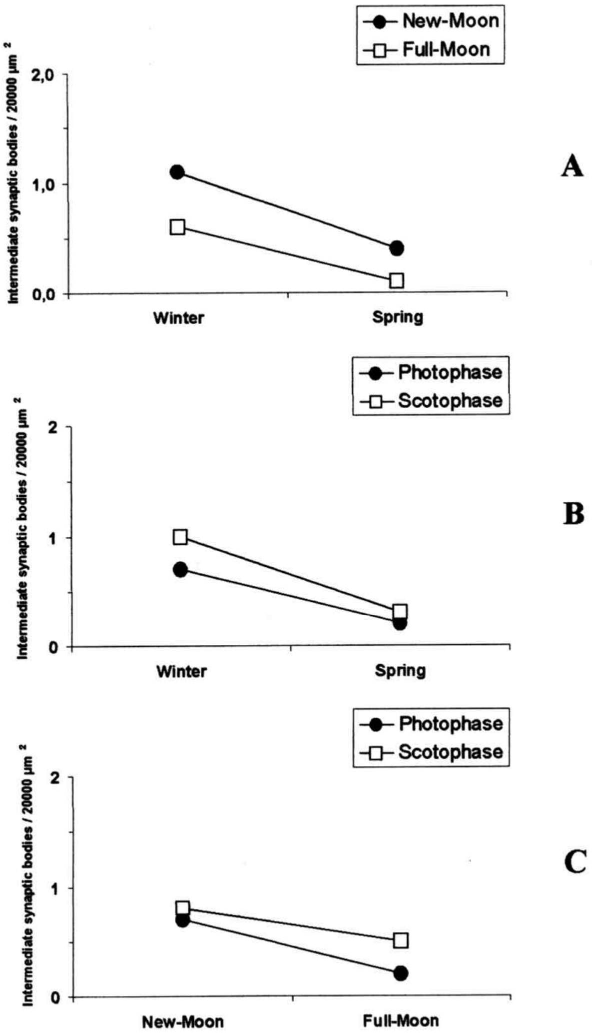

Fig. 7. Influence of the interactions between the external factors on the variations of the number of intermediate synaptic bodies of the pinealocytes. **A.** Seasonal rhythms/lunar phases. **B.** Seasonal rhythms/photophasic rhythms. **C.** Lunar phases/photophasic rhythms.

Table 4. Multiple analysis of the variance. Three-way analysis. Analysis of the external factor influence on the variations of the serum melatonin levels.

SOURCE	df	SUM OF SQUARES	MEAN SQUARE	F-TEST	P VALUE
Seasons (A)	1	5784.025	5784.025	5.391	.0268
Lunary Phases (B)	1	731.025	731.025	.681	.4152
AB	1	664.225	664.225	.619	.4372
Photophases (C)	1	13359.025	13359.025	12.452	.0013
AC	1	180.625	180.625	.168	.6843
BC	1	342.225	342.225	.319	.5762
ABC	1	765.625	765.625	.714	.4045
Error	32	34331.6	1072.862		

cycle effect was not relevant (F-test: 0.681; p<0.415). Considering the joint interaction of the three factors on the melatonin serum levels we observed that there was not a statistically significant (F-test: 0.714; p<0.4045) (Table 4).

Likewise, when we considered the interactions of two of the three external factors (Fig. 9), they were weak and not statistically significant (Table 4).

Discussion

Our results confirm the existence of a dependence relation of the external factors, light-dark, seasonal and lunar cycles, with the SB number and serum melatonin levels fluctuactions. However, the external factors studied do not influence on each type of the SB in the same way.

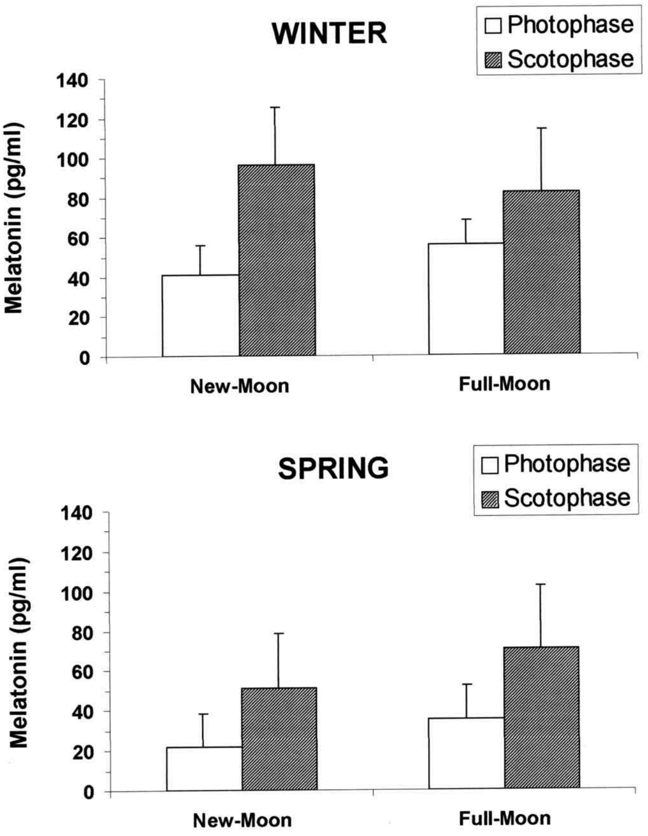

Fig. 8. Histogram of the comparison between the seasonal, photophasic and lunar phases and the level of serum melatonin.

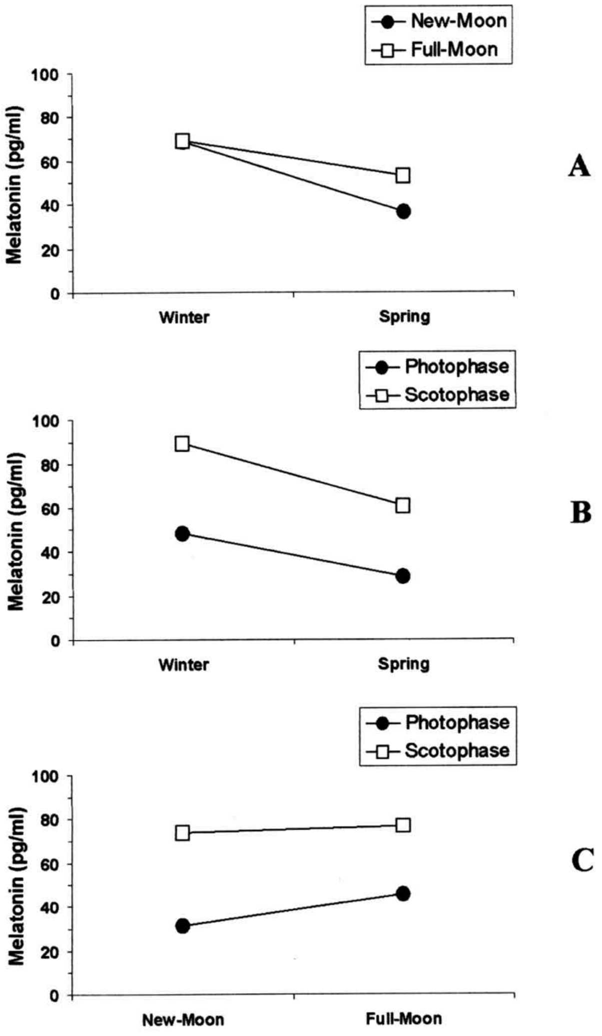

Fig. 9. Influence of the interactions between the external factors on the variations of the level of serum melatonin. **A.** Seasonal rhythms/lunar phases. **B.** Seasonal rhythms/photophasic rhythms. **C.** Lunar phases/photophasic rhythms.

From the study of the SR emphasizes that during the scotophase they increased in both seasons and in both lunar phases, confirming the darkness factor as a strong determinant in the increase of these pinealocyte organelles.

On the other hand, the existence of an inverse correlation between the SR number and the concentration of adrenergic nerve endings in the pineal parenchyma of different mammals (Karasek et al., 1983; Matshuura et al., 1983; González and Alvarez-Uría, 1986) and birds (Robertson et al., 1990) have been highlighted. Likewise, sympathetic denervation leads to an intrapineal increase of the SR number (Romijn, 1975, 1976), an effect that can be counteracted with the administration of noradrenaline or adrenergic agonists (Karasek, 1974; King and Dougherty, 1982; Seidel et al., 1990).

Our results support these data and allow us to establish clearly enough a relation among the SR number, the absence of light and the adrenergic innervation of the pineal gland. The correlation between the two former factors has been reported to have a percentage of 87.2% in winter, the remaining 12.8% being attributed to ‘other factors’ (Martínez-Soriano et al., 1992a,b). The influence of these ‘other factors’ seemed to diminish during summer, when this correlation increased to 97% (Martínez-Soriano et al., 1992b). These results and the other ones presented in this study, suggest that the seasonal factor, separately or jointly with the light-dark one, might occupy those percentages attributed to ‘other factors’.

The SS did not exhibit a predominance in any light-dark, seasonal and lunar phases. Their behaviour showed substantial differences with that of the SR in all the analysed animals. Our results agree with previous studies made on the guinea pig (Lues, 1971; Vollrath et al., 1983), rabbit (Romijn, 1975; Martínez-Soriano et al., 1984, 1999) and even in cultivated pineal tissue from the rat (Karasek and Vollrath, 1982).

However, more recently, studying ultrastructural changes of SR morphology in the retina of the Balb/c mice it has been suggested that both SR and SS structures are the result of a transformation from the former to the latter, at least in the retina, since after the exposure to light during the early photophase (07:00-08:00) the SR undergo transformations aimed at adopting the spherical form (Adly et al., 1999). Circadian studies made about the evolution of these pineal structures in the rabbit (Martínez-Soriano et al., 1984), the guinea pig (Khaledpour and Vollrath, 1987) and the cat (Martínez-Soriano et al., 2000), pointing out an inversely proportional evolution along the day of both structures, could support this hypothesis.

In the case of the ISB, a predominance during the darkness phase, regardless of the seasonal or lunar phase was the most significant finding. However, the scarce and little frequent number of ISB found in the rat, the animal of our study, do not allow the conclusions derived from their evaluation to be very reliable. Following step should be carrying out their study in other animal species where they have been reported to be large enough, such as the rabbit (Martínez-Soriano et al., 1984, 1999) or the hamster (Matsushima et al., 1983), or the comparison between these pineal ISB and the ones observed in the ciliated cells of the inner ear (Smith and Sjöstrand, 1961; Wersäl and Bagger-Sjöback, 1974; Sobkowicz et al., 1986).

The production of the melatonin follows a clearly defined circadian rhythm, with significantly higher values during the nocturnal periods than during the diurnal ones. This fluctuation seems to be determined by endogenous circadian factors synchronised with the environmental light variations (Vollrath, 1981). Apart from this circadian rhythm, the existence of a yearly rhythm of melatonin production has been described. This circannual rhythm would be determined by the gonadal function or vice versa (Reiter et al., 1976). Peaks of melatonin have been detected in serum, in the months of January and February (Arendt et al., 1978), although other authors have not found differences between the months of November, February, June or August (Rollag et al., 1978). Likewise, the existence of a weekly rhythm of melatonin production, with the highest peaks on Saturdays and the lowest ones on Thursdays has been pointed out (Vollrath et al., 1975).

Our results support the influence of the light/dark changes on the serum melatonin levels, showing significantly higher values during the scotophases than during the photophases. However, our results also show clear and significant differences between the winter melatonin levels (February) and the spring ones (April), in discordance with those of Rollag et al. (1978). Similar results in seasonal periods different from those contemplated by us have been given (Cimas et al., 1987). Consequently, and according to other authors (Vollrath et al., 1975; McNulty et al., 1990; McNulty and Prechel, 1992), we could deduce that the external factors light-dark and seasonal cycles significantly influence on the exit variable of the system: the melatonin.

Since the superimposed behaviours of both the melatonin serum levels and the SR number, a functional correlation between both factors has been suggested (Vollrath, 1973; Kennaway et al., 1977; Rollag et al., 1978; Binkley., 1981; Martínez-Soriano et al., 1984; Cimas et al., 1987). Based on previous studies, this suggestion seems unlikely, as the transcriptor protein starts the process of elaboration of both of them at different times: at the end of the diurnal phase in the case of the SR and at the beginning of the nocturnal period in the case of the melatonin (Sousa-Neto et al., 1990; Sthele et al., 1993). However, our results confirm this correlation, as the SR are one the SB that most strongly influence on the serum melatonin evolution. The remained possibilities might be motivated by other different variables, some of them could be the other ones considered in this experiment. Anyway, the analysis and study of the correlations of the external factors with the exit variable does not explain wholly the total variations of melatonin, and so we must guess the existence of another kind of internal variables not considered in our study that influence on the variability of the serum melatonin levels.

As a final consideration, we can add that there is not really any external influence factor capable of determining the variations of the different internal variables of the complete system. The interaction of all the factors determines this aspect: the external factors according to the predominant function of one of them, the photophasic light variations, whereas the internal factors according to the interaction of all of them over the SR, which has the highest incidence on the external variable: the melatonin.

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