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Circadian and seasonal changes of synaptic bodies in different parts of the rabbit pineal gland

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Summary. In the mammalian pineal gland, synaptic bodies (SBs) are poorly understood organelles. Previous studies in rabbits have shown that the organelles are rather heterogeneous in shape, are few in number during the day and increase in number at night. No studies are currently available on seasonal changes in this species and it is unknown whether the biological rhythms are identical in the proximal, intermediate and distal parts of the elongated pineal. To this end, a study was made of 84 rabbits kept under natural lighting conditions to examine numerical variations of the different types of SBs in the proximal, intermediate and distal regions of pineal glands procured at different timepoints of a 24-hour cycle and in each of the four annual seasons. In the present study, rod-like, sphere-like, ovoid, rectangular and triangular SB profiles were distinguished; the first two types being the most abundant. In addition to the well-known circadian changes, with low numbers of SB profiles during the day and high numbers at night, we found pronounced season-related differences as well as differences related to pineal regions. In autumn and winter, nighttime SR profile numbers were significantly higher than in spring and summer. With respect to regional differences it was found that the amplitude of the circadian rhythm increased in a proximo-distal direction in the gland. In autumn the strongly enhanced nocturnal increase was restricted to the distal region of the gland, whereas in winter it was seen in both the distal and the intermediate regions. The regional differences are probably related to the fact that the postganglionic sympathetic fibres, which regulate pineal function, enter the gland distally and proceed rostrally to the proximal region. Taken together, the results show that day- and nightlength are structurally coded in the pineal gland by means of SB numbers. Provided the SBs of the mammalian pineal gland are involved in synaptic processes, the results suggest that synaptic processes are enhanced at night as well as in autumn and winter.

Key words: Pineal gland, Synaptic bodies, Circadian rhythm, Seasonal rhythm, Rabbits

Introduction

The pinealocytes of mammals contain structures generally known as synaptic bodies (SBs). These bodies have an electron-dense, fine-granular core surrounded by a variable number of clear vesicles of varying sizes. SBs may be found both in the cell prolongations and in the perikarya of pinealocytes. SBs have also been reported in a number of sensory organs, including the retina (Sjöstrand, 1958) and the hair cells of the organ of Corti (Smith and Sjöstrand, 1961; Molina and Martinez-Soriano, 1991) and the vestibular apparatus (Wersäll and Bagger-Sjöback, 1974).

In the pineal gland of mammals, SBs are heterogeneous. The most abundant types appear as bar- or rod-shaped structures surrounded by clear vesicles and with a diameter of between 40 and 60 nm; usually termed synaptic ribbons (SRs). The number of SR profiles varies over the 24-hour cycle, being more abundant during the hours of darkness than during the day (Vollrath, 1973). Similar changes have been found in virtually all species studied to date (Kurumado and Mori, 1977; McNulty, 1980, 1981; Matusushima et al., 1983; Martínez-Soriano et al.,1984; Cimas-García et al., 1987). Moreover, SR profiles reveal prominent seasonal changes in number, being low in March and showing a peak in September (Karasek et al., 1988). The number of SR profiles may also undergo changes under experimental conditions (Karasek, 1976; Vollrath and Howe, 1976; Kurumado and Mori, 1980; King and Dougherty, 1982a,b; Kosaras et al., 1983; Martinez-Soriano et al., 1992), and they have been related to the adrenergic innervation of the gland (Romijn, 1975; King and Dougherty, 1982a; Karasek et al., 1983), and to the rhythm of melatonin synthesis (Vollrath, 1973). However, more recent studies have questioned these interpretations (Seidel et al., 1990; Sousa Neto et al., 1990).

The second most frequent synaptic organelles are

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spherical SBs measuring 60 to 220 nm in diameter. They are surrounded by 40-60 nm large vesicles similar to those associated with SRs and are termed synaptic spherules or spheres (SSs). SS profiles have been observed in guinea pig (Vollrath, 1981; Khaledpour and Vollrath, 1987), rabbit (Martinez-Soriano et al., 1984) and hamster (Matsushima et al., 1983). The number of SS profiles varies between species and their fluctuations often differ from those of SRs (Vollrath, 1981; Karasek and Vollrath, 1982; Martinez-Soriano et al., 1984; Khaledpour and Vollrath, 1987), suggesting that they may be functionally different from SRs.

In addition to rod-shaped SRs and round SSs, quadrangular, triangular, ovoid or kidney-like SBs have been reported in certain species such as hamster (Matsushima et al., 1983), rabbit (Martinez-Soriano et al., 1984), guinea pig (Khaledpour and Vollrath, 1987) and cow, sheep and pig (Struwe and Vollrath, 1990). These intermediate forms (Khaledpour and Vollrath, 1987) are present in significantly lower numbers than the previously mentioned shapes, yet exhibit basically similar circadian fluctuations.

Table 1. Data on circadian and seasonal environmental luminosity, mean temperature, relative humidity and atmospheric pressure obtained from the Valencia Meteorological Center (Valencia, S-pain).

Percentage of mean relative humidity

	1985	1986
February	67%	58%
May	68%	64%
June	69%	64%
October	71%	70%

Atmospheric pressure during the days of sacrifice

	5/20/85	6/24/85	10/21/85	2/10/86
07:00 h	760 mm.Hg	763 mm Hg	766.4 mm Hg	762.1 mm Hg
13:00 h	758.8	763.7	766.2	762.4
18:00 h	758.7	763.7	766.2	761.5

Temperatutre in °C during the days of sacrifice

07:00 h	13.2	22.8	15.0	1.2
13:00 h	22.2	23.4	22.8	11.0
18:00 h	18.4	23.0	20.0	8.8

Maximum temperature

	20	24.8	23.6	12.0

Minimum temperature

	9.8	20.4	14.4	0.6

Luminous intensity registered on the Earth´s surface at the moment of sacrifices (measured in cal/min.cm^2)

10:00 h	0.65	0.8	0.4	0.3
14:00 h	1.4	1.3	0.7	0.9
18:00 h	0.7	0.8	0.05	0.05

Transformation of values from previous table (from cal./min.cm^2 to luxes)

10:00 h	455 lux	560 lux	280 lux	210 lux
14:00 h	980 lux	910 lux	525 lux	630 lux
18:00 h	525 lux	595 lux	35 lux	35 lux

Comparative morphological studies have revealed that in mammals the pineal glands may differ widely in shape ranging from cone-shaped (e.g. in humans, ungulates) to elongated organs (e.g. guinea pig, rabbit), with some areas of the pineal complex being greatly reduced (e.g. the intermediate part in rats; for details see Vollrath, 1981). In view of the fact that the nerve supply of the mammalian pineal gland comes basically from two sources, i.e. the sympathetic superior cervical ganglia and the epithalamus, entering the gland from different routes, it cannot be excluded that the cell organelles under consideration may differ in their rhythmicity in the different parts of the pineal.

In the present paper, the study of the SBs in the rabbit (Martinez-Soriano et al., 1984) has been extended to the proximal, intermediate and distal regions of the pineal to find out whether the different types of SBs exhibit similar circadian changes in the different parts of the gland and whether or not seasonal changes can be detected. To this end, the animals were kept under natural conditions of illumination.

Materials and methods

Eighty-four male rabbits (*Oryctolagus cuniculus*) weighing 2600±250 g were used. The animals were zootechnic hybrids of the New Zealand and Californian strains and were supplied by the Valencia Polytechnic Institute (Valencia, Spain). The animals were individually housed in batches of 21 for one week before the experiment, for acclimatisation purposes. Data on circadian and seasonal environmental luminosity, mean temperature, relative humidity and atmospheric pressure (Table 1) was obtained from the Valencia Meteorological Center (Valencia, Spain). Altitude was at sea level, longitude 0° 15' West and latitude 39° 30' North.

Standard diet and water were supplied ad libitum. The rabbits were sacrificed by perfusion fixation every four hours in groups of three, according to the following local time schedule: 06:00(a), 10:00, 14:00, 18:00, 22:00, 02:00 and 06:00(b) hours, in spring (May 20), summer (June 24), autumn (October 21) and winter (February 10), 1985 and 1986. Table 2 shows the conversion of the local time to GMT time.

Sacrifice was carried out following Nembutal anaesthesia (50 mg/kg body weight, given intraperitoneally).

Cardiac perfusion was performed with Karnovsky's (1965) solution, after rinsing with physiological saline. The elongated pineal glands were removed and subdivided into three pieces (proximal, intermediate and distal), of approximately equal length. From each major piece, one small tissue block was selected for further analysis.

The specimens were post-fixed in osmium tetroxide, dehydrated in a graded acetone series and en bloc-stained with a solution containing 0.5% uranyl acetate, 1% phosphotungstic acid, and 70% acetone. After embedding in Epon, 60- to 80-nm sections were prepared for transmission electron microscopy. Contrasting was carried out according to Reynolds (1963).

For the quantification of the synaptic bodies, sections from each tissue block were cut. The area of a grid with the qualitatively best section was selected and the tissue overlying 8 apertures each measuring 65x65 μm was scanned at x12000. The results were expressed in terms of 20,000 μm^2.

Statistical analysis

The data gathered were comparatively analysed through hypothesis contrasting and the evaluation of significance. Statistical significance was considered at $P<0.05$. The variable normality hypothesis was systematically contrasted by non-parametric testing - fundamentally the Kolmogorov-Smirnov approach.

As the study involved continuous quantitative variables, these were compared by Student's test for mean comparison and small samples. An analysis of variance (ANOVA) was performed to establish a block comparison of various arithmetic means obtained for different samples.

The statistical analysis was carried out using a Macintosh II sii computer and StatWorks statistical software complemented by the Multiplan and Cricket Graph II programs.

Moreover, the cosinor method was applied to calculate the acrophases (Nelson et al., 1979).

Table 2. Time conversion table.

DATE OF SACRIFICE	LOCAL TIME	GTM TIME
5/20/1985	10:00	08:00
	14:00	12:00
	18:00	16:00
	22:00	20:00
	02:00	00:00
	06:00	04:00
6/24/1985	10:00	08:00
	14:00	12:00
	18:00	16:00
	22:00	20:00
	02:00	00:00
	06:00	04:00
10/21/1985	10:00	09:00
	14:00	13:00
	18:00	17:00
	22:00	21:00
	02:00	01:00
	06:00	05:00
2/10/1986	10:00	09:00
	14:00	13:00
	18:00	17:00
	22:00	21:00
	02:00	01:00
	06:00	05:00

Results

General observations

In the present study a total of 14 893 SB profiles were analysed. Rod-like SB profiles (SR profiles) predominated (52.5%), followed by round profiles (SS profiles; 32.9%) and variously-shaped profiles (14.6%). The latter group comprised ovoid, triangular and rectangular profiles, roughly equally abundant. The different SB profiles lay singly (Figs. 1-3), in pairs (Fig.4) or in groups of three or more, having the same or different morphology (Figs. 5, 6). In all three regions of the gland, the great majority of SB profiles were found in pinealocyte processes (ca. 85%), the remainder in pinealocyte perikarya. In the pinealocyte processes approximately one quarter of all SB profiles lay near the cell membrane.

Analysis of variance revealed that the factors time of day, season and region of the gland all had significant effects on the different types of SB profiles investigated.

Daily changes

SB profile numbers exhibited statistically significant daily differences in all seasons and in the different parts of the gland investigated. The curves obtained were roughly sinusoidal. SB profile numbers decreased in the first half of the light phase and began to increase between 14:00 and 18:00 h, reaching peaks between 22:00 and 06:00 h. Usually, the first and the second 06:00 h values differed only slightly. When the different types of SB profiles were separately considered, certain differences became apparent. SR profile numbers (Fig. 7) were usually highest at 02:00 h, occasionally at 22:00 h. The acrophases occurred around 04:30 in spring and summer, and at 02:17 in winter. The lowest values were mostly obtained at 14:00 h and occasionally at 10:00 h. The curves of SS profile numbers also showed day/night differences, but they were less regular than those of SR profiles as were the timepoints of the peaks and troughs. Compared to the acrophases of SR profiles, those of SS profiles (Fig. 8) occurred approximately 1 h later, i.e. at 05:24 h in spring and summer and at 03:03 in autumn and winter. Occasionally, two peaks were present. No significant daily changes of SS profile numbers were present in the proximal region of the gland in summer. As can be seen (Figs. 9-11), the ovoid, triangular and rectangular SB profiles also showed daily changes, with higher numbers at night than at day, but in several instances the curves were rather flat. Often, the daily curves of triangular and rectangular SB profiles resembled those of SR profiles, whereas the curves of the ovoid SB profiles resembled those of the SS profiles.

Figs. 7-11 reveal that the amplitude of the daily rhythms of virtually all types of SB profile numbers increased in a proximo-distal direction.

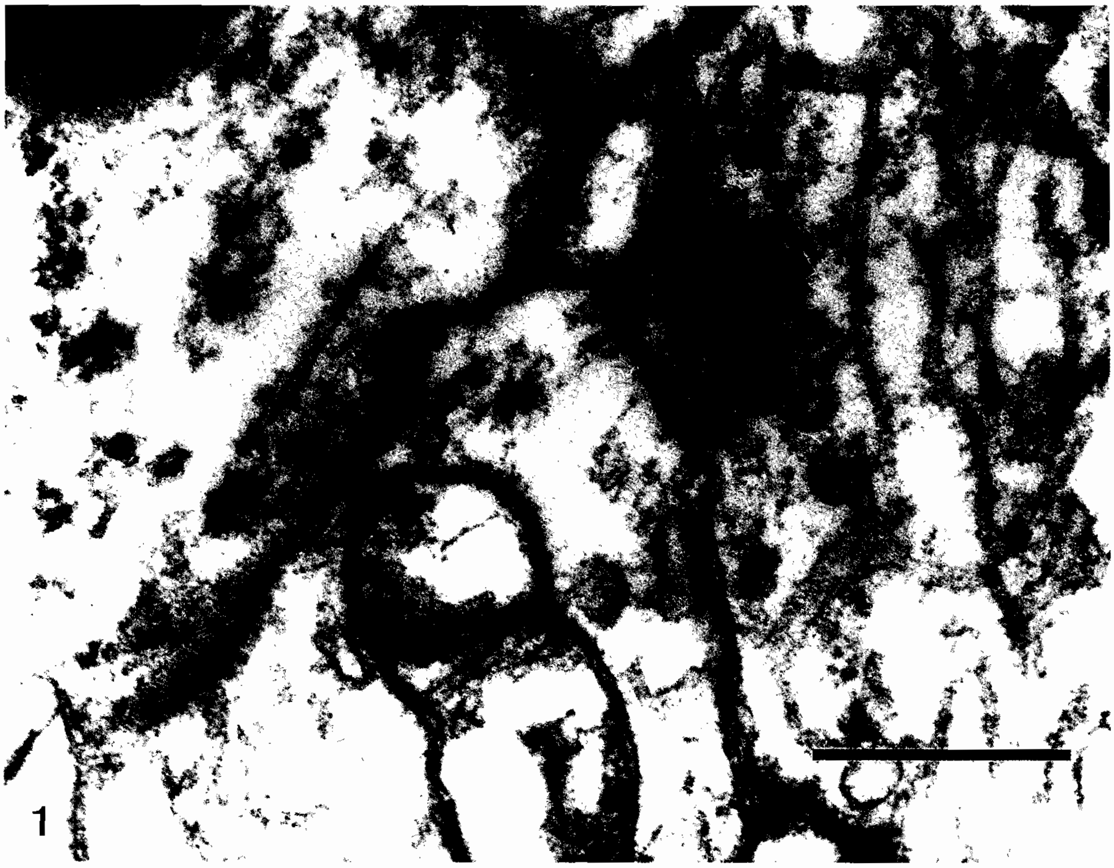

Fig. 1. Synaptic spherule in proximity of membrane (arrow). Bar: 0.5 μm.

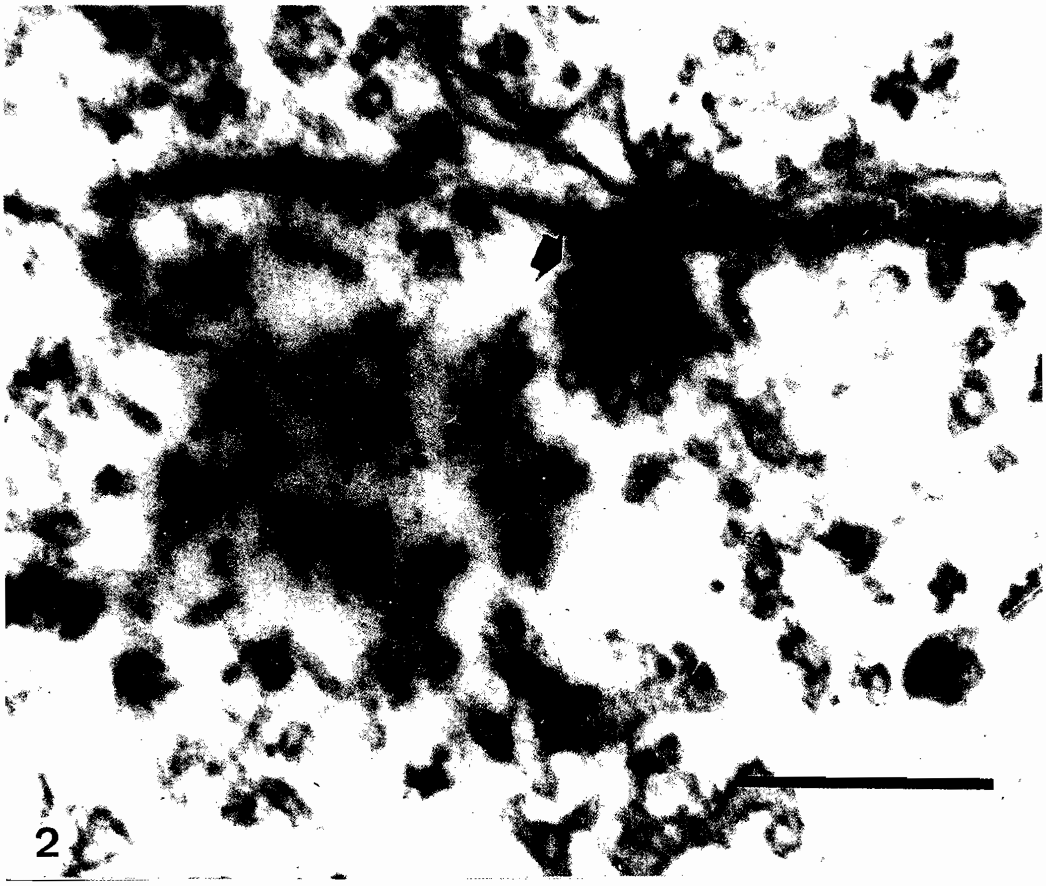

Fig. 2. Triangular shape adhered to membrane (arrow). Bar: 0.5 μm.

Fig. 3. Rectangular shape adhered to membrane (arrow). Bar: 0.5 μm.

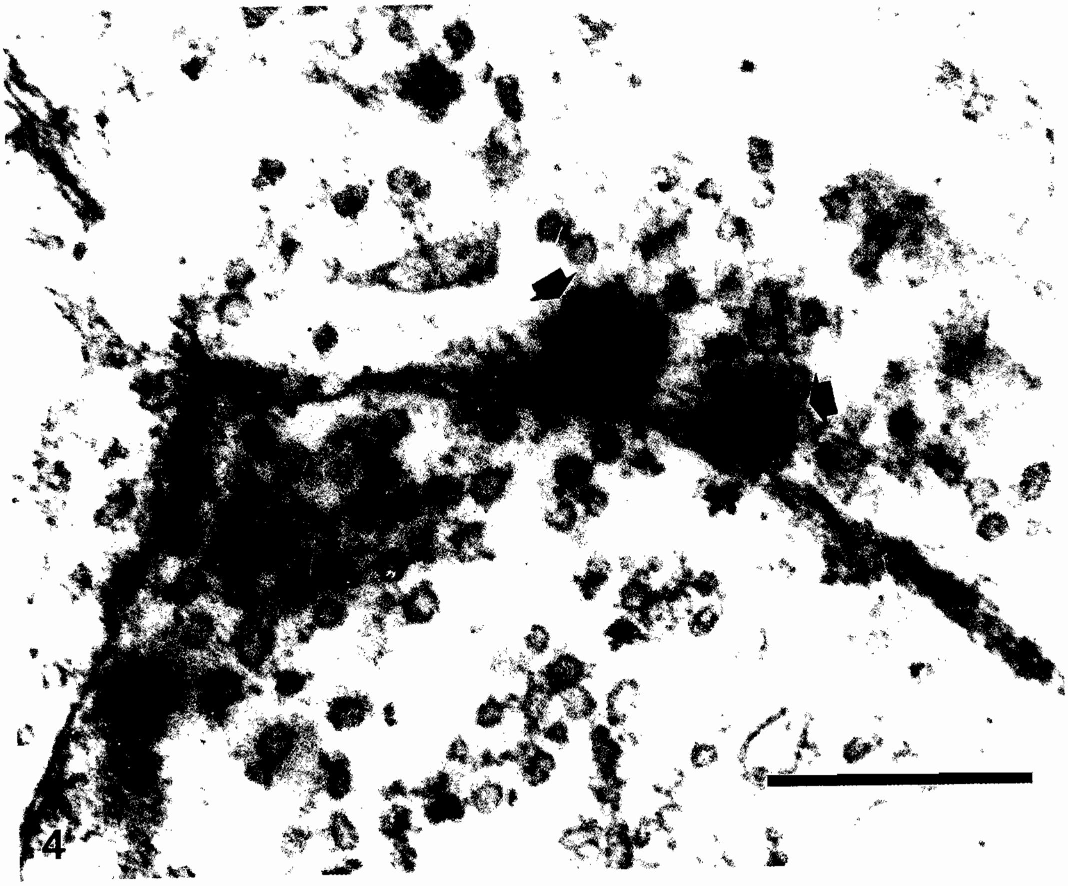

Fig. 4. A pair of intermediate bodies (arrows). Bar: 0.5 μm.

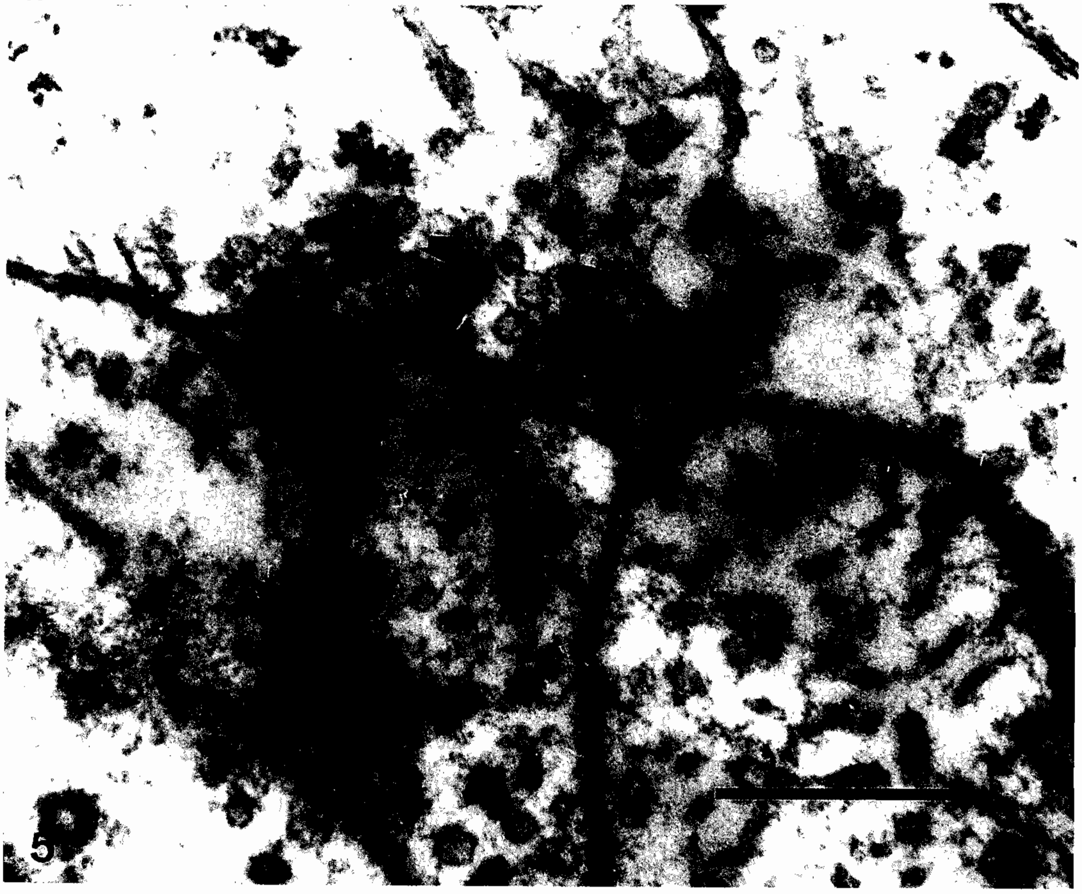

Fig. 5. Field of intermediate forms adhered to membrane (arrows). Bar: 0.5 μm.

Fig. 6. Grouping of intermediate bodies (arrows). Bar: 0.5 μm.

Seasonal changes

SB profile numbers were equal in the animals killed in spring and summer; they showed a clear increase in autumn, followed by a further increase in winter. As can be seen from Figs. 7-11, the seasonal changes were mainly the result of an increase of SR, ovoid, rectangular and triangular SB profile numbers at night, the diurnal minimum being unaffected. Interestingly, SS profile numbers did not show clear-cut and consistent seasonal differences.

Regional differences in the pineal

There was clear statistical evidence for the presence of regional differences in the rabbit pineal gland. With respect to SR profile numbers it was found that they increased in a proximo-distal direction. Statistically significant regional differences were most often seen at 06:00 h and 22:00 h and were more frequent in autumn and winter than in spring and summer.

Regional differences of SS profile numbers were less regular than those of SRs. The overalltendency was that in spring and summer SS profile numbers were higher proximally than distally, whereas in autumn and winter the reverse applied. Most of the statistically significant regional differences were present in summer and across the seasons at 14:00 h.

The other types of SB profiles exhibited regional differences in number only in autumn and winter when they increased in a proximo-distal direction.

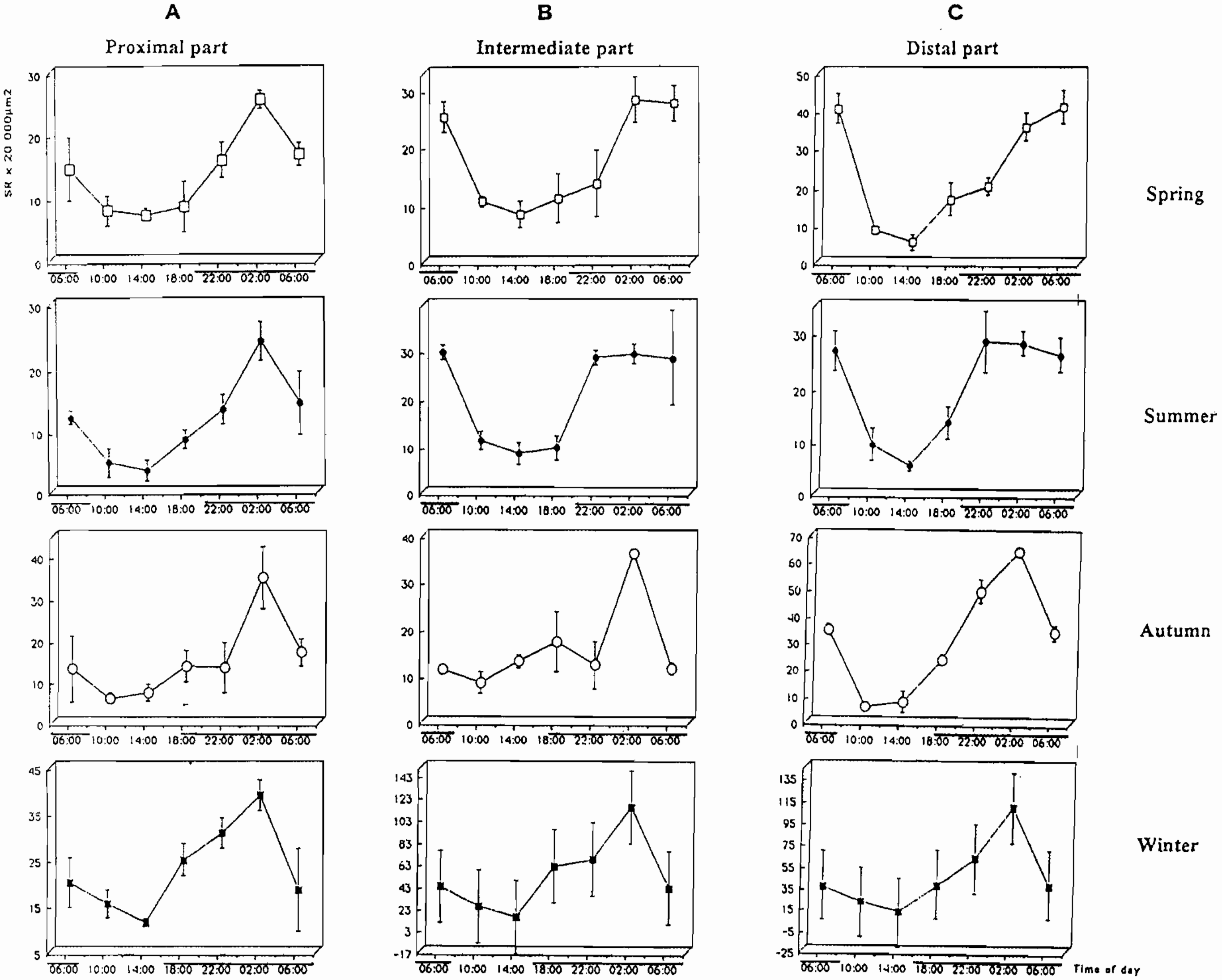

Fig. 7. Synaptic ribbons (SRs) circadian and seasonal evolution in the three (proximal, intermediate and distal) parts. Note that the scales differ in some of the Figs.

Discussion

The results obtained in the present study confirm the existence of a highly heterogeneous population of synaptic body profiles in the rabbit pineal gland (Martínez-Soriano et al., 1984). Apart from the small proportion (1%) of kidney-shaped SB profiles, all the other previously described types were found, but the relative proportions differed to certain degrees. In the present study SR profiles were more abundant (52.5%) than in the previous study (36.3%), whereas SS profiles were less abundant (32.9% vs 45.7%). Ovoid (6.0% vs 8.4%), rectangular (3.9% vs 1.4%) and triangular (4.5% vs. 7.2%) SBs corresponded reasonably well in the two studies. That SR and SS profile numbers differed rather strongly in the two studies, may have to do with the fact that in the previous study the animals were killed in September after having been kept under an artificial light/dark regimen of 12:12, whereas in the present study they were kept outdoors under natural lighting conditions. Since SR profile numbers increase strikingly in autumn and winter, but not the SS profiles (this study), this may explain why SR predominated in this study.

The question arises as to whether the different types of SB profiles are separate entities or not. Without systematic reconstructions based on serial sectioning, the true three-dimensional structure of the SBs is difficult to assess. The few such studies available have shown that the SR profiles are cross sections through thin (30-40 nm thick) plate- or lamella-like organelles (McNulty et al., 1986; Robertson and Dickson, 1987; Jastrow et al.,

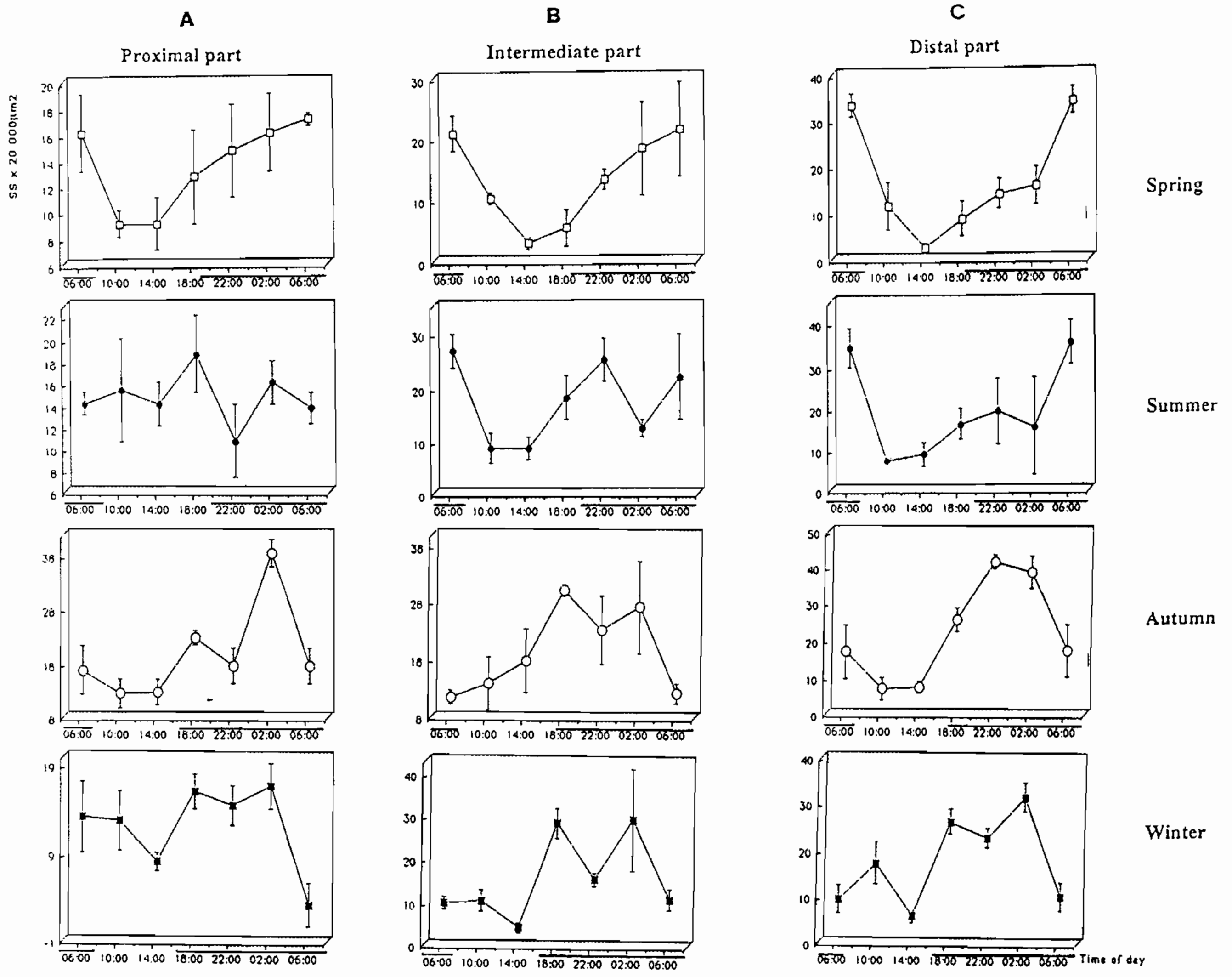

Fig. 8. Synaptic spherules (SSs) circadian and seasonal evolution in the three (proximal, intermediate and distal) part. Note that the scales differ in some of the Figs.

1997). In rare cases, rectangular profiles of longitudinally-sectioned platelike SR have been depicted (King and Dougherty, 1982a,b), showing that the organelle under consideration is indeed plate-shaped. We therefore assume that SR profiles and rectangular profiles are just different appearances of one and the same platelike organelle. That this may be the case is supported by the present observation that SR profile numbers and rectangular profile numbers often showed identical circadian and seasonal changes in a given region of the gland.

The ovoid synaptic profiles are presently interpreted to represent cross or tangential sections through SSs, because the round profiles of the SSs and the ovoid bodies often show corresponding changes in number. However, it cannot be excluded that the ovoid bodies are sections through lentil-like structures. That the rare triangular profiles cannot represent sections through platelike or round SBs is obvious. Perhaps they are cross sections through triangular rod-like SR.

The present study confirms that pineal SB profile numbers undergo circadian rhythmicity (see Bhatnagar, 1994). A new finding is that the amplitude of the rhythms differ in the different regions of the gland, being usually higher in the distal than in the proximal regions. This may have to do with the innervation pattern. There is ample experimental evidence that pineal melatonin formation is regulated by noradrenalin released from intrapineal postganglionic sympathetic fibres. Likewise there is evidence that pineal SR profile numbers are not independent of sympathetic influences. Thus, the classical studies by Romijn (1975) and Romijn et al.

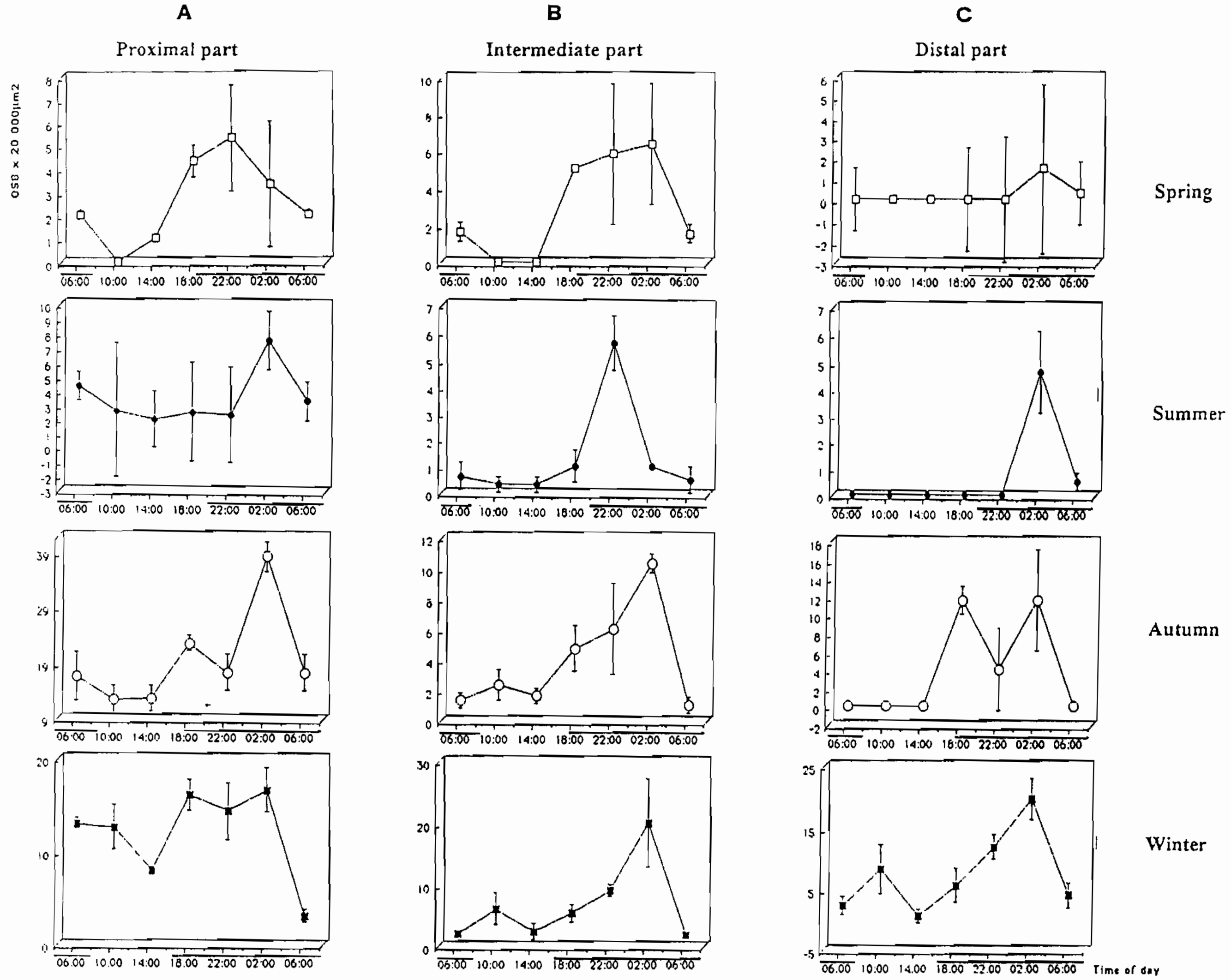

Fig. 9. Ovoid synaptic bodies (OSB) circadian and seasonal evolution in the three (proximal, intermediate and distal) parts. Note that the scales differ in some of the Figs.

(1976) revealed that following surgical or chemical ganglionectomy the number of SRs in the pineal gland parenchyma increases. Also, King and Dougherty (1982a,b) found that after administering an adrenergic agonist (isoproterenol) in sympathectomized rats, the number of SBs decreased. Some years earlier, Karasek (1974) likewise observed in cultured pineal tissue that noradrenalin caused an important decrease in the number of synaptic ribbons. In 1983 Karasek et al. demonstrated an inverse correlation between the number of SRs and the density of adrenergic nerve endings and noradrenalin contents in the pineal glands of different mammalian species. Similar results were reported by González and Alvarez-Uría (1986) in the cat, though it should be noted that more recent studies in the rat have shown that a noradrenergic regulation of pineal SRs is present in young animals only (e.g. 3-week-old), but has disappeared at 8 weeks (Vollrath et al., 1995). According to Romijn (1973) the sympathetic nerve fibres reach the rabbit pineal gland near its distal tip and spread deeply; the innervation density and/or the density of the receptors involved in the regulation of SR numbers could be less pronounced proximally than distally. However, there is also evidence that cholinergic mechanisms are involved in the regulation of pineal SR profile numbers (Gupta et al., 1991) and in this context it is interesting that the rabbit pineal is also innervated by cholinergic parasympathetic neurons. As these appear to be restricted to the distal part of the gland (Romijn, 1973) and assuming that they are also involved in SR regulation, they could account for the fact that the circadian amplitude is larger distally than proximally.

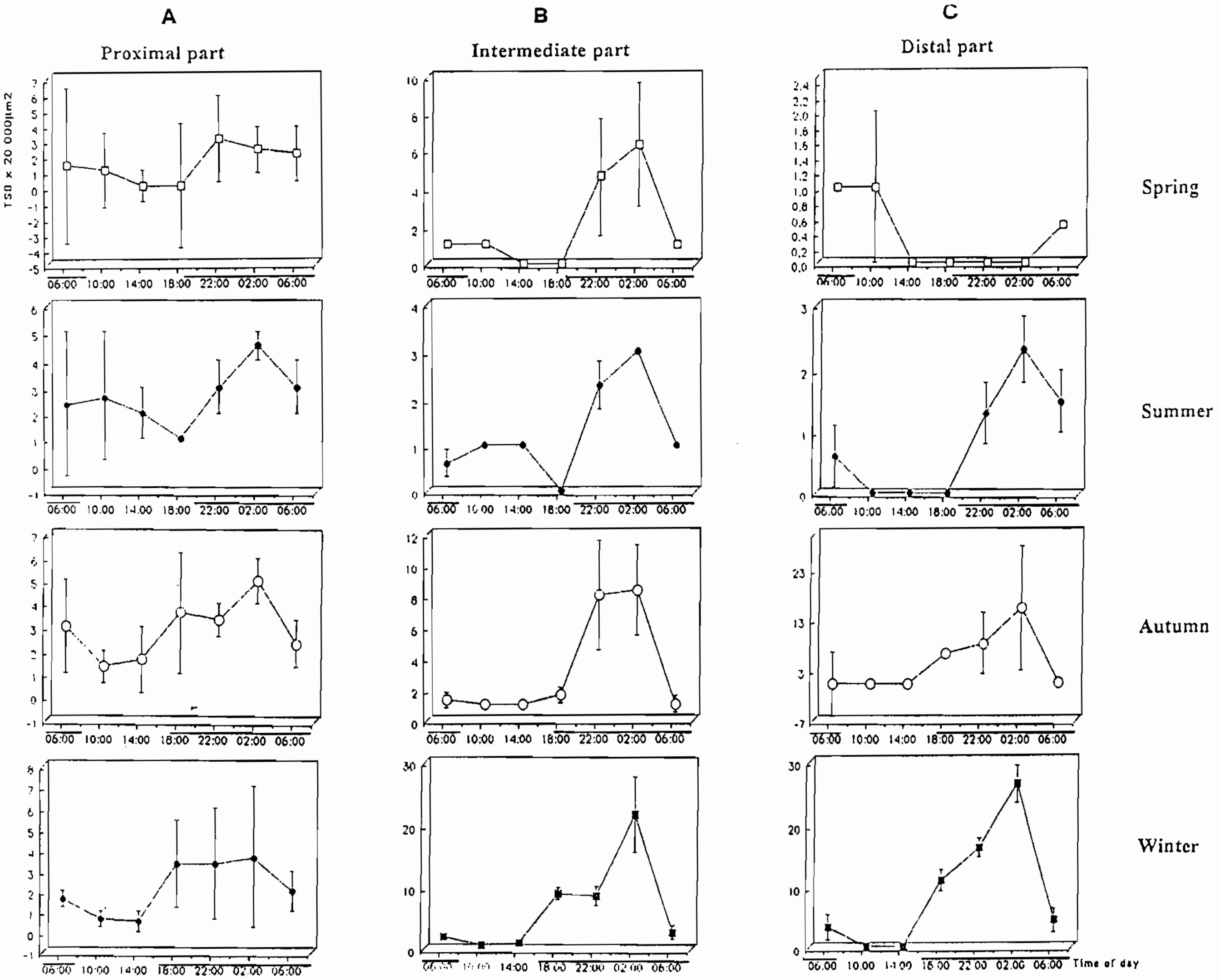

Fig. 10. Triangular synaptic bodies (TSB) circadian and seasonal evolution in the three (proximal, intermediate and distal) parts. Note that the scales differ in some of the Figs.

Perhaps in the rabbit pineal SR numbers are regulated by adrenergic-cholinergic interactions.

The present study shows that, in addition to circadian rhythmicity, there are clear seasonal differences. Reports on seasonal changes are rare. In a two-year study of rats kept under laboratory conditions, Karasek et al. (1988) noted that SR profile numbers exhibited a sinusoidal form over the year, with lowest numbers in April and a maximum in October, the differences being present both at day and night. In the 13-lined ground squirrel, SR profile numbers also exhibited a sinusoidal annual curve, with high numbers from August to October, and a precipitous drop in November (McNulty et al., 1990). The results of the present study are basically in agreement with the previous ones, showing that in autumn and winter SR profile numbers are much larger than in the other seasons. Interestingly, the seasonal differences were not equally pronounced in the different parts of the rabbit pineal gland. In the autumn, the seasonal increase was present in the distal part of the gland only. In the winter it was seen in both the distal and the intermediate parts of the gland, the proximal part remaining unaffected. We attribute these differences to the innervation pattern of the gland (see above).

What is the functional significance of the different types of SBs? With respect to the SRs it has been suggested that, in analogy to other organs where they are present, they are involved in intercellular communication, in the pineal mainly between pinealocytes (Vollrath, 1973). Since in mammals the majority of the sympathetic nerve fibres lie in the

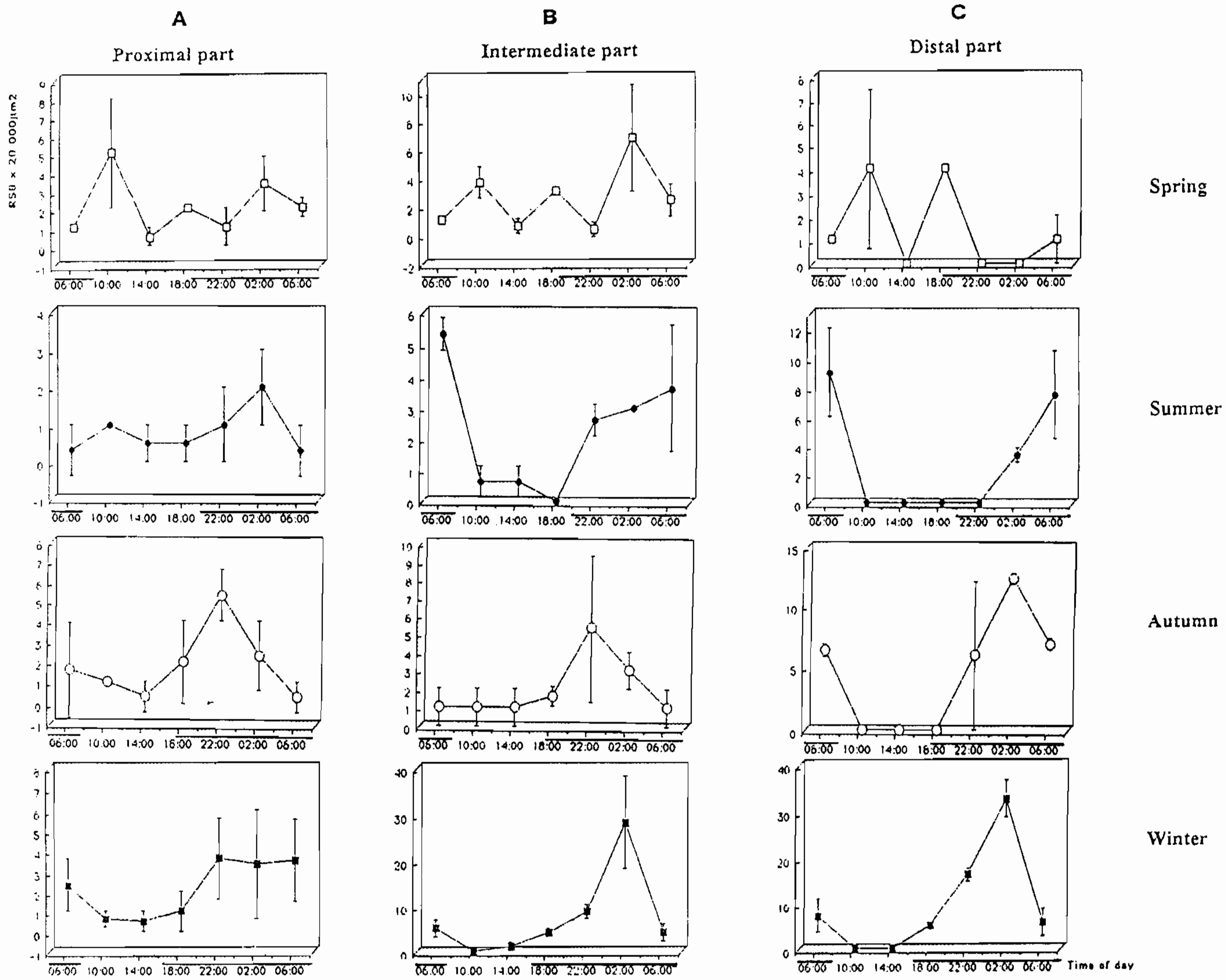

Fig. 11. Rectangular synaptic bodies (RSB) circadian and seasonal evolution in the three (proximal, intermediate and distal) parts. Note that the scales differ in some of the Figs.

perivascular spaces and since direct innervation of pinealocytes is scarce, the SRs may spread neural-derived information from one pinealocyte to the other.

The possible function of the SSs is even less clear. In certain species, they differ from SRs in their response to various physiological and experimental conditions. In the guinea pig, SS numbers increase much more during pregnancy than SR numbers (Lues, 1971) and reveal inverse circadian rhythmicity under physiological conditions (Khaledpour and Vollrath, 1987). Moreover, under continuous illumination, SR profile numbers increase, whereas SS profile numbers decrease (Vollrath, 1986). Sympathectomy in the rabbit induces a much more marked initial increase in SS profile numbers than in the population of SRs (Romijn, 1975). According to Khaledpour and Vollrath (1987), and based on work by Pittendrigh and Daan (1976) and Illnerová and Vanececk (1982), it could be speculated that the different rhythmicities of the SRs and the SSs in the guinea pig might represent the morphological expression of two coupled biological oscillators. This concept may not hold true for the rabbit. In this species, the two types of SBs show a basically corresponding behavior (Martinez Soriano et al., 1984; this study). In the retina, SSs have been described to represent degradation products of SRs (Abe and Yamamoto, 1984). In this study no observations were made that would point in this direction. However, it should be noted that SS profile numbers did not show a distinct increase in autumn and winter as did the SR profile numbers. Moreover, regional differences with respect to SS profile numbers were mostly present in summer and at 14:00 h, suggesting that SS function may be more closely related to photophase and the brighter seasons, whereas SRs appear to play a role mainly at night and in the darker seasons.

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