

The shape of synaptic ribbons in the rat pineal gland

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Abstract. Under the transmission electron microscope, synaptic ribbons (SRs) of the mammalian pineal gland appear as rod-like organelles. Their three-dimensional structure is not precisely known. In the present study, pineal SRs were investigated using serial sections obtained from rats killed at noon and midnight. The shape of the SRs was reconstructed based on SR profile length and the number of sections in which the profiles were contained. The results obtained show that SRs are basically flat plate-like structures with polymorphic lateral edges. Reconstructions of SRs revealed that they had average dimensions of $300 \times 150 \times 35$ nm and were 19.3% larger at night than at day; the difference in SR size points to perhaps major differences in synaptic function between day and night.

Key words: Pineal gland – Synaptic ribbons – Morphometry – Reconstruction – Surface area – Rat (Sprague Dawley)

Introduction

In vertebrates, synaptic ribbons (SRs) are conspicuous organelles of afferent synapses of retina, inner ear, lateral line organ and pineal gland. In electron-microscopic sections they appear as electron-dense rod-like profiles measuring about 150–200 nm in length with a thickness of 30–40 nm, surrounded by electron-lucent vesicles. Three-dimensional reconstructions of SRs from serial sections in retina (Sjöstrand 1974, 1976; McCartney and Dickson 1985), organ of Corti (Sobkowicz et al. 1982) and pineal gland (McNulty et al. 1986; Robertson and Dickson 1987) have clearly demonstrated the presence of plate-like SRs. However, applying a goniometer to pineal tissue, it was found that singly lying SRs were rod-

like, whereas SRs lying in groups were plate-like (Theron et al. 1981). King and Dougherty (1982a) assume that most, if not all, SRs are plate-like or discoid. McNulty and Fox (1992) distinguished ribbon-, rod- or plate-like SR_r and a second population of spherical or punctate shape SR_{sp} . Moreover, intermediate-type structures were observed. In their opinion many of the intermediate forms represent SR_r or SR_{sp} cut in different planes. However, because of the presence of round ribbon profiles having a diameter identical to the thickness of the SRs and based on mathematical calculations it was concluded that most or all of the SR are rod-like (rat; Vollrath et al. 1983). To clarify this point, in the present investigation rat pineal SRs were reconstructed from serial sections to reveal their shape. Since SRs increase in length at night (McNulty 1981; Riemann 1990) material from rats killed at day and night was studied to see whether the change in size is accompanied by changes in shape.

Materials and methods

Eleven male Sprague-Dawley rats (2 months old) were kept under standard laboratory conditions (LD 12:12, lights on at 0700 hours; fluorescent strip lights Osram L 65/25 weiss universal; room temperature $22 \pm 2^\circ$ C; relative humidity 50%; Altromin and water ad libitum). Under anaesthesia with ether, five of them were killed at midnight under dim red light and six at noon (September). Pineal glands were quickly removed and fixed by immersion fixation in Karnovsky's fluid (4% paraformaldehyde, 5% glutaraldehyde, 0.1 M Na-cacodylate buffer, pH 7.4) at 4° C overnight. After washing in 0.1 M Na-cacodylate buffer, pH 7.4, with 4.3% saccharose, tissue was post-osmicated (2% OsO_4 in 0.1 M Na-cacodylate buffer, pH 7.4), block-stained in 0.5% uranyl acetate plus 1% phosphotungstic acid in 70% acetone overnight, dehydrated in acetone and flat-embedded in Epon. For ease of recognition of selected tissue areas to be scrutinised, the tissue blocks were trimmed in a way that the sections obtained were roughly wedge-shaped and that their margins exhibited identification notches. From each pineal between 11 and 40 serial sections (50-nm section thickness) were cut with diamond knives on a Reichert Ultratome III. The thickness of the sections was verified by measuring the transverse diameter of SR profiles tilted from $+45^\circ$ to -45° , which showed that

This paper is dedicated to Professor Andreas Oksche on the occasion of his 70th birthday

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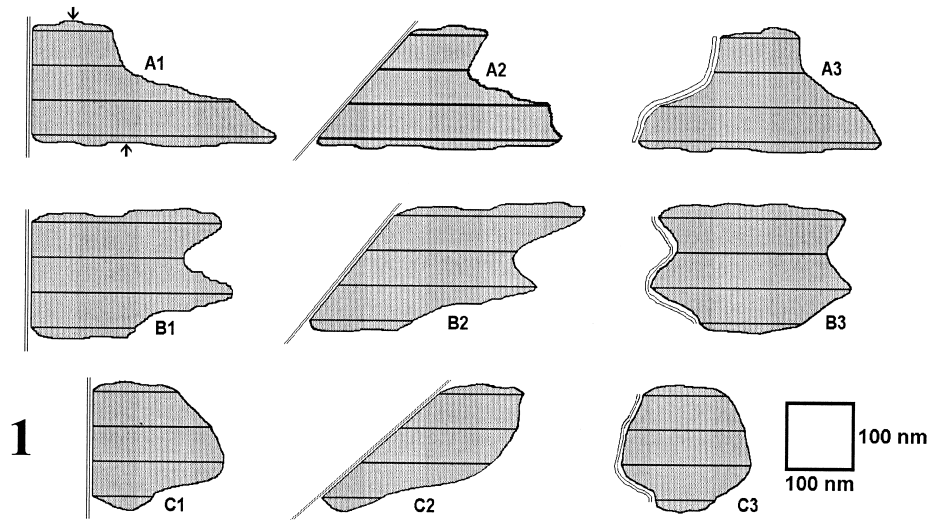


Fig. 1. Three synaptic ribbons (A, B, C) reconstructed following three different principles (1, 2, 3). The faint double line left to the synaptic ribbons represents the cell membranes. For further details, see text

Fig. 2. Possible shapes of 40 randomly selected synaptic ribbons reconstructed from profile lengths measured on serial sections of Sprague-Dawley rat pineals removed at noon (a) or midnight (b). Note the clear increase in size comparing night- to day-time synaptic ribbons. Cell membranes (see Fig. 1) would also lie to the left of the structures shown here

the diameter was indeed 50 nm. Sections were mounted on Formvar-coated one-hole grids. The sections were stained in 8% uranyl acetate in aqua dest. for 1 min, followed by lead citrate according to Reynolds (1963) for 5 min. The grids were then coded so that the investigator was unaware of the experimental background of the tissue. The sections were examined with a Zeiss electron microscope EM 109.

Tissue examination was started using the median section of a series, and the SR profiles encountered were followed in the adjacent sections until they disappeared. For ease of orientation, prominent tissue components (e.g. a nerve fibre, a blood capillary or a strongly indented pinealocyte nucleus) were selected and served as landmarks aiding in the identification of SR profiles on adjacent sections. Magnifications of $\times 100$; $\times 3150$ and $\times 20000$ were applied. A distinction was made between (1) rod-like SR profiles (30–40 nm in thickness), (2) round SR profiles and (3) profiles of varying shapes larger than 50 nm in diameter, termed intermediate forms.

When a SR profile was encountered, it was classified and its size was measured on the screen of the electron microscope with the help of a cursor linked to a personal computer using IDMS System Rev. 2.01 software. Later, for each animal the profiles of 10 randomly selected individual SRs, lying singly or in groups, were reconstructed based on the size measurements of the profile and the number of sections of known thickness in which the profiles were present.

To this end, SR profile lengths were drawn to scale as straight lines and all the profile lines of a given ribbon were superimposed, the distance between the lines being equivalent to the section thickness (50 nm; data were transformed into pixels). The superimposition of the lines was complicated because of the fact that (1) the exact shape of the SR is not known, (2) the lengths of the profiles of a given SR may vary in adjacent sections suggesting a polymorphism of the SR, and (3) the exact course in space of the cell membrane near the SR could not be objectively assessed, making it questionable to use the latter as a "reference point" for constructing the cell-membrane-related border of the respective SR. Therefore, in preliminary tests, assuming a constant distance between the SR and the cell membrane, three principles for reconstruction were applied, resulting in different possible forms of SR shape:

1. The cell membrane is a straight vertical line (Fig. 1: A1, B1, C1).
2. The straight cell membrane is tilted (Fig. 1: A2, B2, C2).
3. SR profile lengths are centred along a straight vertical axis resulting in an irregular course of the cell membrane (Fig. 1: A3, B3, C3).

Since it is unlikely that any of these reconstruction principles used reflects the true shape of SRs, for the definitive reconstructions of SRs the straight lines representing measured profile lengths were randomly superimposed so that the starting points of the next line did not deviate by more than one-half of the length of the previous line. Since the great majority of the profiles of a given SR varied only slightly in length and since it is unlikely that the boundary of the SR near the cell membrane is absolutely straight, the starting points of the lines representing the confines of the SR near the cell membrane were randomly superimposed within a limit of 50 nm. Thus a matrix was realised whose edge points were connected to each other by curved lines using Paintbrush for Windows software. Since it is unlikely that the upper and lower borders of SRs are straight lines, when the lateral edges show protuberances, an additional contour line was added (Fig. 1, A1 arrows) with an average distance of up to 25 nm above the first and below the last drawn profile length of SRs. The surface areas of the SRs shown in Fig. 1 were determined by the IDMS System Rev. 2.01 Software-equipped computer system to find out which way the three different methods of reconstruction and the drawing of contours of the reconstructed SRs affected surface area measurements. Table 1 shows that applying these three methods (e.g. A1, A2 and A3) surface area differed only slightly. Figure 2a, b was created by random use of the three methods described above

to give a most probable representation of SR shapes. The third dimension, not shown in Fig. 2a, b, was the plate's thickness of about 30–40 nm. Surface area was determined for the 40 SRs reconstructed for day and night animals. It should be noted that the data obtained reflect only one of the two flat faces of a given SR. Thus, the total surface area would be twice this number plus the surface area of the small edges. Moreover, from the reconstructed SRs the width-to-length ratios were determined, the length referring to the long axis and the width to the short axis of the SRs. In this context it should be noted that the measured lengths of the SR profiles are not necessarily identical with the lengths of the reconstructed SRs. Irregular or spherical SRs with high variance in thickness in consecutive sections cannot be accurately documented with this method and were therefore not reconstructed in this study.

The measured profile lengths were tested for differences between day and night, using the Mann-Whitney rank sum test.

Results

As often described in the literature, light and dark pinealocytes could be distinguished, the light cells outnumbering the dark cells. Both pinealocyte types consisted of a perikaryon and an unknown number of cell processes. SR profiles were restricted to the light pinealocytes. Usually the profiles lay singly and roughly perpendicular to the presynaptic membrane. Some appeared as pairs of parallel-lying rods (Fig. 3). Occasionally groups of up to eight SR profiles were observed in which some SR profiles were situated distant from the cell membrane. The postsynaptic elements were perikarya or less frequently cell processes of the light pinealocytes. Occasionally dark cells or perivascular spaces lay postsynaptically.

The great majority of the SR profiles observed had the shape of straight rods. On average, SR profiles could be followed over 5 sections. Profiles of a given SR often different in length between sections, ranging from 50 to 720 nm. Serial sectioning revealed that rod-like SR profiles appeared also rod-like in adjacent sections. Reconstructions showed that the SRs were thin plates, with an average length of ~ 300 nm, ~ 150 nm width and ~ 35 nm thickness. As can be seen in Fig. 2a, b, the lateral (thin) edges of the SRs are not straight but rather polymorphic. The width-to-length ratios range from 1:1 to 1:5. In very few cases two small SR profiles lying in line had matching large SR profiles, indicating the presence of a pore (Fig. 2a, *). The pores observed measured 40–70 nm in diameter. Pores measuring 45–130 nm in diameter have been found in ribbons of human retinal bipolar cells (Foos et al. 1969). Seldom reconstructed SRs were curved or crescent shaped (Fig. 2a, ☼), as was also described for SRs in retina (Sjöstrand 1974, 1976; McCartney and Dickson 1985; Rao-Mirotnik et al. 1995) and the organ of Corti (Sobkowicz et al. 1982). C- and v-shaped ribbon profiles, spheres and intermediate structures were rare (cf. McNulty et al. 1987; Bhatnagar 1994). Most of the structures resembling disks on one section turned out to have an irregular or, less commonly, plate-like shape in adjacent sections.

SR profile length was 145 ± 4 nm ($n=389$) at noon and 185 ± 6 nm ($n=368$) at midnight, representing a 27.6% in-

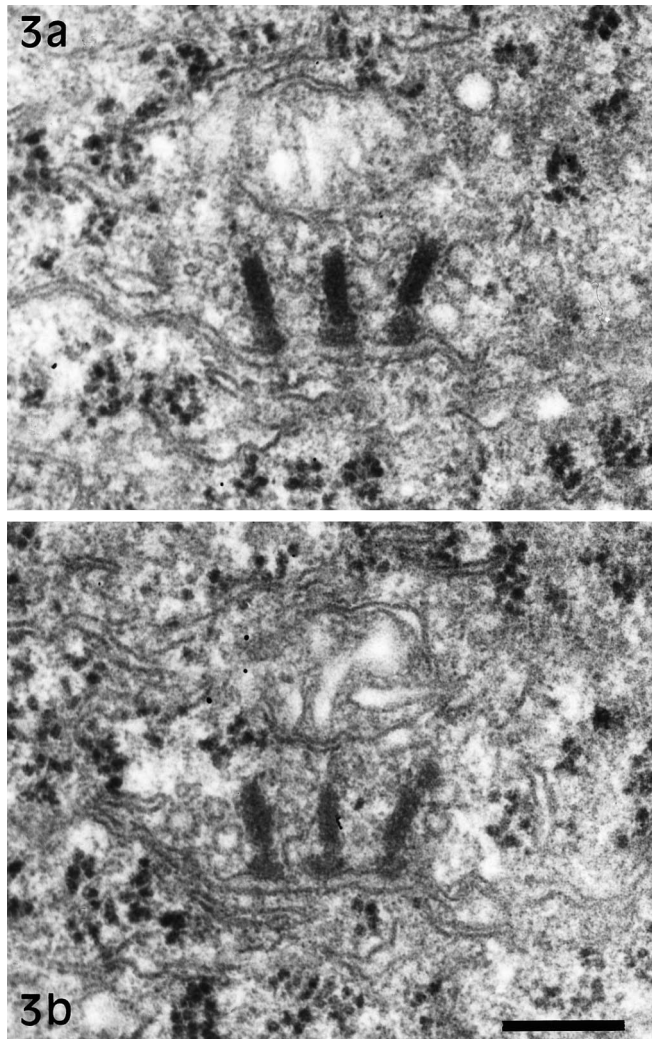


Fig. 3a, b. Field of three neighbouring pineal synaptic ribbons from two adjacent serial sections. Note that the rod-like profiles in **b** are less electron-dense than in **a**, indicating that the plates are cut close to their edges. $\times 60000$. Bar: $0.25 \mu\text{m}$

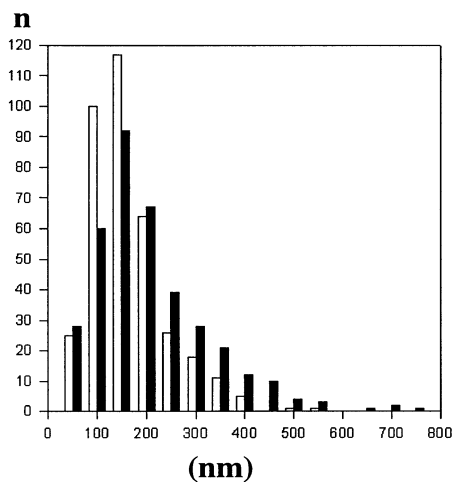


Fig. 4. Frequency of measured lengths of pineal synaptic ribbon profiles encountered at noon (\square , $n=368$) and midnight (\blacksquare , $n=368$). Note that the shorter synaptic ribbon profiles predominate at noon, whereas longer profiles are conspicuous at midnight

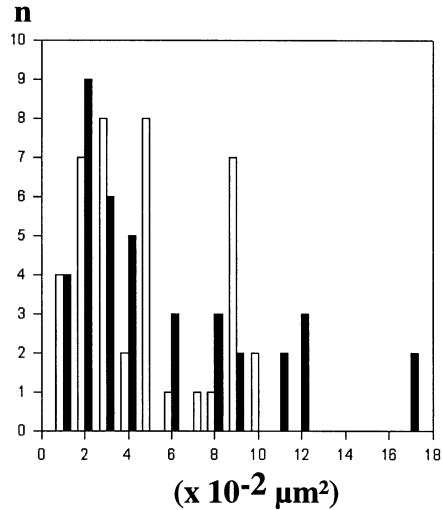


Fig. 5. Surface area of reconstructed pineal synaptic ribbons in Sprague-Dawley rats sacrificed at noon (\square , $n=40$) or midnight (\blacksquare , $n=40$). Areas were determined from the faces shown in Fig. 2a, b and refer only to one face. Note that the largest surface areas occur at 2400 hours

Table 1. Calculation of the surface areas (given in μm^2) of three synaptic ribbons (A, B, C), using three different principles of reconstruction. Note that the surface areas differ very little

Database	Method 1	Method 2	Method 3	Mean	SD
A	0.04616	0.04622	0.04636	0.04625	0.000059
B	0.05156	0.05100	0.05145	0.05134	0.000171
C	0.03363	0.03285	0.03314	0.03320	0.000228

crease ($P < 0.001$). Related to surface area, the increase was 19.3%. Average surface area during daytime was $0.042 \mu\text{m}^2$ and $0.0501 \mu\text{m}^2$ at night. Based on the assumption that all SRs shown here have the same thickness ($\sim 35 \text{ nm}$), the average volume would be $1.47 \times 10^{-3} \mu\text{m}^3$ at noon and $1.75 \times 10^{-3} \mu\text{m}^3$ at midnight, denoting an increase of $0.28 \times 10^{-3} \mu\text{m}^3$ or 19.3%. A mean volume of a SR would be $\sim 1.6 \times 10^{-3} \mu\text{m}^3$. Frequency histograms show that SR profiles measuring up to 150 nm in length were more abundant during daytime than at night, whereas those exceeding 250 nm showed the reverse (Fig. 4). Larger surface areas occur at 2400 hours (Fig. 5).

Discussion

As pointed out in the ‘Introduction’, the three-dimensional shape of pineal SRs is poorly understood. Principally, plate- and rod-like SRs have been thought to exist. In the present study, reconstructions of SRs based on serial sections revealed that virtually all the SRs encountered in the rat pineal gland were plate-like. Plate-like SRs have been unequivocally identified using serial section reconstructions in the pineal glands of rhesus monkey (McNulty et al. 1986) and chicken (Robertson and Dickson 1987) and by fortuitous sagittal sections

through SRs in the rat (King and Dougherty 1982a; McNulty and Fox 1992).

The finding that in the rat pineal virtually all the SRs observed were plate-like should not be taken as evidence that this is also the case in other species. Applying a goniometer to SRs, both McNulty et al. (1986) and Theron et al. (1981) noted that some SR profiles did not change appreciably when tilted, suggesting the presence of rod-like structures. Interestingly, Theron et al. (1981) pointed out that singly lying SRs were rod-like, whereas in ribbon fields rod-like structures and oval plates were jointly present. These observations show that it is premature to discount the possibility of rod-like SR being present in certain species. To be absolutely certain about the three-dimensional shape of SRs it is imperative to use serial sections. In our view it is not sufficient to carry out goniometer tilting experiments only, because in sections with a thickness near the thickness of the profile under question, i.e. 30–50 nm, even sections through plates would change very little, if at all, giving a rod-like impression. The presence of rod-like SRs has also been deduced for the guinea-pig pineal based on numerical relations between round versus rod-like profiles (Vollrath et al. 1983), but this conclusion may have to be revised in the light of the present findings.

While the present method of reconstruction shows clearly that the SRs under consideration are plate-like, it should not be overlooked that the method used is based on two objective criteria only, i.e. profile length and section thickness. Since there are no objectively assessable points of reference in the tissue for the SRs, reconstructions cannot be unbiased, because there is no objective method for superimposing the measured profiles. The method used in the present study does not account for torsions of SRs (Sobkowicz et al. 1982), the reconstructions do not show the thickness of the plates, which is rather constant anyhow, the synaptic vesicles surrounding the SRs are not visualised and the exact position within space is not reflected. Nevertheless, the reconstructions demonstrate that the plates are of variable size and shape, have polymorphic edges and their basic shape is roughly rectangular with width-to-length ratios between 1.1 and 1:5. With respect to the latter observation it is relevant that McNulty (1980) described width-to-length ratios of 2:5 and 4:5 for the plate-like SRs in the goldfish pineal. In view of the crescent shape of SRs in retinal rod SRs (Rao-Mirotznik et al. 1995) it is interesting to note that one of the reconstructed structures (Fig. 2a, \star) was similar in shape.

An important finding of the present study is that the SRs were larger at midnight than at noon, with no change in their basic shape. The increase in SR profile length found in this investigation (27.6%) is comparable to that described by Riemann (1990; 31%) and Spiwox-Becker (1995; 20.7%) in Sprague-Dawley rats. SR length increased by 66% in the Chinese hamster (Matsushima et al. 1983) and by 73% in the goldfish (McNulty 1981). Our findings corroborate the concept that SRs are dynamic structures.

The data obtained raise the important issue of how the increase in size of SRs influences counting results of

SR profiles. In many studies a numerical increase of SR profiles under various physiological and experimental conditions has been described (Lues 1971; Karasek 1976; Vollrath 1981; Khaledpour and Vollrath 1987; Karasek et al. 1988; Karasek 1992; McNulty and Fox 1992; Bhatnagar 1994). The nocturnal increase lies in the range between 2- and 6.7-fold (for review see McNulty and Fox 1992). According to our results approximately one-third of the nocturnal increase could be accounted for by the nocturnal enlargement of SRs. Hence, to obtain reliable data on SR density, the use of method which is not influenced by the size of the structures to be assessed would be required, e.g. the disector method (Sterio 1984). Moreover, changes in pineal volume have to be taken into consideration.

The enlargement of SR at night as shown in this study raises the question of their growth mechanism. Since the thickness of the rod-like SRs does not change from day to night, it appears that SRs grow by apposition at their thin edges. Perhaps this is the reason for the presence of the protuberances at this site. The formation of SRs during development is obscure and there is no morphological evidence as to how SRs grow. SRs have a high protein content (Bunt 1971; Krstić 1976) and consist of globular subunits 4–6 nm in diameter (Usukura and Yamada 1987). In this context, we hypothesise that precursor material from the cytosol may either aggregate near a SR and then fuse with it, or that the precursor material fuses directly with the SR, forming globular subunits.

Functional considerations

The nocturnal increase in size of SRs raises the question of how this relates to the function of the SRs and to that of the pineal gland as a whole. As pointed out in the 'Introduction', two hypotheses of pineal SR function prevail, i.e. the conveyor belt hypothesis (Bunt 1971) and the β -adrenoreceptor regulation hypothesis (King and Dougherty 1982a, b).

According to Bunt (1971) SRs transport synaptic vesicles (SVs) to the cell membrane for exocytosis. The increase of SR size at night could mean either a widening or an elongation of the conveyor belt. Under the assumption of a constant speed of the conveyor belt, a wider belt would increase the number of SVs released from the belt followed by exocytosis, resulting in enhanced intercellular communication (Vollrath 1973, 1981; Reiter 1977; Bhatnagar 1994), while an increase in belt length away from the cell membrane would increase the transport time of the vesicles. In view of the concept that the SRs may contribute substances to the SVs (Osborne and Thornhill 1972), there would be more time available for interaction between the electron-dense SR core and the adjacent vesicles. This interaction could also be relevant with respect to the recycling and reloading of exocytosed SVs. From our reconstructions of SRs the impression gained is that both the length and the width increase.

King and Dougherty (1982a, b) hypothesise that SRs regulate the number of plasmalemma-related β -adrenore-

gic receptors by externalising newly synthesised or internally stored receptors or, conversely, by internalising adrenergic receptors present along the cell membrane. According to their concept SR development is related to decreasing β -receptor density, implying that SRs increase in number following receptor internalisation leading to β -adrenergic subsensitivity. In our view there is insufficient evidence to relate SR profile numbers to pineal β -adrenergic receptor density in rats. Under the usual light/dark conditions, SR profile number has a maximum in the middle of the dark phase (cf. McNulty and Fox 1992; Bhatnagar 1994) but receptor binding studies reveal a peak at the end of the light period (Romero et al. 1975) or in the middle of the dark phase (Gonzales-Brito et al. 1988). Data on nocturnally rising β -receptor mRNA levels (Carter 1993) do not solve the issue since it is not known how much time elapses between transcription, translation and incorporation of the receptors into the plasmalemma. Depriving the pineal of its sympathetic input by ganglionectomy or continuous light leads to pineal β -adrenoceptor supersensitivity (Craft et al. 1985) and increased SR profile numbers (Vollrath and Huss 1973; Romijn 1975; Vollrath 1986; Reuss 1989; McNulty and Fox 1992), whereas King and Dougherty's concept would require decreased SR profile numbers. Moreover, SRs are rather infrequent compared to the large cell surface so that their influence on internalisation and externalisation of β -adrenoceptors is rather limited and restricted to relatively small membrane areas. Finally, the King and Dougherty hypothesis would imply a change in function of the SRs from a synaptic structure to a storage organelle of β -receptors. Wherever SRs are present (retina, inner ear, lateral line organ), they are involved in synaptic processes, releasing neurotransmitters by exocytosis. We therefore favour the hypothesis that SRs in the mammalian pineal gland are likewise components of chemical synapses (Hopsu and Arstila 1965; Vollrath and Huss 1973; Vollrath 1973).

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